THE ROLE OF EXOGENOUS ANTIOXIDANTS IN ENHANCING REPRODUCTIVE FUNCTION AND PERFORMANCE

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The Role of Exogenous Antioxidants in Enhancing Reproductive Function and Performance

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PREFACE

When we say global warming and pandemic, it is a reality accepted by everyone that many hazards, risks and dead ends await our planet. In our world, which is polluted with many chemical and industrial wastes, it is gaining importance to fight the risks that threaten nature, people, animals and all live organisms. Especially stressful conditions are something we have to fight the most. The risks surrounding our environment, air pollution, malnutrition, chemical, radioactive and biological risks penetrate into the cells of living organisms and reduce the quality of life. One of these biological risks discovered today is the phenomenon of "Oxidative stress". It is an undisputed fact by all relevant scientists that excessive "Reactive Oxygen Species (ROS)" that arise as a result of biochemical reactions in living organisms now threaten cellular mechanisms. In addition to deadly diseases such as cardiovascular diseases and cancer, the effects of antioxidant compounds on fertility, whose protective effects against free radicals, which can play an important role in many diseases, have been discovered and continue to be discovered, have begun to be investigated and very important data have been obtained. The addition of antioxidants, whose effects on fertility in mammals have been investigated, to culture media in in vitro embryo production systems as well as parenteral and oral use has come to the fore and studies on this subject have increased.

We believe that this book will make important contributions to scientists and students who are interested in the subject today, where antioxidants are gaining importance.

We would like to thank the distinguished scientists who contributed to this book.

Best regards

EDITORS
Prof.Dr.Yavuz ÖZTÜRKLER
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CONTENTS

CHAPTER I

USE OF ANTIOXIDANTS TO IMPROVE THE REPRODUCTIVE PERFORMANCE IN MODEL LABORATORY ANIMALS

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1. Introduction

The majority of laboratory animals are used for understanding the causes, diagnosis, and treatment of various diseases in animals and humans. Beyond biomedical research, laboratory animals are also reared as pets, game animals, and in the pet trade. The most popular laboratory animals are rodents, such as mice and rats. Rabbits, gerbils, guinea pigs, and hamsters are

also a member of laboratory animals (Bailey, 2014). During the use of laboratory animals for biomedical research, it is considered necessary to manage and maintain a particular strain for a long-term period under pet batteries (Jamsai and O'Bryan, 2011). For this purpose, the understanding of their reproductive traits, physical and behavior is of utmost importance to rebreed the laboratory animals and to maintain the particular a strain. In case of the pet rearing, the pet vendors usually deal with old aged, salvaged, or sterile animals as these nonproductive laboratory animals live for longer durations. Each species of laboratory animal possesses its specific features for management and reproduction but is usually managed in similar manners in breeding batteries. The selection of laboratory animals is based on the reproductive attributes of shorter gestation and proliferative nature in their short lifespan. These characteristics provide the serial and quickest way to complete the particular biomedical trials in several generations of lab animals. The second most advantage of laboratory animal keeping is to maintain them in a small area by controlling the environmental conditions. However, any deviation in physical or behavioral status emerged due to the environment greatly influencing the reproductive performances (Abedal-Majed and Cupp, 2019).

The male and female reproductive issues in humans are studied by using laboratory animals. Considering sex determination as a predominant aspect in laboratory animals prior to trial, is vital to study male and female-specific anomalies (Shah et al., 2016). Under nutritional, environmental, or managemental effects, the altered biological processes lead to oxidative stress, in turn, lower the fertility of male and female laboratory animals by following different mechanisms. During the diseased conditions, oxidative stress is a common phenomenon in all animal species and a similar trend of oxidative stress is observed in laboratory animals. The resemblance of biological processes between laboratory animals to other species and humans, and rearing advantages are key elements to observe the physiological or pathological exerted oxidative stress. In addition, the amelioration of physiological and pathological induced oxidative stress is also studied in different laboratory animals used as model experimental species. Different reproductive issues are emerged in males and females because of oxidative stress alike to other body systems. The influence of oxidative stress on reproductive physiology, hormonal patterns, cellular functions, and molecular expression in male and female laboratory animals are being observed for decades (Matzuk and Lamb, 2008).

This chapter aimed to highlight the physiological and pathological oxidative stress effects on the reproductive performance of male and female laboratory animals and how the utilization of various agents as antioxidants, plays a role to overcome oxidative stress in different reproductive anomalies.

2. ROS Production in Response to Internal and External Factors

Oxidative stress is a condition in which an oxidant-generating system outperforms an antioxidant defense system, a process involved in many disorders, including male factor infertility and/or subfertility in laboratory animals. Reactive oxygen species (ROS) are products of normal cellular metabolism and are produced during intercellular and intracellular signaling enzymatic processes. ROS are intermediate of oxidative stress and several different factors are involved in the production of ROS. These are byproducts of aerobic metabolism triggered by internal and external factors and reactive nitrogen species (RNS) are produced from the electron transport chain of mitochondria. A free radical is a molecule or atom that has an unpaired electron and is extremely reactive to achieve an electrically stable state. ROS are free radical derivatives of compounds containing oxygen (O_2). Not all ROS, however, are real free radicals. H_2O_2, for example, does not have an unpaired electron but is extremely reactive. Peroxyl (ROO) and hydroxyl (OH) radicals, superoxide (O_2) anion, and H_2O_2 are some of the clinically significant ROS discovered in infertility. Nitrogen molecules such as nitric oxide (NO) and peroxynitrite anion (ONOO), while not strictly ROS, appear to have a role in oxidation and reduction reactions in infertility. Lipids in membranes and carbohydrates in nucleic acids are two common compounds that accept the unpaired electron. When ROS levels exceed the antioxidant carrying capacity, this might result in cellular and DNA damage (Rahal et al., 2014; Pizzino et al., 2017).

In lab animals physiological and environmental stress is inevitable, and it is also well known that cage environment extremely affects the quality of life of experimental model animals. With the passage of time, researchers are gaining more knowledge about the humane handling and care of experimental animals. Moreover, animal welfare has grown over time and clinical trials must submit ethical approval before publishing research work. But there are hidden variables that cause a significant level of stress in lab animals, and it is difficult to translate into scientific words. The laboratory environment has many stressors like poor temperature, light and air regulation, which is further worsened by physical and social stressors (Gaskill and Garner, 2017; Bailey, 2018). From a

physiological point of view, a balanced level of ROS helps to achieve various biological functions in reproduction. Various cellular processes need balanced levels of ROS, for example, capacitation and acrosomal reactionon the male side and folliculogenesis, ovulation and fertilization on the female side. However, high levels of ROS or insufficiency of antioxidants can negatively affect sperm and ova survival and function (Opuwari and Henkel, 2016).

2.1. Production of ROS in Response to Internal Factors

The role of reactive oxygen species (ROS) as exacerbating or main factor in a variety of maladies, including ischemia, excitotoxicity, neurodegenerative disorders, and aging, is generally acknowledged and extensively addressed elsewhere. Recent evidence suggests that ROS, notably mitochondria-generated ROS, are engaged in physiological signaling cascades that regulate diverse cellular and organ functions, with H_2O_2 serving as a key messenger molecule. Reproduction requires higher levels of energy and due to increased metabolism ROS are produced (Blount et al., 2016). A variety of intrinsic factors are involved in oxidative stress like the age of the animal, breed, species, behavior, social rank, how these animals breed, and gender of the animal. For example, male zebra finches (Taeniopygia guttata), are more prone to oxidative stress than females due to more struggle in breeding (Alonso-Alvarez et al., 2004). Similarly, increased oxidative stress may reduce the life expectancy of male animals compared to females like in tarantula (Brachypelma albopilosa), where females live 18 years more than males (Criscuolo et al., 2010). Such a huge difference in life expectancy of male and female animals can be due to metabolic rate and method of breeding, where males are more exposed to oxidative stress than females.

2.2. ROS Production in Response to External Factors

Extrinsic or external factors e.g., seasonality, pollution, experimental conditions, laboratory assays, diet, light exposure, and infectious agents may influence the oxidative status of the individual lab animals. For lab animals, the room temperature must be ideal as spermatogenesis is strongly influenced by heat stress. A recent study reported that heat stress has a primary role in negatively affecting spermatogenesis and testicular histology in mice (Kastelic et al., 2019). Similarly, when rats were exposed to the higher temperature of 45C, the testicular mass and epididymal weights were reduced (Kheradmand et al., 2011), such changes in testicular mass and lower sperm production can be caused by

the higher rate of apoptosis and atrophy of seminiferous tubules (Kanter et al., 2013). It is also known that day-old mice and adult virgin mice need higher thermal environment; however, male mice develop aggressive behavior at that temperature, and therefore, there is no solution like one size fits all.

In lab animals, seasonality and exposure to light have a significant role in sperm production, for example in rhesus monkey higher sperm production and exocrine function was found in the autumn season (Wickings and Nieschlag, 1980). Moreover, constant exposure to light in Wistar rats (non-seasonal breeder) increases steroli cell population and function which subsequently increased daily sperm production (Rocha et al., 1999). The environment has a very important role in the life of experimental lab animals and it can directly affect the male reproductive organ structure and function. In the same way, the daily life routine of lab animals can significantly influence sperm production and quality, for example when rats were exposed to daily swimming exercise, their sperm production and quality decreased rapidly and reduced hypothalamic function (GnRH), and activity of SOD, CAT and GPx (Arisha and Moustafa, 2019). One of the external factors that have a major influence on reproduction is heavy metals, this factor causes a significant decrease in weight of reproductive organs and fertility (Ait Hamadouche, 2009).

Modern reproductive technologies known as ARTs also cause the production of ROS and oxidative stress, different techniques of semen storage involve centrifugation and manipulation for extended periods. These all steps of freezing and thawing cause thermal changes, production of ROS and increased susceptibility to oxidative stress (Aitken and Drevet, 2020).

Various types of infections from outside (ascending infections) and then the production of immunologic defense cells (leukocytes) also harm the sperm and starts a cascade of oxidative stress pathways (Collodel et al., 2015). During bacterial infections, the metabolites and toxins directly bind with sperm cells and initiate ROS production. Such infections might spread to lab animals during semen collection and careless handling of animals.

2.3. ROS Production in Male Lab Animals its Physiological or Pathological Effects on Spermatogenesis, Sperm Capacitation and Fertilization

The majority of reproductive functions in male animals i.e. sperm cells production and maturation, DNA and chromatin condensation, sperm transport, hyper-activation, maturation, and acrosome reaction are mainly controlled by redox systems (R. Aitken et al., 2003; Silva et al., 2020). In male animals, most

ROS production is inside sperm cells, and it is present mostly in infertile semen samples. A cascade of biochemical events controls the development of male and female gametes, each cell has been gifted with its defense mechanisms against oxidative stress. However, sperm cells are weaker in defense and during different phases of maturation when ROS are produced. During oxidative stress, molecules with unpaired electrons are highly reactive and act as oxidants. Various physiological reactions like Haber-Weiss and Fenton reactions generate hydroxyl radicals which is reactive radical. Similarly, leakage of the electron transport chain causes a reduction of stable molecules or free radicals. These all sources at a cell level generate a huge source of ROS. The classification of ROS can be primary radicals (superoxide anion), which turn into secondary radicals such as hydroxyl, hydrogen peroxide, and peroxyl. Then those ROS which contains nitrogen is termed tertiary forms. RNS is equally important as ROS because these are involved in spermatogenesis and steroidogenesis (Doshi et al., 2012). Most RNS are formed from NO by catalyzing L-arginine with nitric oxide synthase (NOS). Three forms of NOS are reported in inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS), and endothelial nitric oxide synthase (eNOS) (Doshi et al., 2012). In testes, there is a special subclass which is testes-specific TnNOS involved in steroidogenesis by Leydig cells and NO production in males. Similarly, other forms are involved in maintaining and degenerating (apoptosis) germ cell lines in the seminiferous epithelium (Agarwal et al., 2012). In this way, RNS has an evident role in Sertoli-Leydig cell function and higher concentrations can cause damaging reactions. These effects may range from changing the structure of membranes to lipid peroxidation and nitrosation. Sertoli cells are the main players in spermatogenesis and regulate ROS production with help of different antioxidants in rats (Hipler et al., 2000). ROS are known to impair ovarian and testicular steroidogenesis and are involved in a range of pathologic diseases of the testis. Oxidative stress is defined as the disruption of redox signaling and regulation as well as an imbalance in favor of prooxidant species.

The two testes, which create sperm, are the principal sex organs of the male reproductive system. The testis has seminiferous tubules which are made-up of germinal epithelium and peritubular tissue. The epithelium is made-up of two fundamental cell types: somatic and germinal cells. Germ cells, including spermatogonial stem cells and differentiated cells generated during and after meiosis, are primary and secondary spermatocytes and spermatids at distinct developmental stages. Sertoli cells are involved in phagocytosis, the secretion

of testicular fluid for sperm transport, the creation of endocrine and paracrine chemicals that govern spermatogenesis, and the secretion of the androgen-binding protein. The Leydig cell is a polyhedral epithelioid cell with a single ovoid nucleus, one to three nucleoli, and an abundance of dark-staining peripheral heterochromatin. Many membrane-bound lipid droplets and a substantial volume of the smooth endoplasmic reticulum can be found in the acidophilic cytoplasm. The primary source of androgens in males is the testicular Leydig cells. The early growth of gonads requires more energy than the development of ovaries. The presence of many mitochondria in male germ cells emphasizes the role of mitochondria in testicular metabolism. Carbohydrate metabolism, including glycolysis and mitochondrial oxidative phosphorylation, is required for germ cell survival in the adult testis.

ROS production in the male reproductive system:

Spermatogonia, mature sperm and somatic Sertoli cells have strong glycolytic activity, whereas spermatocytes and spermatids use mitochondrial oxidative phosphorylation to make adenosine triphosphate (ATP). Three kinds of mitochondria are recognized during spermatogenesis:

- in Sertoli cells
- in spermatogonia
- in preleptotene, leptotene spermatocyte

In the mitochondria, in zygotene spermatocytes, the intermediate form exists; in pachytene spermatocytes, secondary spermatocytes, and early spermatids, the condensed form exists. Studies have shown that low and verified concentrations of ROS play a pivotal role in sperm physiological processes such as capacitation, hyperactivation, acrosome reactions, and signaling processes to provide suitable fertilization but an increase in oxidative stress leads to male infertility by inducing peroxidative damage to the sperm plasma membrane, DNA damage, and apoptosis. ROS levels must be kept stable to guarantee proper physiological activity while limiting pathological damage to the spermatozoa. ROS is considered to disrupt fertility by influencing sperm membranes and DNA. In addition, ROS impair sperm motility and ability to fuse with the oocyte as well as paternal genomic contribution to the embryo; in fact, sperm are vulnerable to oxidative stress-induced damage due to the high proportion of PUFA in their cytoplasm with low concentrations of scavenging enzymes.

In male animals, the primary source of ROS is semen containing sperm cells and RNS are produced from accessory sex gland secretions and immature and mature forms of spermatozoa. The male reproductive system produces different forms of RNS starting from leydig cells, seminiferous tubules, ejaculatory duct, pelvic plexus, and penile muscles. For example, improper development of sperm cells causes the retention of the cytoplasmic droplet and it is positively correlated with higher oxidative stress (Rengan et al., 2012). This incomplete development of sperm cells can negatively affect sperm motility, morphology, and mitochondrial function which might result in infertility. Furthermore, abnormal spermatogenesis causes badly affect mitochondrial function and increases ROS production. The second major source of endogenous ROS is accessory sex glands (prostate and seminal vesicle) secretions which include leukocytes, and neutrophils (Agarwal et al., 2014). For example, during cases of infection, the leukocytes number increases in semen and termed leukocytospermia, this condition causes poor quality of semen, apoptosis and infertility (Agarwal et al., 2008). The induction to an apoptotic cascade is characterized by rapid loss of spermatozoa motility, even more generation of ROS, caspase activation in the cytosol, and oxidative sperm DNA damage. In short, the apoptotic process, accelerated by oxidative stress in the male genital tract, is the main pathway leading to DNA damage.

Lab animals can develop infections/diseases of reproduction organs during handling and inappropriate semen collection and such conditions can rise production of ROS due to bacterial toxins and leukocytes offering protection against external invaders. High levels of ROS, DNA fragmentation and lipid peroxidation have found in cases of infected lab animals. Similarly, in the human study, it has found that higher levels of pro-inflammatory cytokines and lipid peroxidation was present in the presence of inflammation and leukocytes (Martínez et al., 2007).

2.4. ROS Production in Female Lab Animals and its Physiological or Pathological Effects on Oogenesis, Folliculogenesis, Steroidogenesis, Fertilization, Embryogenesis and Pregnancy

2.4.1. Physiological effects

The half of reproductive function is contributed from the female side which starts from the production of oocytes to the maintenance of pregnancy, contrary to male animals, females have the additional responsibility of birth and production of different hormones. In contrast to male animals, most of reproductive organs

are located inside the pelvic cavity in female. Ovaries are an essential part of the female reproductive system acting as endocrine and exocrine organs at the same time. The size and shape of ovaries are variable in lab animals but the functions are nearly similar i.e. production of female gametes and regulate the cyclicity so that repeated opportunities of pregnancy can be created for male animals. ROS has a major role in most of reproductive functions like folliculogenesis, ovulation, steroidogenesis, fertilization, recognition of pregnancy, major apoptotic pathways, and the formation of the placenta (Silva et al., 2020). ROS are by-products of aerobic metabolism and act as secondary messengers in various cellular processes. A specific level of ROS i.e. 60-80ng/ oocyte is required for proper maturation and resumption of meiosis from diplotene arrest. This observation was confirmed by a study where the addition of antioxidants stopped the maturation process in rat oocytes, and a higher level of ROS i.e. >80ng caused apoptosis of oocytes (Chaube et al., 2005; Pandey et al., 2010). Ovaries are the main players in female reproduction and due to higher metabolism during folliculogenesis and ovulation, and ROS is produced more than required levels. If the production of ROS is not controlled then oxidative stress directly affects the quality of oocytes and reduces the rate of fertilization and pregnancy in lab animals (Nasr-Esfahani and Johnson, 1991). Studies have reported that higher level of oxidative stress reduces ovarian function and causes apoptosis and cell cycle arrest (Goud et al., 2008; Tiwari et al., 2015). Hypoxia is considered an important factor in the early days of pregnancy for embryonic life and the formation of the placenta in mice (Daikoku et al., 2003).

2.4.2. Pathological Effects

Reproductive organs and hormonal functions together to regulate folliculogenesis, estrous cycle, ovulation, and pregnancy; however, both internal and external stress can baldy influence hormone levels and fertility. It is believed that oxidative stress is a pathogenic and major cause of infertility in females. During uncontrolled or aggressive production of ROS damage starts at the cellular level called cell injury. It involves the degradation of cell proteins, lipid peroxidation of the cell membrane, and DNA damage. Oxidative stress due to physical stress or environment can have long-lasting effects on the female reproductive system which cause infertility and ultimately sterility. The amount and time of exposure to oxidative stress are

important, short-term stress is controlled with a set of antioxidants but high amounts of oxidative stress severely damage the physiological functions of reproduction and fertilization. Mitochondria are the first affected cell organelle ROS as histones are not present in mitochondrial DNA, proteins and lipids of mitochondria are susceptible to oxidative stress. Therefore, ROS is mainly produced in mitochondria causing abnormal generation of ATP and metabolic disorders (Catt and Henman, 2000). If the mitochondria are not functional, the oocytes, and embryos are prone to injury and ultimately leads to infertility (Kowaltowski and Vercesi, 1999).

Most female lab animals ovulate multiple times and any hormonal or metabolic disturbance can delay the ovulation process, on the other hand, as the age of the animal increases the oxidative stress grows after repeated ovulation (Miyo, 2010). During folliculogenesis, four stages (recruitment, selection, dominance, and atresia) regulate the development and maturation of oocytes. The last stage termed atresia involves the loss of follicular granulosa cells and theca cells, and oxidative stress is one of the factors which initiates this process of atresia (Meng, 2020). A study by Want et al. (2012) confirmed that mice injected with arsenic sodium showed lower SOD and GSH-Px levels, and oxidative stress caused lower growth of follicles with high follicular atresia. This was further confirmed by Zaho et al. (2014) where ovarian tissue of mice treated with oxidants and proapoptotic genes were activated which displayed apoptosis of granulosa cells. Oxidative stress causes the activation and upregulation of FoxO1 protein, and various proapoptotic genes (Bim, FasL, Puma, and TRAIL) are activated ultimately resulting in follicular atresia in mice (Shen et al., 2012). Before, the step of fertilization oogonium passes through various stages of meiosis, and maturation promoting factor is the cell-cycle factor that resumes meiosis. Oxidative stress can directly stop the maturation-promoting factor by inhibiting tyrosine phosphatases and thereby disturbing oocytes meiosis (Jia, 2019). ROS are produced under conditions of stress and damage the membrane lipids producing lipid radicals i.e. alkyls and peroxyls (El-Beltagi and Mohamed, 2013). In general, oxidative stress acts on the cell membrane and starts a set of self-propagating chemical reactions known as lipid peroxidation. This reaction directly changes the structure and function of the oocyte membrane, thereby, the permeability and membrane fluidity are directly influenced leading to abnormal fertilization (Rao et al., 2009). The oxidative breakdown of lipids is referred to as lipid peroxidation. Free radicals 'steal' electrons from lipids in sperm cell membranes, producing cell damage such as changes in structure, accumulation,

and dynamics of lipid membranes; this results in a free radical chain reaction mechanism that produces highly reactive chemicals. The lipid peroxidation cascade can be divided into three stages: initiation, propagation, and termination. During gametogenesis, the DNA lack histones and remains in free form, there are several steps during gamete production, which are exposed to ROS. Though gametogenesis occurs in a very stable environment to avoid any mutation in germ cells, oxidative stress can affect one or multiple stages, transcription, translation, and replication. In a lab environment, gametes are more prone to oxidative stress (IVF) than natural environment *(In-vivo).* In case, ROS are higher at danger levels, this can cause cell arrest and embryonic death in the early phase of pregnancy. Most of the oxidative stress is generated inside the ovary where follicles and steroid hormones are produced. The ovary acts as an endocrine organ and produces steroid hormones. During hormone production, hydrogen peroxide and superoxide anion in the follicles influence oocyte quality (Nasr-Esfahani and Johnson, 1991). Repeated oxidative stress causes permanent damage to oocytes in mice, and causes abnormal ovulation due to changes in DNA, proteins, and lipids (Chao et al., 2005). This stimulation leads to an inflammatory reaction and activation of cyclooxygenase 2 further damaging the ovaries. ROS have a greater impact on gametogenesis and at the individual level reduce sperm motility and fusion capacity with the oocyte (Irvine, 1996). During advanced reproductive techniques embryos might exhibit abnormal development due to genetic abnormalities or oxidative stress, this developmental arrest has been observed at the 2-cell stage in mice due to hydrogen peroxide (Nasr-Esfahani and Johnson, 1991). Similarly, embryonic development depends on M-phase promoting factor and SOD1, therefore, any of these two factors' downregulation can slow down embryonic development especially it was documented in older mice (Tatone et al., 2006). Oxidative stress may potentially compromise DNA integrity resulting in aberrant sperm function. DNA damage manifests itself as many modifications such as single-strand or double-strand fragmentation, the formation of basic sites, changes in purine, pyrimidine, deoxyribose, and DNA cross DNA cross-linking. These changes can cause gene transcription to begin or halt, increase telomeric DNA degradation, epigenetic changes, replication mistakes, and GC

to TA transversions. ROS forms oxidized DNA base adducts inside DNA strands, such as 8-hydroxy-2′-deoxyguanosine (8-OHdG) which compromises sperm chromatin integrity and makes sperm DNA sensitive to oxidative stress. The apoptotic pathway consists of highly intricate, sophisticated, energy-dependent, sophisticated cascade mechanisms. These are regulated by two key modulators, called the intrinsic mitochondrial pathway and the extrinsic receptor complex pathway. This process of apoptosis eliminates abnormal germ cells and inhibits overproduction in ovaries during the process of proliferation. Various studies on lab animals have confirmed that supplementation of antioxidants reduces oxidative stress and overcomes the harmful effects of ROS. A recent study confirmed that lower levels of oxidative stress increase the reproductive capacity in mice producing larger litters (Stier et al., 2012). Furthermore, this study also implied that oxidative stress directly affects the conception rate in female laboratory mice and it has no influence on reproductive effort associated with rearing (i.e. lactation). It is also worth mentioning that oxidative cost of reproduction is mostly focused on pre-breeding phase, and there is insufficient data on the post-breeding oxidative stress cost like gestation and lactation of the female animal.

3. Use of Enzymatic and Non-Enzymatic Antioxidants to Combat the ROS Induced Reproductive Issues

3.1. Mechanism of Action of the Enzymatic Antioxidants

Enzymatic antioxidants are the key protective compounds in the reproductive system against ROS. The major enzymatic antioxidants (SOD and CAT) act combine to remove the ROS molecules. The SOD is found in three forms SOD1 (present in the cytoplasm with Cu or Zn metal factors), SOD2, (mitochondrial type a Mn-cofactor) and SOD3 (extracellular type having Cu-Zn cofactor) different compartments of cells and catalyze the dismutation of O^{-2} to O^{2} and H_2O_2 making it comparatively stable compound. In contrast, CAT is intracellular and contains a heme group that covert the relatively unstable molecule H_2O_2 into nonreactive water (H_2O) and oxygen (O_2) molecules. Hence, SOD and CAT work together in reducing oxidative stress by the removal of ROS molecules. Glutathione peroxidase (GPx) and Glutathione reductase (GR) are important enzymatic antioxidants that maintain the homeostasis of glutathione (GSH) for lowering oxidative stress. The GPx is a selenium-dependent antioxidant which

converts GSH to its oxidized state (GSSG) and hydroperoxides to the water molecule. The GR is a dimeric flavoprotein antioxidant that reduces oxidized GSH (GSSG) back to GSH (Lü et al., 2010; Aziz et al., 2019).

3.2. Mechanism of Action of the Non-Enzymatic Antioxidants

Nonenzymatic antioxidants also play a vital role in oxidative stress balance. These are of particular interest because they are currently being used in vivo as the treatment for male and female infertility due to their ability to break up the chain reaction of lipid peroxidation (Moussa et al., 2019). Ascorbic acid/ Vitamin C possesses oxidant scavenging properties. Due to the hydrophilic property of vitamin C, it acts at the plasma level rather than the lipid bilayers of cells. Vitamin C is a powerful antioxidant that removes the superoxide radicals and oxygen species by donating a hydrogen atom to make a relatively stable ascorbyl-free radical. α-Tocopherol is commonly known as vitamin E and belongs to the class of fat-soluble vitamins. Vitamin E act as an antioxidant due to the presence of a hydroxyl group in its phenolic group that donates a hydrogen atom and neutralizes the free radicals. Following the reaction of the hydrogen atom and free radicals, a non-radical product and a vitamin E radical are formed. Later, this vitamin E radical acts over either free radical lipid or form the vitamin C. In this way, vitamin E neutralizes the peroxyl radicals and blocks polyunsaturated fatty acids peroxidation which protects the cellular membranes (Irato et al., 2021). β-carotene is also one of the antioxidants members from the vitamins class and it possesses lipophilic propertis hence it is present in the lipid bilayer. It scavenges the singlet oxygen, superoxide, and peroxyl radicals. Due to provitamin activity, the carotene transfers the active radicals into electrons by donating hydrogen atoms to radicals or bonding to radicals. Lycopene is a member of the carotenoid family with potent ROS scavenging capacity. It has potency to quench the singleton oxygen multiple times than vitamin E or C. In addition, it maintains the glutathione levels by regulating the activities of glutathione peroxidase and reductase. L-Carnitine is a water-soluble antioxidant molecule that plays a key role in metabolism, principally mitochondrial fatty acids oxidation. It is synthesized in the liver and kidney by methionine and lysine. It scavenges the hydrogen peroxide and superoxide radicals and gives a relatively stable product of chelate transition metal ions. N-acetyl-cysteine is also of potent antioxidant that scavenges particularly, thiols and hydroxyl radicals and acts as a precursor to glutathione. Glutathione is one of the most abundantly nonenzymatic antioxidants existing in class endogenously. Its

biosynthesis occurs in the liver by deriving from animal and plant dietary sources. The presence of the thiol group in the glutathione molecule makes it an antioxidant. It plays a key role in enzymatic (GPx and GR) and non-enzymatic (vitamins C and E) antioxidants (Nimse and Pal, 2015).

The selenium is used for the synthesis of selenoprotein and serves as the sole co-factor for GPx, thioredoxin reductase, and iodothyronine deiodinases that work as intracellular antioxidants for scavenging the ROS molecules. Zinc as a trace element, it has a key role in the redox process. It is a cofactor for SOD and metallothioneins which help to neutralize the superoxide and hydroxyl radicals (Zoidis et al., 2018). Polyphenols are strong antioxidants that scavenge free radicals by donating an electron or hydrogen atom. It contains several groups such as phenolic acids (benzoic acid, cinnamic acid) and flavonoids (quercetin, kaempferol, naringenin, taxifolin, genistein, rutin, and resveratrol) (Serra et al., 2021; Hashem et al., 2020; Gessner et al., 2017).

3.3. Antioxidants: A Therapeutic Approach for Male Infertility

Antioxidants are used in male infertility as a single sole product to keep their potency in the biological process. The use of GSH has a beneficial impact on sperm kinetics, sperm morphology, and sperm count. Vitamin C provision maintains the semen parameters and sperm DNA integrity against ROS-induced disorders. Vitamin E is helpful to treat the condition of asthenozoospermia and increase motility and fertilizing capacity as a result of reduced lipid peroxidation. Carnitine improved sperm concentration and motility in normal and as well as asthenozoospermic individuals. Selenium increases the GPx activity leading to high motility and sperm count in infertility issues. N-acetylcysteine is also effective to enhance semen parameters by lowering oxidative stress during idiopathic infertility. Use of Zinc sulfate help to improve semen quality (motility and count) in asthenozoospermia-suffering individuals. An improvement in sperm membrane integrity is the main aspect following Zn provision in asthenozoospermia condition which show the antioxidant effect of Zn. The involvement of carotenoids in the integrity of cell membranes and spermatogenesis, hence, the use of carotenoids is useful to sperm parameters (Adewoyin et al., 2017).

The antioxidant system of semen is composed of enzymatic and non–enzymatic antioxidant capacity molecules. Keeping in view of this synergetic mechanism, a combination of antioxidants is used to improve male infertility issues. Combining vitamins C and E improved the DNA fragmentation of

sperm, sperm motility, and concentration. Selenium and vitamin E combination increased sperm motility, maturation, and viability by regulating spermatogenesis and epididymal milieu against ROS-induced disorders. The combination of selenium and N-acetyl-cysteine help to improve the serum testosterone level, in turn, increases sperm concentration, motility, and morphology during infertility. The menevit, a combination of vitamin C, vitamin E, folate, garlic, lycopene, selenium, and zinc), improve semen quality in sub-fertile individuals. The combination of vitamins C, E and Zn is beneficial in asthenozoospermic individuals to improve semen quality against oxidative stress (Ahmadi et al., 2016; Ali et al., 2021; Cilio et al., 2022).

3.4. Use of Antioxidants in Female Infertility Conditions

In females, both enzymatic and nonenzymatic antioxidants are helpful to minimize oxidative stress due to excessive ROS production. Antioxidant supplementation like vitamin C, GSH, folic acid, melatonin, etc. protects oocyte against the harmful effects of ROS. Vitamin C supplementation helps to cure endometriosis in females, luteal defects, improving fertilization. Vitamin E improves epithelial growth in blood vessels of CL and endometrium, and lowers the ROS increment during gestational diabetes mellitus. Vitamin A, E, and beta-carotene help in recurrent pregnancy loss. GSH improves cervical mucus for sperm penetration in turn improve ovulation and reduces oxidative stress during the pathological conditions of endometriosis, PCOS, and recurrent pregnancy loss. The use of polyphenolic antioxidants (quercetin, resveratrol, rutin, etc.) from plant products may be beneficial for reducing oxidative stress in subfertility cases, particularly PCOS, gestational diabetes mellitus, and endometriosis. Some miscellaneous antioxidants like L-arginine role in successful implantation, Myo-inositol in ovarian function, and PUFAs in prostaglandin, and steroid hormone regulation, are noted (Ruder et al., 2008; Smits et al., 2018; Agarwal et al., 2018; Nath and Roy, 2020; Bhardwaj et al., 2021).

3.5. Fertility in Male Lab Animals After Different Antioxidant Supplements Under Environmental and Toxicological Stressors

The use of antioxidants plays a pivotal role in male and female reproductive health and fertility. The exposure of males and females to external and internal stressors induce oxidative stress which in turn adversely affects the male and female gametes, gametogenesis, reproductive tract functionalities, and hormonal biosynthesis that may lead to subfertility. To highlight the effects of

different antioxidants on fertility under physiological or pathological induced oxidative stress, laboratory animals have been used. In male infertility cases, the rat is extensively selected as a model animal compared to mice, rabbits, or guinea pigs. The laboratory animals are used as simulants to various male subfertility conditions and different aspects like hypophyseal-pituitary-gonadal axis regulation, steroidogenesis, spermatogenesis, sperm maturation, accessory sex glands integrity, testicular and epididymal functions, testicular antioxidant activities, and expression of specific genes/proteins, etc. is documented using exogenous antioxidants against oxidative stress in physiological (aging, exercise, noise, radiations, obesity, disturbances in sleep, transportation, temperature exposure, anxiety) or pathological (heavy metal exposure, chemicals exposure, prolonged use of medicines, diseases, pathological condition) conditions. Dietary antioxidants in form of flavonoids, vitamins, and trace elements are the most appropriate choice to combat oxidative stress conditions. The vitamin C alone or in combination with vitamin E is effective to improve the antioxidant testicular microenvironment, spermatogenesis, expression of antiapoptotic factors, gonadotropin and steroid hormones synthesis, and sperm parameters when male rats are challenged against oxidative stress due to forced swimming (Vijayprasad et al., 2014), noise (Fathollahi et al., 2013), sleep deprivation (Rizk et al., 2020), prolonged hyperthermia (Qari et al., 2021) and electromagnetic radiation exposure (Al-Damegh, 2012). To highlight deleterious effects of heat stress and ameliorative effects of antioxidants, the rabbits, mice, and guinea pigs are also candidate laboratory animal models. It is observed that polyphenols such as Soursop juice (Jimoh et al., 2021), extra-virgin olive oil, betaine and ginger (El-Ratel et al., 2021), moringa leaves (El-Desoky et al., 2017), baobab fruit pulp (Anoh et al., 2018), quercetin (Naseer et al., 2018, 2020), guava leaves (Ngoula et al., 2017), ginseng (Kopalli et al., 2019) etc. are considered suitable antioxidants to alleviate the heat stress-induced male infertility **(Table 01).**

In the current era, the expansion of industries is a great challenge. The industry effluent contains different forms of toxins that not only affect the climate but also the surrounding population. Along emergence of respiratory, renal, cardiovascular, and digestive issues from these toxins, male subfertility is also a common problem in the exposed population. To highlight the fertility-associated risks in the exposed population to industry chemicals, laboratory animals are used to observe the deleterious effects of toxicants, particularly oxidative stress. The antioxidants from different origin of vitamins, minerals and plant extracts are used to overcome the fertility issues linked to industry toxicants. It

is observed that vitamin C is the most potent and economical antioxidant that caters the oxidative stress solely or in combined action with other antioxidants. Male infertility due to oxidative stress induced by lead and arsenic (Raeeszadeh et al., 2021), nickel (Kong et al., 2019), boldenone undecylenate (Behairy et al., 2020), chlorpyrifos (Shittu et al., 2012), and carbamazepine (Akorede et al., 2020) in rats is ameliorated by alone vitamin C. The nitrate (Attia et al., 2013) in rabbits, tyrosine nitration toxicity in mice (Scarlata et al., 2020), di-(2-ethylhexyl) phthalate (Shen et al., 2018), and Ivermectin induced testicular oxidative is mitigated by vitamin C along in combination to vitamin E or A, Se and probiotics. The glutathione is useful to increase the fertility of male rabbits after exposure to plant fungicide, mancozeb-induced testicular toxicity (Elsharkawy et al., 2019). In addition, the use of pentoxifylline and silymarin in rats as antioxidants mitigate male infertility due to oxidative stress induced by antineoplastic drugs (cisplatin, streptozotocin etc).

Zinc and selenium as trace minerals antioxidants alone or jointly are effective to alleviate male infertility induced by different toxicant i.e. streptozotocin (El-Hakim et al., 2021), doxorubicin (Kabel, 2018), NSAIDS (Sharma et al., 2020; Owumi et al., 2020), monosodium glutamate (Hamza and Diab, 2020), etc. or pathological conditions (diabetes, Sahu et al., 2020; varicocele; Taghizadeh et al., 2017). The rats and mice are usually used as simulant models to observe the effects of zinc and selenium on the activities of antioxidants, oxidants in testes, hormonal synthesis, testicular pathophysiology, and sperm parameters **(Table 02)**.

In addition, to vitamins and trace minerals, the use of polyphenols as antioxidants to treat male infertility is the up-raising trend in medical science. These compounds are derived from plant leaves, barks, pulps, seeds, juices, or extracts of whole plants. Each compound contains a variety of medicinal uses like an anti-diabetic, pro or anti-apoptotic, anti-inflammatory, antineoplastic, and antiviral properties. The teucrium polium, garlic extract, and mangifera indica counteract male infertility induced by insecticides (carbon tetrachloride, cypermethrin, or acetamiprid) in rats and guinea pigs (Rahmouni et al., 2019; Assayed et al., 2010; Guiekep et al., 2019).

The protective effect of polyphenolic compounds in male infertility induced by heavy metals is helpful to improve the histopathology of testes and epididymis, to maintain reproductive hormones and antioxidant enzyme activities, and sperm variables. Amongst polyphenols, chenopodium album Linn. (Jahan et al., 2019), elagi acid (Mehrzadi et al., 2018), caffeic acid (Erboga

et al., 2016), Cucumin (Sudjarwo et al., 2017), and chrysin (Ileriturk et al., 2021) are beneficial in mercury, lead, arsenic, and cadmium-induced testicular toxicity in rat model animals. Moreover, propolis (Kumari et al., 2017) and roselle and ginger (Amin and Hamza, 2006) provide ameliorative effects against antineoplastic drugs-induced infertility in rats. The oxidative stress induced by varicose and obesity is also combated by alpha-lipoic acid (Shaygannia et al., 2018) and grape seed proanthocyanidin extracts in subfertile rats (Wang et al., 2019). After the extensive use of polyphenolics in research trials to curtail infertility issues in males, polyphenols are a choice other than vitamins and trace minerals.

3.6. Female Lab Animals Fertility Outcomes Following Antioxidant Supplementation

The oxidative stress in females influences physiological processes like oocyte development, follicle dynamics, CL formation, fertilization events, placenta functioning, and embryonic development. The onset of cellular oxidative stress brought damaging effects in reproductive organs in turn resulting in subfertility. The excessive oxidative stress is catered by different dietary antioxidants to normalize the female reproductive processes. It has been observed the use of antioxidants in numerous female reproductive disorders and complications such as polycystic ovarian syndrome, pre-eclampsia, aging, chronic stress, heat stress, diabetes, recurrent pregnancy failure, etc. simulating in rat, rabbit, and mice species, is an economical and alternative therapy. The dietary antioxidant supplementation of rutin (Jahan et al., 2016), genistein (Rajaei et al., 2019), CoQ10 (Xu et al., 2019), and MitoQ (Aljunaidy et al., 2018) in PCOS, preeclampsia, and IUGR conditions, are useful to improve the serum antioxidant activities, restoration of hormonal synthesis, and placental activity. The fertility issues associated with aging, heat stress, and chronic stress is alleviated using a variety of phenolic antioxidants like vitex agnus-castus fruit, portulaca oleracea (Ahangarpour et al., 2016a, b,), quercetin (Naseer et al., 2017), vitamin C and baobab fruit pulp (Anoh et al., 2018), rhodiola rosea root extract (Kadioglu et al., 2020), using mice, rabbit, and rat animal models. Additionally, infertility induced by chemotherapic agents in rat models is mitigated by the provision of resveratrol (Ozcan et al., 2015), CoQ10 (Ozcan et al., 2016), quercetin and vitamin E (Samare-Najaf et al., 2020). Use of vitamin C (Kong et al., 2016) and resveratrol (Banu et al., 2016) improve antioxidant levels, minimize follicular apoptosis and maintain the reproductive hormones in nickel and chromium-

exposed rats **(Table 03)**. Use of nutritional antioxidants for the restoration of unexplained female reproductive issues is a remarkable option in laboratory animals. However, there is a need for extensive and focal research points using laboratory animals as targeted models, to mitigate the role of ROS in normal reproductive events, short- or long-term infertility issues in females.

4. Concluding Remarks and Perspective

The following concluding points were based on available literature:

- Turbulences due to excessive oxidative stress at the testicular and ovarian level, and reproductive tracts of the male and female lead to alteration in gametes integrity, hormonal synthesis, and finally conception rate when conditions are simulated using laboratory animals.
- Extensive research work is available in laboratory animal models about the effect and safety of antioxidant therapy to restore and prophylactic treatment in male and female infertility issues.
- Comparing the oxidative stress level and reproductive variables pre- and post-therapy, provide the effectiveness of any protocol for future indications.
- The antioxidant compounds are used as single or in combination; but considering a single ideal antioxidant for different male and female reproductive disorders is not promising because of the severity, multiple etiologies and nature of the condition.
- Along with vitamins and trace minerals, the option of polyphenol is an alternate choice to the combat oxidative stress-related male and female infertility disorders.

Future studies are required to determine the dose, duration, and protocol using a more appropriate animal model for the ailment of male and female infertility in humans. Fertility outcomes in form of conception, carrying the fetus for the full-term, and morphology of embryo/neonates should be necessarily considered following utilizing the antioxidants in infertile male and female animal models.

Table 1. Effect of antioxidants on reproductive status of male laboratory animals under external stressors

Physiological condition	Animal species	Antioxidant and Dosage	Biological activity	Physiological action	Reference
Forced swimming	Rat	Vitamin C (20mg/kg)	Antioxidant	Improves the stress induced reproductive infertility	(117)
Noise stress	Rat	Vitamin C (125mg/kg) and E (75mg/kg)	Antioxidant	Improve the FSH, LH, testosterone and pregnancy	(46)
Prolonged whole body hyperthermia	Rat	Vitamin C and E (100mg/kg)	Antioxidant	Maintain serum antioxidant level, prevent testes tissue damage	(92)
Sleep deprivation stress	Rat	Vitamin C (100mg/kg)	Antioxidant	Enhance the testicular structure and function in sleep deprivation	(99)
Electromagnetic radiation	Rat	Vitamin C and E (40 and 2.5 mg/kg)	Antioxidant	Restore testicular architecture and enzymatic activity	(14)
Extreme heat stress	Rabbit	Soursop juice (2.22 ml/kg)	Antioxidant	Enhance sperm quality and mitigate lipid peroxidation	(60)
Heat stress	Rabbit	Extra virgin olive oil (300mg/kg), betaine (1000mg/kg) and ginger (200mg/kg)	Antioxidant	Improve sexual desire, semen quality and oxidative stress	(43)
Heat stress	Rabbit	Moringa leaves ethanolic extract (50mg/kg)	Antioxidant	Improve heat tolerance, oxidative status and semen quality	(41)
Heat stress	Rabbit	Bi-carbonate buffer, Vitamin C and Baobab fruit pulp	Antioxidant	Vitamin C and Baobab fruit pulp improve testosterone	(19)
Heat stress	Rabbit	Quercetin	Antioxidant	maintain the sperm quality and lower the oxidative stress	(81)
Heat stress	Rabbit	Quercetin	Antioxidant	maintain testes function and apoptosis	(82)
Heat stress	Guinea pig	Guava (*Psidium guajava*) leaves essential oil	Antioxidant and antibacterial	Increase sperm motility and count, reduce sperms defects with increase in antioxidant activity	(84)
Heat stress	Rat	Korean Red Ginseng	Antioxidant	Therapeutic effect in testicular hyperthermia	(69)

Table 02. Effect of antioxidants on reproductive performance of male laboratory animals under toxicological conditions

Pathological condition and oxidant	Animal species	Antioxidant and Dosage	Biological activity	Physiological action	Reference
Nitrate induced toxicity	Male rabbits	vitamin C & E (200 ppm), Se (0.2 ppm) and probiotic (1000 ppm)	Antioxidant	Increased testosterone and fertility	(22)
Lead and Arsenic Induced toxicity	Rat	Nano-Vitamin C (200mg/kg)	Antioxidant	Testosterone, LH, and FSH	(93)
Di-(2-ethylhexyl) phthalate-induced blood-testis barrier disruption by p38 MAPK	Immature rats	vitamin C (200mg/kg) & E (100 mg/kg)	Antioxidant	Maintain the spermatogenesis and BTB disruption by inhibiting p38 MAPK signaling pathway	(109)
Nickel nanoparticle induced reproductive toxicity	Rat	Vitamin C (1gm/L)	Antioxidant	Inhibit the apoptosis of testicular cells induced by oxidative stress	(68)
Chronic chlorpyrifos-induced oxidative stress	Rat	Vitamin C (100mg/kg)	Antioxidant	Decreased lipoperoxidation in testes and pituitary glands	(110)
Boldenone undecylenate induced testicular oxidative	Rat	Vitamin C (120 mg/kg)	Antioxidant	Improve sperm related function but no effect on hormonal regulation	(29)
Ivermectin-induced toxicity	Rat	Vitamin C & A (1.25 mg/kg; 8000IU/kg)	Antioxidant	Promising effect against oxidant-antioxidant imbalance	(85)
Carbamazepine -induced reproductive toxicity	Rat	Vitamin C (100 mg/kg)	Antioxidant	Maintain the oxidative changes, sex hormones, sperm characteristics, pituitary and testicular changes	(13)
Fructose-induced diabetic oxidative stress	Rat	Ca, Mg, vitamin C and E	Antioxidant	Provide better ameliorative benefits for seminal variable	(28)

Pathological condition and oxidant	Animal species	Antioxidant and Dosage	Biological activity	Physiological action	Reference
Tyrosine Nitration-Associated Infertility	Mice	Tocopherols and Ascorbic Acid	Antioxidant	Maintain the motility, lipid peroxidation, DNA oxidation and tyrosine nitration	(104)
Mancozeb impaired fertility	Rabbit	Glutathione	Antioxidant	Increased FSH, LH, testosterone, levels with improved steroidogenesis	(44)
Varicocele	Rat	Zinc	Antioxidant	Maintain the testes function	(114)
Streptozotocin-Induced Diabetic	Rat	Zinc and selenium	Antioxidant	Reduce sperm abnormalities, improve sperm motility, and sexual hormones, minimize testicular oxidative damage, and steroidogenesis-related genes regulation	(42)
Diabetes-induced stress	Rat	Zinc and selenium combination	Antioxidant	Minimize testicular and epididymal damage	(102)
Doxorubicin induced toxicity	Rat	Zinc and alogliptin (20 and 10mg/kg)	Antioxidant and antidiabetic	Restore testicular functions, sperm characteristics, hormonal profile and antioxidant defenses	(61)
NSAID induced toxicity	Rat	Selenium (0.5mg/kg)	Antioxidant	Selenoproteins ameliorates Ibuprofen induced male reproductive toxicity	(107)
Monosodium glutamate-induced toxicity	Mice	Selenium nanoparticles (1.7 ppm)	Antioxidant	Improve antioxidant enzymes and decrease lipid peroxidation markers	(51)
diclofenac-induced toxicity	Rat	Selenium (0.25mg/kg)	Antioxidant	Maintain sperm count and motility, testicular function enzymes and levels of luteinizing hormone and testosterone	(87)
Cisplatin-induced Testicular Toxicity	Rat	Pentoxifylline (75 and 150mg/kg)	Antioxidant	Improve sperm, testis parameters, testosterone, LH, and FSH	(45)
Streptozotocin-induced diabetic	Rat	Silymarin	Antioxidant and liver tonic	Improve sperm parameters, reproductive performances, decrease LPO, and increase antioxidant enzymes	(66)
Carbon tetrachloride-induced toxicity	rats	Teucrium polium	Antioxidant, anti-inflammatory and anti-rheumatic	Improve sperm parameters, testicular texture, antioxidant enzymes by lowering MDA	(95)

Pathological condition and oxidant	Animal species	Antioxidant and Dosage	Biological activity	Physiological action	Reference
Cypermethrin- induced teratogenicity	Rat	Garlic extract (50mg/ml) and vitamin C (20mg/ kg)	Immune booster, antioxidant, antihypertensive	Reduction in foetal malformations induced by cypermethrin	(21)
Acetamiprid induced toxicity	Guinea pig	Mangifera indica (100-200mg/kg)	Antioxidant,	Reduce reaction time, sperm anomalies and testicular MDA,	(50)
Mercuric chloride-induced oxidative stress	Rat	Chenopodium album Linn. and vitamin C	Antioxidant and medicinal	Ameliorate the antioxidant enzyme activity, daily sperm production, and DNA damage	(57)
Arsenic induced toxicity	Rat	Elagic acid	Antioxidant	Reduce the arsenic accumulation in testes and oxidative stress parameters. Improve the serum testosterone level, testicular antioxidant markers and histological parameters	(76)
Lead acetate induced toxicity	Rat	Curcumin	Antioxidant	Improve pathological changes in testes, increase sperm count, motility, viability, increase SOD, GPx, and decrease MDA	(113)
Lead acetate	Rat	Chrysin (30mg/kg)	Antioxidant, anti-inflammatory	Relieves oxidative stress and maintain sperm parameters	(54)
Mitomycin C-induced testicular toxicity	Mice	Propolis (400mg/ kg)	Antioxidant	decrease oxidative stress, reduce DNA damage and restore testicular testosterone and inhibin	(71)
Ciplastin induced toxicity	Rat	Roselle and ginger, (1g/kg)	Antioxidant and anti-inflammatory	Increased level of testicular antioxidants	(18)
Varicocele	Rat	Alpha-Lipoic Acid	Antioxidant	Reduce the effects of elevated testicular temperature and increased oxidative stress	(108)
High-fat diet induced testicular oxidative stress	Rat	Grape seed proanthocyanidin extract	Antioxidant	Decrease testicular oxidative stress and reduce the apoptosis in spermatogenic cell	(118)

Table 03. Effect of antioxidants on reproductive performance of male laboratory animals under toxicological conditions

Pathological condition and oxidant	Animal species	Antioxidant and Dosage	Biological activity	Physiological action	Reference
Induced polycystic ovary syndrome	Rat	Rutin (100-150mg/kg)	Antioxidant	Better antioxidant and lipid profiles, decrease in the value of C reactive protein, restoration of estrous phase and decrease cystic follicles	(58)
Induced polycystic ovary syndrome	Rat	Genistein (0.2mg/kg)	Antioxidant	Improve ovarian tissue morphology, oxidant and antioxidant	(96)
Induced preeclampsia	Rat	CoQ10	Antioxidant	enhancing the function of mitochondria in the placenta	(120)
IUGR induced by hypoxia	Rat	MitoQ (125 uM, IP)	Antioxidant	Decrease hypoxia in placentas of male and female fetuses and improve diastolic dysfunction	(16)
d-galactose aging	Mice	Vitex agnus-castus fruit (600mg/kg)	Antioxidant	Reduce oxidative stress, atrophy of the endometrium, premature-aging female, and postmenopausal syndrome.	(7)
d-galactose aging	Mice	Portulaca oleracea (200mg/kg)	Antioxidant	Decrease follicles degeneration, atrophy of uterine wall and endometrial glands, and MDA contents. Improve hormonal level and antioxidant activity	(8)
Heat stress	Female rabbit	Quercetin	Antioxidant	quercetin provision improves the follicular development, minimize granulosa cells apoptosis, and maintain the oocyte competence	(80)
Heat stress	Rabbit	Bi-carbonate buffer, Vitamin C and Baobab fruit pulp	Antioxidant	Vitamin C and Baobab fruit pulp improve progesterone	(19)
Compulsory immobilization stress	Rat	Rhodiola rosea root extract (50mg/kg)	Antioxidant and anti-inflammatory	Prevent the increase in oxidative parameters and proinflammatory cytokine levels in ovarian tissue	(62)

Pathological condition and oxidant	Animal species	Antioxidant and Dosage	Biological activity	Physiological action	Reference
Ciplastin induced Oxidative stress	Rat	Resveratrol (10mg/kg)	Antioxidant	Maintain anti-Müllerian hormone and atretic and antral follicle counts	(88)
Ciplastin induced Oxidative stress	Rat	Coenzyme Q10 (150mg/kg)	Antioxidant	Protect ovarian reserve by counteracting both physiological and mitochondrial ovarian ageing	(89)
Doxorubicin-Induced Infertility	Rat	Quercetin (20mg/kg) and Vitamin E (200mg/kg)	Antioxidant	Improve ovarian function by lowering apoptosis, expressing ovarian aromatase and ER-α gene, increase estrogen and progesterone levels, increase ALP and decrease osteocalcin	(103)
Nickel nanoparticle induced oxidative stress	Rat	Vitamin C (1g/L)	Antioxidant	Increase antioxidants enzymes and antiapoptotic factor Bcl-2, decrease the ovarian ROS, MDA, and NO levels, and proapoptotic factor Bax, Fas, Cyt c, Bax, and Bid protein.	(68)
Chromium-toxicity	Rat	Resveratrol (10mg/kg)	Antioxidant	Mitigate the apoptosis in follicle and restore E2 levels	(27)

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CHAPTER II

THE ROLE OF EXOGENOUS ANTIOXIDANTS IN ENHANCING REPRODUCTIVE FUNCTION AND PERFORMANCE IN HUMANS

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1. Introduction

Nutritional supplements have been suggested as potential treatments for enhanging reproductive function and performance in humans. Exogenous antioxidants are one of the most important nutritional supplements in this regard and current in the literature. The role of exogenous antioxidants in enhancıng reproductıve function and performance in humans are discussed in this chapter.

2. Exogen Aniıoxidants Affect on Male Sexual Function Introduction

Erectile dysfunction (ED) and ejaculation (ejaculation, anorgasmia, premature ejaculation, delayed ejaculation, asthenic ejaculation) disorders are male sexual dysfunctions. (1)

2.1. Erectile Dysfunction

The co-activation of neuronal, vascular, hormonal, and smooth muscle cells causes an erection. ED is described as the inability to achieve and/or sustain a decent erection at the level required for sexual performance. (1,2)

With ageing, ED becomes more prevalent. It reaches a pinnacle, especially after the fifth decade. According to studies, the prevalence of ED ranges from 30% to 52%. (1)

Risk for ED; age, cigarette/cigar smoking, cigarette exposure, diabetes, excessive, dispensary, general, year. (1)

In its etiology, vascular causes (all diseases that decrease penile blood flow), neurogenic causes (Multiple Sclerosis, Parkinson's, medulla spinalis trauma, cerebrovascular events, peripheral neuropathy, disc herniation, iatrogenic pelvic nerve damage), hormonal causes (hypogonadism, hyperprolactinemia, hypothyroidism, hyperthyroidism, adrenal diseases), medications, surgical causes (radical prostatectomy, radical cystectomy, rectal surgery, pelvic radiotherapy, and cryotherapy), psychological causes play a role. (1-3)

Risk factors are rectified, lifestyle adjustments are implemented, and oral medication (phosphodiesterase type 5 inhibitors) is administered as the first step in treatment. Intracavernous injection, intraurethral medicines, vacuum devices, and extracorporeal shock wave therapy are used in second-line treatments. Surgical procedures (penile renovascular surgery, penile prosthesis implantation) are used as the third and last treatment step. (2,3)

2.2. Pathophysiologies Causing Erectile Dysfunction

2.2.1. Phosphodiesterase Enzyme Inhibition

As a result of sexual stimulation, nitric oxide synthase (NOS) produces nitric oxide (NO), which causes a penile erection. The enzyme guanylyl cyclase is activated when NO diffuses from the cell membrane to the smooth muscles. Guanylyl cyclase catalyzes the conversion of guanosine-5'-triphosphate (GTP) to 3'-5'-cyclic guanosine monophosphate, a second messenger (cGMP). Cyclic GMP activates the cGMP-dependent protein kinase (PKG) step, which

phosphorylates relaxation-related proteins. Furthermore, cGMP reduces intracellular calcium ion (Ca2+) concentrations. Smooth muscle relaxation occurs in the arterial and trabecular circulations. Arterial dilatation causes increased blood flow to the penis, which leads to a penile erection. Increasing cyclic GMP degradation, the PDE5 enzyme promotes arteriolar vasoconstriction and penile detumescence. As a result, inhibiting PDE5 activity enhances erections by raising cGMP levels. (4)

2.2.2. Nitric Oxide Synthetase Decomposition

The endothelium produces NO, a potent vasodilator and free radical. Endothelial NOS generates NO via utilizing cofactors such as tetrahydrobiopterin (BH4), flavin adenine dinucleotide, flavin mononucleotide, and calmodulin, as well as L-arginine and oxygen molecules. The major cofactor in this process, BH4, is vital in producing NO by NOS. The oxidation of BH4 to dihydrobiopterin (BH2) by oxidative stress promotes NOS cleavage. This unbound NOS also creates more superoxide anions instead of NO, causing the NO level to fall. These are unstable radicals (superoxide and NO), and their reaction produces peroxynitrite (ONOO), which is a much more stable radical and a potent oxidant.

Peroxynitrite also generates powerful oxidants, including hydroxyl and nitrogen dioxide (NO2) radicals, which cause oxidative stress and ED. (5-7)

2.2.3. Insulin Signaling Path

Insulin binding to an insulin receptor on endothelial cells results in phosphorylation of insulin receptor substrate 1. (IRS-1). Phosphorylated IRS causes phosphoinositide-3 kinase (PI3K) phosphorylation, which leads to protein kinase B activation (Akt). Endothelial NOS is phosphorylated and activated by Akt. Phosphorylated NOS increases NO production and causes vasodilation. On the other hand, insulin resistance activates protein kinase C (PKC), which lowers IRS phosphorylation and inactivates PI3K. As a result, NO pre-production is inhibited, and vasodilation is reduced. (8)

2.2.4. Superoxide Production Caused by Glucose Oxidation

Hyperglycemia, or high blood sugar levels, stimulates glucose oxidation. The glycolysis pathway is activated by glucose oxidation, resulting in the synthesis of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2), which are employed as energy-producing substrates by the mitochondrial electron transport chain. In turn, NADH and FADH2 send

electrons to complex ubiquinone oxidoreductase and complex III cytochrome c reductase, increasing superoxide production. Excessively increased levels of superoxide caused unbound NOS and subsequently ED.(8)

2.2.5. The Renin-Angiotensin System (RAS)

The renin-angiotensin system (RAS) regulates blood pressure as well as fluid and electrolyte balance. Angiotensinogen produced from the liver transforms released renin to angiotensin I. (Ang I). The lungs' angiotensin-converting enzyme (ACE) converts Ang I to angiotensin II (Ang II). Ang II, the primary component of the RAS, causes a tonic contraction of the smooth muscles of the corpus cavernosum during penile erection. Increased Ang II production, on the other hand, is linked to ED. Chronically higher Ang II levels activate the angiotensin type 1 receptor (AT1R), causing salt and water retention and vasoconstriction by the kidneys, followed by a drop in endothelial NO concentration and high blood pressure, and eventually ED. (9-12)

2.2.6. Acetylcholine Esterase Pathway

Acetylcholine (ACh) is a parasympathetic neurotransmitter produced by the cholinergic nerve in the smooth muscle cells of the penis corpus cavernosum. ACh relaxes the smooth muscle of the corpus cavernosum and improves erection by boosting endothelial NO generation. Acetylcholinesterase (AChE) decreases acetylcholine levels by converting it to acetate and choline. It eventually leads to ED. The suppression of AChE activity increases ACh levels, leading to enhanced penile erection.(10-13-14)

2.3. Ejaculatıon Dısorders

The ejection of sperm from the urethra is accomplished through the synchronized contraction and relaxation of neural activity and muscles.

The complete lack of antegrade or retrograde ejaculation is referred to as anejaculation—the absence of vesiculoseminalis, prostate, and ejaculatory ducts in the urethra results in sperm emission.

Anorgasmia is the inability to experience orgasm, which can result in anejaculation.

The mildest form of anorgasmia is delayed ejaculation. It might be either psychogenic or organic (incomplete spinal nerve damage, iatrogenic nerve damage of the penis, and drugs).

The passage of sperm via the bladder neck into the bladder is known as retrograde ejaculation. Absence of antegrade ejaculation, either wholly or partially.

Primary (lifelong) premature ejaculation is described as present and continuous since the first intercourse experience, before and/or less than one minute after vaginal penetration. Secondary (acquired) premature ejaculation occurs when the ejaculation latency time decreases significantly (ejaculation in three minutes or less). (1-3)

2.4. Antıoxidants in Exogen Affect Male Sexual Function

2.4.1. Resveratrol

Polyphenols are an antioxidant family comprised of flavonoids, anthocyanins, phenolic acids, lignans, and stilbenes. Resveratrol (3,4',5-trihydroxystilbene) is a polyphenolic molecule found in grapes, wine, peanuts, and blueberries that belongs to the stilbene subgroup. Resveratrol was identified in grapes in 1976 as a phytoalexin. (15) In China and Japan, resveratrol was dubbed Kojo-kon (Kojo-on: Also known as Itadori tea) in 1982; it has been claimed that it is discovered in dried "Polygonum cuspidatum" used in skin infections, fungal infections, heart, liver, and vascular diseases. (16)

Three different antioxidant mechanisms explain the natural antioxidant role of resveratrol. One strategy is to compete with coenzyme Q and diminish the oxidative chain complex at the location of ROS production. The other is to trap the superoxide radical produced in the mitochondria, and the final is to limit lipid peroxidation caused by the Fenton reaction products. (17)

Yazir et al. found that chronic stress raised circulating corticosterone, inflammatory markers in both serum and corpus cavernosum, decreased eNOS and nNOS expression in the corpus cavernosum of rats, and decreased serum testosterone levels. It has been demonstrated that resveratrol therapy probe improves depression-like behaviours, decreases inflammatory markers that increase due to chronic stress, enhances eNOS and nNOS expression released by the corpus cavernosum, and raises serum testosterone levels. (18) Resveratrol has been demonstrated to prevent ED caused by persistent stress. Yazir et al. arrived at a similar outcome in a rabbit investigation. (19)

Yu et al. looked into the impact of diabetes on ED in rats. Resveratrol is an activator of the silent information regulator 2 related enzyme 1 (SIRT-1). SIRT-1 was discovered to be secreted from cavernous tissue and reduced in

diabetic rabbits. The injection of resveratrol has been demonstrated to boost SIRT-1 release and control erectile function. (20)

Murat et al. discovered that resveratrol had both protective and therapeutic effects on endothelium-dependent relaxations in hypercholesterolemic rabbit corpus cavernosum. (21)

2.4.2. Ellagic Acid

Ellagic acid (EA) is a gallic acid dimeric derivative. It can be found in various fruits and vegetables, such as walnuts, peanuts, pomegranates, and blueberries. It has antioxidant, anti-diabetic, anti-inflammatory, anti-carcinogenic, and antimutagenic effects. (22)

Goswami et al. discovered that using ellagic acid in conjunction with sildenafil improved sexual function in diabetic male rats compared to normal rats. (23)

2.4.3. Vitamin E (α-tocopherol)

Vitamin E refers to all eight distinct vitamin types with comparable structures. These are trimethyl, dimethyl, and monomethyl tocopherols, as well as the equivalent tocotrienols. Plants can only make it. Tocopherol protects the fatty acids in the cell membrane as an antioxidant. Tocopherols can affect membrane characteristics, receptors placed in specific membrane areas, and signal pathways by shielding oxidizable lipids. (24) In 2006, Kondoh et al. presented a preliminary investigation on the effect of vitamin E on the therapy of erectile dysfunction. A patient with ED who did not respond effectively to PDE-5 was given 300 mg of alpha-tocopherol. The IIEF5 was used to assess the patients. These clinical findings are the first to demonstrate the impact of vitamin E on the efficiency of a PDE-5 inhibitor. (25) In another investigation, Mai and Amira investigated ageing-related vitamin E on ageing-related ED in rats. They concluded that further research into the effect of low-dose sildenafil and vitamin E combinations on age-related ED is warranted. In these investigations, vitamin E was observed to enhance the therapeutic impact of the PDE5 inhibitor. (26,27)

2.4.4. Folic Acid

Folic acid is a water-soluble vitamin of the B group. It participates in one-carbon metabolism in the body, providing the single-carbon unit required for purine and thymidylate production, as well as the methylation of key biological components such as phospholipids, proteins, DNA, and neurotransmitters. (28)

In 2021, Zhang et al. released a meta-analysis on the link between serum folic acid and ED. This study, which looked at nine trials, concluded that folic acid is an independent risk factor for ED and that folic acid supplementation will help with ED treatment. (29)

Male sexual dysfunction is a common health issue in society that has psychosocial consequences if left untreated. Exogenous antioxidants have been studied for their influence on male sexual dysfunction. However, the majority of the studies have been conducted on animals, and more detailed and extensive research on this subject is required.

3. Female Sexual Dysfunction And Exogen Antioxidant Use

The health of women largely depends on female sexual function, which is influenced by a variety of psychosexual, environmental, and biological factors. (30) Female sexual dysfunctions (FSD) are classified into four types in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V), the standard reference for psychiatric and behavioral disorders.

1. Orgasmic disorders,
2. Sexual interest or arousal disorders (FSIAD),
3. Genito-pelvic pain or penetration pain disorder
4. Problems related to a substance or drug use.

The disease must have been identified if the symptoms caused severe distress for at least six months with no other plausible explanation. (31) Despite the possibility of co-occurring, these disorders are thought to be distinct and diagnosable.

3.1. Epidemiology

Several studies have estimated the prevalence of FSD to be anywhere between 25.8 and 91.0%. (31) 3.64% of women reported having problems with desire, 31% with arousal, 35% with orgasm, and 26% with sexual pain, according to Hayes and colleagues. (32) A study found that 5.8% of the sampled population in the United Kingdom reported symptoms that met the diagnostic standards for FSD, and that 15.5% of women had complaints related to FSD at some point in their lives. The most prevalent sexual complaint, in line with other epidemiological studies, was a lack of sexual desire. (33) 45% of Finnish women between the ages of 18 and 74 who participated in the study said they

had lessened sexual desire. (34) For those under the age of 25, this rate is 20%; however, for those between the ages of 55 and 74, it can range from 70% to 80%. The findings of studies conducted in Sweden and Australia are comparable. Most FSDs seem to increase in incidence with age. The most common problem is a lack of sexual desire, followed by a lapse in orgasmic pleasure (11% of the Australian sample) and an absence of orgasmic gratification (10%). (35,36) The prevalence of sexual complaints among women of all ages is between 40% and 50%, according to a 2015 consensus statement from the fourth ICSM that examined different assessment methods. (37)

3.2. Pathophysiology

The motivation and reward systems, which include the hypothalamus, ventral striatum, amygdala, insula, and orbitofrontal cortex, are activated by sexual desire. (38) The ventromedial and amygdalar regions activation decreases during the arousal stage of the sexual response. The vulva, clitoris, and vagina physically swell as a result of increases in sympathetic activity, which also results in an increase in vascular blood flow to the genitals, a rise in temperature and secretions, relaxation of the pelvic floor, and an increase in pudendal and genitofemoral nerve conduction. (39)

Reduced sexual desire may be the result of certain psychosocial factors, such as physical or emotional abuse, severe life stresses, or other psychological dynamics that affect desire. FSIAD should not be blamed for such situational causes of decline or difficulty in sexual function. Interventions in psychotherapy for the determined cause may be helpful in this case. Sexual dysfunction brought on by medical, psychiatric, or drug- or substance-related side effects is also incompatible with the FSIAD diagnosis and needs to be assessed separately.

3.3. Treatment And Use Of Exogenous Antioxidants

Hormonal therapy and non-hormonal therapy are the two main categories of current treatments for sexual interest or arousal disorders. (31)

Hormonal treatment of FSIAD includes:

- Hormone replacement with systemic or vaginal estrogen
- Androgen supplement
- Tibolone, a selective estrogen receptor modulator (SERM)
- Ospemifene (SERM)

Non-hormonal treatment of FSIAD includes:

- Flibanserin
- Sildenafil
- Herbal treatments such as Ginkgo biloba extract (GBE) or ArginMax
- Eros-clitoral device
- Psychotherapy
- Bupropion

Some researchers have even suggested trying dietary supplements as a treatment for sexual dysfunction. Short-term or chronic usage of 300 mg/day GBE 11 is not supported by experimental data from a randomized, placebo-controlled, active comparative research. A total of 108 women between the ages of 22 and 73 with low sexual desire participated in a randomized, controlled clinical experiment where they were given ArginMax (a combination of ginseng, ginkgo, damiana, L-arginine, multivitamins, and minerals). (40,41) In comparison to the placebo group, roughly 72% of supplement-treated women after 4 weeks reported a significant rise in sexual desire (68%) and overall sexual satisfaction (72%). In addition, there was an increase in sexual desire, a decrease in vaginal dryness, an increase in sexual encounters, and an increase in sexual satisfaction.

Another study found that the exogenous antioxidants ginseng and vitamin E are beneficial for FSD. With the exception of desire and satisfaction, all aspects of sexual function improved when ginseng and vitamin E supplements were taken, but these effects were not statistically different from placebo. Supplement users demonstrated a significant improvement in these final two domains. (42)

FSD is a common health problem that can cause serious psychosocial problems. It is an area that has remained in the background compared to male sexual dysfunction and has not been adequately studied. It is an inevitable fact that more studies are needed in the field of FSD treatment in general. The same need applies to the treatment of FSD with exogenous antioxidant supplements.

4. The Use of Antioxidants in Female Infertility

Infertility is defined as the inability to achieve pregnancy despite unprotected, regular sexual intercourse for 12 months period. (43) While this period is considered to be 12 months for healthy, young couples, the American Society

for Reproductive Medicine *(ASRM)* recommends that this should be considered 6 months for women older than 35. (44) The prevalence of infertility varies between 7-28% depending on the age of the women. (45)

Since the most important factor affecting ovarian reserve and oocyte quality is the age of the woman, the prevalence of infertility increases with age. Infertility can cause mental, physical and psychological disorders in women. Therefore, it constitutes one of the important reasons for applications to the gynecology outpatient clinic. Studies have shown that only 85% of couples are successful in achieving pregnancy within the first year, while 15% are unsuccessful, and it is recommended that these couples should be evaluated further in terms of infertility. (45)

Ovulatory disorders *(25%)*, endometriosis *(15%)*, pelvic adhesions *(12%),* tubal/uterine anomalies *(11%),* and hyperprolactinemia *(7%)* are the major etiological diseases of female infertility. Disruptions in any of the stages of ovulation, fertilization, embryo development, embryo transfer and implantation cause infertility in women. (46) Today, a wide range of both medical and surgical treatments are used to prevent infertility. In this review, the role of antioxidant therapies in female infertility have been elaborated.

The rate of infertility has been increasing in both men and women over the past years. Various environmental toxins cause sub-fertility, spontaneous abortion, low birth weight, folliculogenesis and oocyte damage in women. (47)

One of the important reasons for the increase in infertility rates in both men and women is determined as the negative effects of oxidative stress on fertility. Oxidative stress is the disruption of the balance between free oxygen radicals and protective antioxidants in the body in favor of free oxygen radicals. (48)

Since free radicals are unstable and highly reactive molecules, they become stable by attracting electrons from nucleic acids, lipids, proteins and carbohydrates, and cause serious cellular damage. (49,50) Free radicals are divided into two as free oxygen radicals and free nitrogen radicals. Superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl ion (OH^-) (51) are classified as free oxygen radicals, while examples of free nitrogen radicals are nitric oxide (NO^-). (52)

Oxidative stress causes infertility in women by directly affecting oocyte, embryo and implantation through various mechanisms such as cell membrane lipid peroxidation and cellular protein oxidation. (53) In addition, reactive oxygen radicals damages oocyte DNA, which causes apoptosis of oocytes. (54)

The increased free oxygen radicals in the follicular fluid may affect fertility by inhibiting the antioxidant mechanism in the follicular fluid and disrupting the supporting role of the endometrium, which provides the development and implantation of the embryo. (54)

One of the most important issues to be emphasized in this stiuation is embryo development and endometrial receptivity for a successful implantation which is n negatively affected via oxidative stress. (13) However, in previous literature it was reported that oxidative stress was associated with infertility depend on various conditions such as endometriosis, hydrosalpenx, and polycystic ovary syndrome. (53) Free radicals are involved in many physiologic events like oocyte maturation, steroidogenesis, (56) ovulation, embryo development, implantation, pregnancy, (57) normal labor, (58,59) and menopause while they are also associated with pathological events such as preterm labor, (60,61) PCOS, endometriosis, and infertility. The most important issue to be emphasized is whether the antioxidant capacity is capable of neutralizing free radicals at a sufficient level. In cases where the antioxidant capacity is insufficient, many pathological conditions that cause infertility may occur.

The antioxidant system neutralizes free radicals and protects the reproductive system components and all other tissues from the damaging effects of oxidative stress. Antioxidants are divided into two classes, enzymatic and non-enzymatic, (62) Enzymatic antioxidants are natural antioxidants and they neutralize hydrogen peroxide to water and alcohol and prevent cell structures from being damaged by these radicals. Examples of enzymatic antioxidants can be stated as superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase. (63) Non-enzymatic antioxidants are synthetic and are taken into the human body via diet. Examples of these are vitamin C, vitamin E, zinc, selenium, glutathione, taurine, hypotaurine, beta carotene and carotene. (62,63) Vitamin C is an important antioxidant that plays a key role in the inhibition of the peroxidation and the recycling process of oxidized vitamin E and glutathione.

Taurine and hypotaurine are predominantly found in follicular and tubal fluids and protect the embryo against oxidative stress. (64) Glutathione is found in the oocyte and tubal fluid and plays a major role in the development of the zygote up to the morula and blastocyst stage. (65) Herbal medicines with antioxidants protect germ cells from apoptosis caused by oxidative stress. Therefore, herbal medicines containing antioxidants can be used as complementary alternative treatments to support fertility. (66,67)

Although antioxidant treatments seem beneficial on fertility, attention should be paid to the duration of use and overdose in treatment. It should be kept in mind that antioxidants can cause reductive stress in the body if they are utilized for a long time and in high doses, which can be as dangerous as oxidative stress. (68) It should be kept in mind that long-term and high-dose use of antioxidants cause many diseases such as muscular dystrophy, (69) cardiomyopathy, (70) pulmonary hypertension, (71) Parkinson's, (72) rheumatoid arthritis, (73) insulin resistance and metabolic syndrome, (74) and cancer. (75) The only exception to these can be emphasized as melatonin. It has been reported that melatonin has a very low potential for side effects, even when used for a long time and in high doses. Therefore, more academic studies are needed on the use of antioxidants in female infertility.

Experts from the European Society of Human Reproductive and Embryology and the American Society of Reproductive Medicine (76) state that antioxidants are important in the prevention and treatment of female infertility, but there are many issues that need urgent clarification in this area.

5. Male Infertılıty And The Contrıbutıon Of Exogenous Antıoxıdants To Fertılıty Intraduction

Infertility is a major fertility problem affecting 15% of couples of reproductive age, and half of these cases are due to male-related abnormalities.

The essential element of male fertility is spermatozoa. The ability to fertilize spermatozoa depends on its healthy life cycle. This means that, first, healthy sperm must be produced, then it must be in a healthy environment. Additionally, they must have an excellent working mechanism in order to perform the expected fertilization function. Among these three issues, reactive oxygen species (ROS) play an essential role in proving the healthy physiological balance of fertility. Deterioration of the common ROS mechanism leads to pathological outcomes, and oxidative stress (OS) develops. (77-83)

The presence of OS may lead to a severe deterioration in the ingredient of the fertilization fluid (semen), which protects and transfers the spermatozoa under appropriate conditions. Therefore, OS may lead to male infertility by reducing fertilization capacity. (80-84)

Antioxidants, which play an important role in balancing ROS and preventing OS, are endogenous molecules that are physiologically manufactured and run by the body. When the endogenous antioxidant is insufficient, exogenous antioxidants (XAO) can be used to prevent the pathological process of OS. The

results of the clinical studies reveal that, if OS could be prevented by the use of XAO, fertility rate could be increased in infertile men. (84,86)

The role of XAO in the increased fertility rate is generally accepted in the medical world. Unfortunately, the etiopathogenesis of the effect of XAO has not been fully explained and there are not enough prospective randomized studies. (86-90)

Therefore, the use of XAO as a therapeutic agent in male infertility still has not been determined by any criteria such as indication, dose and duration. In conclusion, XAO has not gained widespread use in treatment protocols currently all over the World. (90-93)

5.1. Physiology Of ROS (Reactive Oxygen Species) Function On Semen

5.1.1. Effect of ROS on Fertility Physiology

While high ROS ratios in semen are harmful, the effects of low and balanced ROS concentrations on sperm physiology are vital. ROS provide morphological remodeling of spermatozoa whith intracellular pathways. This leads to chemotaxis. On the otherhand, excessive amounts of ROS may be harmfull on spermatozoa. (77,94)

Sperm maturation: Mechanisms of cellular signal transduction that regulate sperm maturation in the epididymis are affected by ROS levels. In addition, appropriate ROS levels strengthen the disulfide bonds in the membrane structure that protect DNA and mitochondria from damage. (93-98)

Intercellular communication and sperm hyperactivation: ROS activates multiple enzymes such as adenylate cyclase, cyclic adenosine monophosphate (cAMP), protein kinase A (PKA). PKA mediates the motion of sperm flagella which occurs as sperm-specific hyperactivation. (92-97)

Capacitation (successful sperm penetration into the zona pellucida), Acrosome reaction and penetration: The sperm become fully ready for the acrosome reaction with releasing the exocytotic proteolytic enzymes resulting from phosphorylation supported by ROS, so spermatozoa binding to the zona pellucida properly. Similarly, ROS supports sperm-oocyte fusion by blocking phospholipase A2 deactivation also. (93-97)

5.1.2. Endogenous Antioxidant Mechanisms

ROS in spermatozoa are regulate thr plasma membrane and mitochondria by adenine dinucleotide phosphate (NADPH) oxidase system. (77, 94-97)

5.2. ROS İmbalance And Pathophysiology Of OS (Oxidative Stress)

5.2.1. OS Definition

OS occurs with the breakdown of oxidative balance in semen. This imbalance generally occurs in two ways: Either ROS is high, or antioxidants are lacking.

The primary ROS in spermatozoa is superoxide (O2–). O2– also provides the production of hydrogen peroxide (H2O2. When molecules of OH- levels rise, Lipid peroxidation (LPO) begins. (77,79,91,97)

5.2.2. OS With An Excess Amount Of ROS

In idiopathic male infertility, ROS may be responsible for 30-80% of the disorder. (77-83)

a- Sources of ROS in the ejaculate: The seminal fluid (ejaculate) consists of secretions which are generated by the prostate, seminal vesicle, Cowper's gland, and testicular tissue.

Leukocytes: The rate of ROS may increase as much as a hundredfold when the number of leukocytes increases in the ejaculate as a response to either infection or inflammation. In clinical terms, Leukocytospermia is characterized by the presence in the semen of more than 1 million peroxidase-positive leukocytes which have harmful effects on sperm.

Sertoli cells: Clinical trials have shown that Sertoli cells have the ability to produce ROS. However, physiologic or pathologic levels of Sertoli cells in semen has not yet been determined by authorities for diagnostic criteria.

Immature sperm cells: Excess cytoplasm must be excreted from mature sperm at the end of spermatogenesis. If not, this excessive cytoplasm increases the risk of OS by activating the NADPH system due to the production of excess ROS.

b- OS-related illness necessitating surgery: Testicular hyperthermia and hypoxia induced by varicocele are considered to cause testicular dysfunction through OS. It has been reported that seminal ROS levels are directly related to the degree of varicocele. And the varicocelectomy improves the fertility rates through balancing seminal ROS levels, namely protecting semen from OS.

Cryptorchidism, like varicocele, may lead to hyperthermia in testicular tissue and cause OS. In addition, disruption of the blood testicular barrier (BTB) may occur as a result of cryptorchidism surgery. Damage of BTB may be the cause of OS for spermatozoa along with autoimmunity. (96-101)

Biopsy of testicle also may lead to damage of BTB, causing OS

c- Prostatit: Nonbacterial chronic prostatitis, the most common type of prostatitis, leads to chronic inflammation in the prostate gland which promotes both autoimmune events and OS through leukocytes.

d- OS with Systemic Diseases: Many systemic diseases can cause OS by affecting the reproductive organs.

Systemic Infections may increase the number of leukocytes in the seminal fluid. This condition has been observed in infections of many viruses, as well as chronic infective diseases such as Tuberculosis, Malaria and Chagas disease. Autoimmune/inflammatory diseases can also increase seminal leukocytes. (96-104)

Diabetes has many systemic adverse effects on reproductive systems. Many studies have found that DNA fragmentation caused by OS is increased in men with diabetes compared to men without diabetes.

Many systemic diseases, such as Renal Failure, chronic kidney diseases, hemoglobinopathies (and related excessive blood transfusions), and hyperhomocysteinemia have been identified as reasons for the seminal OS. (104-106)

e- OS along with COVID-19 infection: Recent studies have shown that COVID-19 infection may cause deterioration of the process of spermatogenesis by inducing apoptosis.This deterioration is thought to be likely through the pathway of OS. Additionally, findings showed that the risk of infertility increases in men with COVID-19, due to the formation of antisperm antibodies, inflammation of reproductive organs and negative effects of fever.

f- OS from exogenous factors: Radiation (even spread from electronic devices) and radiofrequency can effect levels of ROS in human semen. Well-known environmental toxins (phthalates in plastic objects, metals such as cadmium, chromium, lead, manganese, and mercury), smoking, and alcohol use (due to conversion to acetaldehyde in the body) may increase ROS production. Thus, these factors can adversely affect fertility at all stages.

Negative lifestyle choices such as poor diet, lack of exercise (sedentary life), insomnia, long working hours, hectic lifestyle and increased mental and physical stress can affect sperm quality negatively by increasing likelihood of seminal OS. This impact can be observed directly or indirectly through systemic chronic diseases. (106-112)

g- OS status in assisted reproductive techniques (ART): Exogenous factors may act as negative effect on semen in the course of ART. These factors include exposure to visible light, the unhealthy composition of the culture medium,

(related to pH, temperature, oxygen concentration, etc.), the effect of centrifuge during spermatozoa preparation, type of ART technique including gamete or embryo processing and type of cryopreservation technique (freezing/thawing process). (77,78,83-85,92-96,106-116,107-117,116-123,126-132)

5.3. Pathophysiology

Vital biomolecules such as proteins, are affected harmfully when beginning deterioration of the homeostatic balance of ROS.

Lipid peroxidation (LPO): Spermatozoa are delicate to oxidative injury due to containing high lipid levels in their cell membrane. LPO is composed of a three-step process that runs as an autocatalytic mechanism. In the first stage, hydrogen atoms are released, free radicals occur, and subsequently, lipid radicals and peroxyl radicals are formed. This reaction chain expands if metals are present. In the advanced stage, cytotoxic aldehydes form due to the breakdown of hydroperoxide. In the result stage, as a stable final product, malondialdehyde (MDA) is formed and the coaction chain is terminated (Because it is the final harvest, MDA is used to detect as a key biochemical marker in order to analyze and monitor the level of peroxidative damage affecting spermatozoa). (95-98)

DNA damage: ROS increases sperm nuclear DNA fragmentation disrupting chromatin cross-linking which causes chromosomal microdeletions. Additionally, ROS make mutations in mitochondrial DNA (mtDNA) via LPO. As a result sperm motility reduces due to inhibited energy production. (88-91)

Apoptosis: ROS can activates apoptotic caspases. (77,85,86,93,96,108-118)

5.4. Searching For OS Findings

5.4.1. Routine Sperm Analysis

The presence of asthenozoospermia is an excellent diagnostic tool for OS since it is an important indicator of infertility.

Seminal hyperviscosity, an indicator of an increase in the amount of seminal plasma malondialdehyde (MDA), show the presence of the OS as well as a decrease in seminal plasma antioxidant status. It may also show the presence of Ureaplasma urealyticum infection. (112-117)

Leukocytospermia, another predicting factor of OS. It is easy to confuse immature spermatozoa with round cells in semen. Thus, it is necessary to make sure that the round cells are not immature spermatozoa through special tests.

Teratospermia and cytoplasmic droplets are indicators of a possible trigger of uncontrolled ROS production. (118)

Hypoosmotic swelling test (HOST) is predict the presence of OS. (118-123)

5.4.2. Other Tests

The amount of extracellular and intracellular ROS is measured by chemiluminescence.

Luminol usable to measure of levels of OS.

LPO markers are detected by using thiobarbituric acid (TBA).

The seminal oxidation-reduction potential (ORP) provides a comprehensive OS measurement. Galvanostatic electron measurement has recently been developed for ORP. (124-130)

Seminal MDA and Nitric oxide (NO) were found to be related to sperm DNA fragmentation and Acrosomal anomaly. (124, 131-135)

5.5. OS Prevention / Exogenous Antioxidants (XAO)

5.5.1. Reduction Of ROS Production / Lifestyle Changes

Bad habits (such as smoking), occupational and personal stress, toxins exposure (pollution, heavy metals, etc.), presence of high temperature of testicles (saunas, presence of varicocele etc.), and chronic diseases should be taken in the patient's story meticulously because they can reason for OS. It is important to identify and eliminate such risks for treating male infertility. (77,96)

5.5.2. In vivo Antioxidants

Antioxidants reduce the formation of ROS or eliminate it to stop the oxidative chain reaction

a- Presence of endogenous antioxidants: Antioksidants, that present in the plasma and spermatozoa, can be classified as preventative and purificatory. Preventative antioxidants (such as transferrin) prevent the formation of ROS either by chelation of metals or by binding of some molecules. Purificatory antioxidants (such as vitamin E) eliminate ROS that are already produced.

There are different antioksidants classification as enzymatic and non-enzymatic. Naturals ones are enzims, others are vitamins (C, E and B vitamins), amino acids (carnitine, cysteine, taurine, hypotaurine), carotenoids, pentoxifylline, metals and albumin. (77,80,98,115)

b- XAO supplementation: Reason for high level of ROS is unclear in the seminal fluid. Either production of ROS is increas or clearance capacity of ROS decrease (or both). If high semen ROS levels are antioxidant supplements will be very beneficial.

Antioxidants either inhibit the formation of ROS by acting as pro-oxidant which block pro-oxidative chain reactions, or purify the ROS. As a result, they can neutralize the cell and its micro-environment and protect it from oxidative damage.

Results of varies clinical studies shows a significant reduction in OS or DNA damage and improvement in the rate of asthenospermia after treatment with antioxidants. (77,80,87,96,101-107,101-104)

5.5.3. Tips For Antioxidant Therapy

There are not enough studies that evaluate the risk of treatment of large amounts of Antioxidants. However, therapy of infertility by antioxidants has recently become popular because these therapeutic agents do not have serious side effects.

Ideally, Antioxidants;

1- should both reach high amounts in the seminal fluid and compensate for the inadequacy of vital elements
2- should both increase the cleaning skill and decrease the ROS levels in semen.
3- should not suppress the ROS levels in semen totally since low ROS levels may harmfull sperm functions.(78-107)

a- Effect of XAO on sperm dysfunction: Published randomized-placebo-controlled studies on dietary antioxidants show that antioxidants have beneficial effects on sperm function for fertility mostly. While researchers have not sufficiently attended to the identification of subgroups who benefited the most from antioxidant therapy. (78)

b- Effect of XAO on sperm DNA damage: Sperm DNA damage, a cause of infertility, is found smaller number than lack of spermatozoa concentration and motility, among causes of infertility, Clinical studies show any improvement of fertility, but there are not enough studies to make a definitive decision.

c- Substances used as XAO: The most studied XAO are vitamins, minerals, L-carnitine, coenzyme-Q10 (CoQ10), melatonin, and N-acetyl cysteine.

Although the results of studies on these substances are encouraging, the statistical superiority of the controlled group over the placebo group is not clear. In addition, these studies have not clearly measured seminal oxidative stress and seminal vitamin levels and the level of oxidative DNA damage was not used as a monitoring criterion for response to antioxidant therapy. (78,105,108)

d- Other XAO who underwent clinical research:

Letrozole, Nerve growth factor (NGF): Functions of sperm were significantly increased after letrozole treatment.

Ubiquinol: Ubiquinol is the reduced form of CoQ10 and acts as a powerful antioxidant in the body. Improves sperm flagellum morphology.

Nitric oxide (NO): OS stabilizing role has been determined on NO.

Herbal extracts: Many extracts are used in clinical trials recently. For example, epigallocatechin-3-gallate (EGCG) and Camellia sinensis L. has yielded improvement in sperm parameters. Although there have been increasing studies in this field in recent years, the impact of EGCG is not widely accepted. It is rarely used in routine clinical treatment partly because there is no product that has acquired and turned into medicine. (89-93)

5.5.4. Assisted Reproductive Techniques (Arts) And In Vitro Antioxidant Use

Antioxidants are used in two ways for assisted reproductive techniques (ARTs). First, they can be prescribed to the subfertile couple as an oral supplement a few months before the ART cycle. Second, antioxidants can be used as an in vitro supplement during ART to minimize sources of ROS. (130-135)

a- Protect the semen sample: Due to the delicaty of human spermatozoa, the use of antioxidants in vitro is very important in ARTs. There are multiple studies on the protective action of antioxidants in spermatozoa from harmful effects of ROS throughout both semen preparation and oosit incubation. This protection is more important for sperm samples prepared from infertile men as they are more susceptible to oxidative damage during semen processing than samples from fertile men.

b- Cell damage: Studies have revealed that hydrogen peroxide is the most toxic ROS for spermatozoa. Thus, semen must be protected from the negative effect of hydrogen peroxide. Antioxidants prevent motility of spermatozoa from harmful effects of exogenous ROS, However, the impact of superoxide dismutase is limited in prevention. (136-140)

c- DNA Damage: If there is any DNA damage on spermatozoa, ART are less victorious. XAO can protect the nature of DNA from the negative effects

of exogenous ROS. Thus, the use of XAO can be crucial for the success of ART.

d- Gentle semen handling protocols: Although XAO effectively protects sperm from exogenous ROS, it has a limited effect to endogenous ROS which can be produced in sperm. Fortunately, a small but significant effect of albumin has been found to be protective against endogenous ROS. The prevention of ROS formation becomes much more important than the inhibition of ROS.

e- Cryopreservation: The role of antioxidants in protecting sperm during cryopreservation and thawing has been investigated. Studies focused on sperm protection from OS-related motility decrease and DNA damage following cryopreservation and thawing. In these studies, the antioxidants Pentoxifylline, vitamin E, vitamin C, rebamipide catalase, resveratrol, and genistein were examined. , and different results were obtained.(140-144)

Overall, results show that antioxidants are usually forcefull in saving sperm from the OS-resulting harmfully effects. However, the mode of cryopreservation is also important in protection from OS. (78,107,115,119-124)

The negative influence of oxidative stress on male infertility has been well understood by numerous studies in recent years. Additionally, treatment with exogenous antioxidants replacement therapies has been increased to reduce the negative effect of OS. Although practices have shown that fertility rates can be increased with the use of antioxidants, they are still insufficient to support strong evidence for the routine use of antioxidants. The variety of antioxidants used for treatment has been increasingly day by day. Furthermore clinical results are encouraging for further studies on this the impact of antioxidants. Based on our review of the literature, we argue that, in order for antioxidants to enter into routine clinical use, more comprehensive clinical studies are needed.

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CHAPTER III

EFFECTS OF ANTIOXIDANT COMPOUNDS ON FERTILITY IN SMALL RUMINANTS

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1. Introduction

For continued profitability and productivity in lamb breeding, maintaining optimal reproduction is the most important factor. ROS is one of the most influential factors influencing productivity. Productivity is diminished by oxidative stress caused by an increase in reactive oxygen species (ROS). In in vivo applications, hormonal injections, and changes in nutritional strategy (vitamin-mineral supplements, etc.) are used for this purpose. In in vitro applications, the medium is supplemented with exogenous antioxidants to increase productivity.

2. Antioxidants

There is a balance between ROS and antioxidants in a healthy organism. When this equilibrium is disturbed, oxidative stress develops. Oxidative stress occurs when oxidant substances, particularly ROS, outnumber antioxidants.

Stress can impair numerous functions and result in low fertility or infertility (1). ROS's principal components are hydroxyl (OH), hydrogen peroxide (H2O2), and superoxide (O2-) (2). As prooxidant molecules, ROS and reactive nitrogen species (RNS) are involved in aerobic metabolism. NO is the most fundamental component of RNS. Free radicals are like a sword with two edges. Free radicals are required for sperm activity (capacitation, acrosome reaction, and hyperactivation), fertilization, and embryo development at physiological levels. However, sperm and oocyte damage, deformities and abortions, intrauterine growth retardation, and infertility may result from concentrations above physiological levels. Under physiological conditions, the body's natural antioxidants are sufficient to neutralize free radicals. Under pathophysiologic conditions, endogenous (natural) antioxidants cannot prevent an excessive increase in free radicals. Therefore, external antioxidant intake is required (3).

Multiple antioxidant enzymes play a crucial role in follicle and oocyte maturation, implantation, embryogenesis, and embryonic development by regulating ROS (4, 5). A uterus that does not support pregnancy may result from irregular ovarian steroid secretion and/or insufficient release of antioxidant enzyme activities (6). Antioxidant enzymes regulate endometrial function in rodents, guinea pigs, humans, and lambs, ensuring successful conception and pregnancy (7).

Enzymatic antioxidants are distinguished from non-enzymatic antioxidants, (1, 2). Natural antioxidants refer to enzymatic antioxidants. Superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase are natural antioxidants. Synthetic antioxidants are another name for non-enzymatic antioxidants. These dietary antioxidants include vitamins (C and E etc.), selenium, zinc, beta-carotene, and taurine, among others (1). Antioxidants from nature neutralize excess ROS and prevent them from causing cellular damage. These antioxidants convert hydrogen peroxide to alcohol and water (2). Superoxide dismutase, a natural antioxidant, plays a role in cellular protection. Two molecules of superoxide are metabolized into hydrogen peroxide and molecular oxygen by this enzyme (O2). The catalase enzyme transforms hydrogen peroxide into water and oxygen (8).

According to the Data Access Committee of the International Embryo Transfer Association (IETS), millions of animals, including cattle, horses, ovines and pigs, have been born in the last decade thanks to assisted reproductive technologies. In addition, the collection of ovine oocytes with desirable genetic traits from commercially valuable slaughtered donors, combined with the use

of assisted reproductive technologies, has facilitated genetic advancement, and benefited future ovine breeding (9, 10).

The application of assisted reproductive technologies can result in the cessation or reduction of intracellular ROS production, resulting in oxidative stress. This effect reduces the developmental vigor of mammalian oocytes and causes mitochondrial dysfunction. These deficiencies in mammalian resulting in poor pregnancy outcomes, recurrent pregnancy losses, embryonic deaths, and reproductive disorders including low birth weight (11).

Given the associations between oxidative stress and reproductive disorders following embryo transfer, α-tocopherol (12), glutathione (GSH) (13), melatonin (14), N-Acetyl-Cysteine (15), vitamin C (16). In vivo, alpha-ketoglutarate significantly reduced the effects of growth suppression, intestinal mucosal damage, fertility decline, abnormal folliculogenesis and oxidative stress (17).

It has been reported that an increase in ROS may harm and kill the fetus by reducing ATP synthesis (18). The placenta is a tissue that transports nutrients and oxygen and produces numerous peptides and hormones that contribute to the circulation of mother and child. As a result of increased oxidative stress in ewes subjected to nutrient restriction, the antioxidant/prooxidant balance is disrupted, and growth and development in placentomes are altered. Similarly, it has been reported to affect fetal weight, resulting in infants with lower birth weights. Low birthweights also influence fertility success (19).

In ewes consuming grain crop residues and dry pastures, serum total antioxidant capacity decreases over time (20). Before estrus, the increase in metabolic rate and steroidogenic activity causes a rise in ROS (2). According to Kuru et al. (21) and Sonmez et al. (22), using intravaginal sponges for synchronization purposes in small ruminants increases oxidative stress. In addition, increased ROS production during mating has been shown to disrupt the ovulation mechanism, decrease oocyte and embryo quality, and decrease luteal progesterone production (4, 23). Due to these factors, using antioxidants is particularly vital in ewes undergoing estrus and synchronization (20). Embryo implantation also produces a rise in the production of free oxygen radicals. This expansion is required for implantation. However, excessive ROS production induces blastocyst apoptosis and impairs fertility (24).

Therefore, using a suitable antioxidant before and during pregnancy is essential for optimal placental development and function in ewes to reduce embryo mortality, improve lambing rate, and increase newborn viability (18). Generally, ROS are generated by the oocyte, embryo, and environment during

embryonic development (3). In the study conducted by Shkolnik et al. (25), it was determined that LH temporarily increased ROS production prior to ovulation. It was determined that the use of antioxidants during this period had a negative impact on ovulation and that ROS is required for ovulation. Similarly, RNS (Nitric Oxide) has been reported to play a significant role in oocyte meiotic maturation and oocyte cumulus enlargement in lambs (26).

The thioredoxin (thioredoxin peroxidase, thioredoxin, and thioredoxin reductase) system is another antioxidant (8). Glutathione peroxidase and thioredoxin reductase, which are selenoproteins, are the most abundant selenoenzymes that protect cells from damage. Selenoproteins are one of the biological forms of selenium. Moreover, thioredoxin reductase can recycle ascorbic acid, allowing-tocopherol to be converted to its active reduced form (27). Thus, selenium contributes to scavenging excess ROS and enhancing antioxidant status (8). Total antioxidant capacity and post-copulatory progesterone increased in ewes given selenium prior to, during, and after synchronization (20).

The powerful antioxidant melatonin is an amine hormone. The pineal gland primarily produces it. Additionally, it has been reported that it is secreted by tissues other than the pineal gland. An example of one of these tissues is the ovary. It has been reported that this ability diminishes as the diameter of the follicle increases, where it is synthesized by the cumulus-oocyte complex (28). Melatonin can eliminate ROS and RNS. In contrast to other antioxidants, melatonin metabolites have been reported to be capable of scavenging ROS and RNS. This indicates that melatonin can sequentially protect against oxidative stress (29). This sequential reaction may be effective even at very low doses in protecting against oxidative stress. Melatonin levels decrease when the circulation is subjected to intense stress. Since melatonin, which interacts with elevated ROS and RNS, is rapidly metabolized, its circulating concentration decreases while its synthesis concentration remains unchanged (30). It has been reported that melatonin exerts a beneficial effect on cumulus cells by regulating methylation events. It is explained that the cumulus cells play a crucial role in the maturation of the oocyte (including retention during meiosis, resumption of meiosis, and support for cytoplasmic maturation) (31).

Due to insufficient glutathione synthesis, physical damage and oxidative stress may occur in the cumulus cells of lambs prior to puberty. Upon administration of melatonin to prepubescent lambs, it was determined that melatonin has a protective effect on the cells and improves their quality via methylation events at a specific nuclear binding site (ROR) (32).

It was reported that a single injection of 10 mg melatonin was effective in inducing estrus and lambing rate in seasonal anestrus Singharey goats. The progesterone levels of goats injected with 10 or 20 mg of melatonin increased. In these groups, high rates of multiple births were observed. With melatonin administration, serum melatonin levels increased, total antioxidant capacity improved, and malondialdehyde levels decreased (33). Before the breeding season, melatonin implantation in lactating Tahirova ewes reduced the duration of estrus, pregnancy, and lambing. Tolü et al. (34) found that melatonin implantation in lactating Turkish Saanen goats significantly reduced the rejection rate. Melatonin administration is an effective method for advancing the breeding season and boosting fertility in Sarda lambs (35).

Melatonin affects the production of sex steroids by stimulating the expression of genes specific to estrogen production (E2) (36). Spicer et al. (37) reported the inducing effect of tri-iodothyronine (T3) on E2 production. Melatonin and Insulin-like Growth Factor-1 (IGF-1) (38, 39) hormones decrease T3 production, suggesting that these two hormones inhibit E2 synthesis. Melatonin and IGF-1 promote progesterone (P4) release from luteal tissue (40, 41). Therefore, the effects of melatonin and IGF-1 on decreased E2 and increased P4 levels result in a lower E2:P4 ratio, resulting in better maternal recognition of the pregnancy response in sheep (42).

Various chemical and growth factor combinations are frequently used to modify the in vitro maturation environment. The Insulin-Transferrin-Selenium (ITS) combination is one example. Insulin is a hormone that provides glucose and amino acid absorption and has mitogenic properties (37). Transferrin is abundant in the follicular fluid and plays a crucial role in the proliferation of granulosa cells and also reduces the production of free radicals to some extent (43). Selenium is an essential trace element that prevents oxidative damage in cells. The combination of ITS has been recommended in addition to many cell and oocyte culture systems (28). Adding ITS to IVM media can enhance oocyte maturation (44) and boost the yield of high-quality blastocysts (16). Other cytokines or growth factors may also have positive effects on oocyte development. Fibroblast Growth Factor 2 (FGF2), a member of the fibroblast growth factor (FGF) family, has been shown to inhibit granulosa cell apoptosis, accelerate follicular development, support oocyte maturation, and increase embryo development capacity (45, 46). IGF-1 contributes to folliculogenesis, increases follicular diameter, and encourages oocyte maturation (47). Leukemia Inhibitory Factor (LIF) is a member of the interleukin-6 family of cytokines. It

has been reported that LIF can improve embryo development in mice, lambs, goats, cattle, pigs and humans (48). It has been determined that supplementing IVM media with sericin and/or LIF can significantly enhance the developmental competency of lamb oocytes by increasing blastocyst yield by about twofold compared to conventional IVM media (49).

Another antioxidant is Vitamin C, ascorbic acid (AA). It plays a role in protecting cells against oxidative stress. It is reported that it may contribute to the development of embryos, especially cloning and somatic cell nucleus transfer embryos before implantation (50). AA, which has many physiological roles, is the first firewall of mammalian cells against oxidative stress (51). In a study on in vitro maturation of lamb oocytes and embryonal development of mature oocytes, it was observed that the addition of L-Ergothioneine and L-Ascorbic acid to IVM, in vitro fertilization and in vitro culture media had beneficial effects on in vitro maturation of oocytes and embryonal development, especially at the cleavage-to-morula stages (52).

In in vitro fertilization applications, protocatechuic acid (PCA, 3,4 Dihydrobenzoic acid) is one of the antioxidants added to the medium in which secondary oocytes are cultured. The phenolic compound protocatechuic acid is found in fruits, vegetables, cereals, and medicinal plants. In vitro culture of secondary oocytes is a proven method for producing oocytes capable of completing meiosis. However, oxidative stress is a significant issue. This stress disturbs oxidative homeostasis by increasing free oxygen radicals, thereby causing damage to cell membranes and DNA, enzyme inactivation, and cell death. According to reports, adding PCA to the culture medium as an antioxidant for secondary oocytes promotes follicular survival, DNA integrity, and meiotic development competence in cultured oocytes; PCA is a potent antioxidant (53).

3. Vitamins and Minerals

Ruminants are frequently exposed to severe deficiencies of trace elements such as copper, cobalt, selenium, iodine, manganese and zinc. Fertility in cattle is associated with enzymatic dysfunctions resulting from these deficiencies. The deficiency of a single mineral may include reproductive failure (54). There is a reciprocal relationship between trace elements and estrogen and progesterone levels in lamb and goat serum. Lamb and goats with inactive ovaries have low serum Se and Zn levels (55).

Copper deficiency in the diet or a general dysfunction resulting from copper deficiency affects reproductive functions directly. Copper deficiency has been associated with several clinical symptoms (54).

A semi-purified diet low in copper content decreased conception rates in goats. 50% of pregnant goats with copper deficiency abort their young. The death of the fetus occurs between the second and fifth months of pregnancy. Aborted fetuses formed mummified fetuses, and placental degeneration with hemorrhagic or necrotic lesions was observed (56). Cell proliferation and high metabolic rate lead to the formation of ROS in the embryo and fetus (2).

Vitamin A plays an important role in reproduction (57). Vitamins A and E play a synergistic role in reducing the effect of ROS. By transporting these vitamins to the fetus through the placenta, the fetus is protected from the destructive effects of lipid peroxidation. Reduction of ROS activity and inhibition of lipid peroxidation by vitamins A and E prevent oxidative deterioration by protecting mitochondrial integrity in the placenta (58). In a study conducted in Tuj ewes, it was observed that vitamin A and E applications, in addition to passive immunization with testosterone antibodies, increased pregnancy and lambing rates. In this study, it was determined that progesterone concentrations during pregnancy were higher than in the untreated groups. Vitamin A and E injections significantly decreased plasma MDA levels. In this study, the antioxidant effects of vitamins A and E, together with testosterone antibody injection, decreased free radical levels and increased pregnancy rates and the number of pups born. The combination of vitamin E and testosterone antibodies resulted in an increase in oxidative stress and a decrease in GSH levels during mating and pregnancy (59).

It was reported that oral administration of vitamin A would be an essential supplement for increasing reproductive performance in subtropical Rahmani lamb. Additionally, oral administration of vitamin A and vitamin C increased progesterone concentration. It was observed that giving vitamins A and C to ewes during early pregnancy and at the time of mating increased the birth weight of lambs (60).

A month before calving, supplementing pregnant goats with 50 IU of vitamin A twice a week improves their health by boosting their immunity and reducing the stress caused by calving. It also enhances the offspring's growth performance and vitality by increasing birth weight, live weight gain, and decreasing hypothermia and mortality (61).

Beta-carotene is a precursor of vitamin A and affects fertility. Beta-carotene is reported to affect follicle size and follicle quality (62). Due to beta-carotene deficiency, delayed ovulation, decreased fertilization rate, small corpus luteum formation, decreased progesterone concentration, and increased embryonic and fetal mortality have been observed (63, 64). A positive correlation was

reported between beta-carotene concentration and corpus luteum diameter, and progesterone concentration (65). Beta-carotene was found to protect the steroidogenic cells of the ovary and uterus against oxidative damage (66). It was determined that beta-carotene and vitamin A injections in Tuj ewes had a preventive effect on the increase of lipid peroxidation levels in the lambs of these ewes. It was also observed that free radicals were suppressed. In ewes whose antioxidant defense system is supported, the transfer of protective substances to lambs provides lamb survival and high growth rates (67).

In a study conducted in Tuj ewes outside the breeding season, multiple pregnancies were obtained with testosterone antibody, beta-carotene and vitamin E injections in addition to synchronization applications, but these rates were not at the desired level (68). In another study conducted in Turkey, it was reported that β-carotene or Vit E+Se injection before estrus had no positive effect on fertility in ewes whose estrus was induced by intravaginal progesterone sponge+PMSG (400 IU)+PGF_{2a} application during anoestrus period (69). Applications of beta-carotene at 20-day intervals increased pregnancy rate, offspring productivity, and twinning rate in ewes undergoing their first insemination (70). It was determined that beta-carotene+Vitamin E applications prior to Ovsynch and Cosynch synchronization programs in cows significantly increased plasma-carotene levels and that this increase led to an increase in pregnancy rates after artificial insemination (71). There is evidence that short-term beta-carotene supplementation in goats increases corpus luteum number, luteal tissue volume, progesterone synthesis, and progesterone secretion from luteal tissue. There have been reports that beta-carotene may be beneficial for embryo implantation, particularly in maternal recognition during pregnancy (57).

Vitamin E is an essential vitamin for mammalian reproduction. Intravaginal sponge administration for estrus synchronization in goats causes an increase in oxidative stress. Excessive ROS production can be prevented by vitamin E administration during the preovulatory period and may increase multiple birth rates and litter size. Vitamin E plays an important role in oocyte maturation and development, and a deficiency in oocyte maturation and development may cause an increase in the number of fetal resorptions in the early embryonic period (22). The relationship between maternal and neonatal plasma levels of vitamin E has shown that this vitamin crosses the placenta (72). It has been reported that vitamin E (4 IU vitamin E kg/ml live weight) treatment of ewes in late pregnancy and early lactation, whose nutritional requirements are not fully met, may be a strategy to improve both colostrum (>lactose) and milk (>fat) quality (73).

The effects of vitamin E and multimineral administration on fertility parameters were investigated during the seasonal anoestrus period and synchronized with progesterone and PMSG protocol. In this study, there was no difference between estrus symptoms. However, estrus signs were more pronounced in ewes given vitamin E and multimineral and attracted more rams. Ewes in this group remained immobile and allowed the rams to jump. Ewes that did not receive vitamin E and multi-mineral showed irregular and weak estrus signs. It was revealed that vitamin E and multi-minerals administered to Awassi ewes 14 days before mating increased the rate of multiple births (74). It was reported that simultaneous Se and vitamin E injections with a synchronization program increased pregnancy and lambing rates in Awassi ewes. Vitamin E and Se injection had no effect on twinning, lamb birth weight and lamb sex (75). Vitamin E and Selenium (Sodium Selenite 0.5 mg/ml Selenium and 50 IU vitamin E as DL-α-tocopherol) injections improved serum total antioxidant capacity in ewes with synchronized estrus. It was determined that these applications increased serum progesterone concentration in the period after mating (20).

Periconceptional folic acid (500 μg/lamb) or flaxseed oil supplementation significantly improved the progesterone profile during pregnancy in pregnant Ossimi ewes during the breeding season (76).

Field experience shows that cobalt deficiency impairs reproductive performance in both cattle and ewes (54).

Manganese is essential for normal fertility in ruminants, and a manganese deficiency prevents conception. Because of the generally low manganese content of maize silage, there is a need for constant vigilance against reproductive impairment due to manganese deficiency in livestock whose diets contain high proportions of maize silage and also in manganese-deficient animals on land (54).

Iodine affects reproductive functions due to its vital role in thyroid function. Therefore, reproductive failure in cases of iodine deficiency is probably a secondary symptom of thyroid dysfunctions resulting in irregular estrus, retensio secundinarum, abortion and stillbirth (54).

Zinc deficiency in ruminants, as in other species, causes a more pronounced fertility impairment in males than in females. This effect is severe and seems to be specific to the final stages of spermatozoa maturation (54).

Although iron deficiency is rarely observed in grazing cattle or small ruminants, it is an essential element in ruminants. Iron is abundant in all forages, and a deficiency in adult ruminants seems unlikely. However, in some cases,

ruminant reproduction may be adversely affected by iron deficiency due to low iron availability in some roughages (54).

The main function of vitamin D is to protect skeletal health by regulating the intestinal absorption of calcium and phosphorus, renal excretion, bone formation and mineral mobilization (77). Some studies have shown that vitamin D deficiency negatively affects reproductive performance in lambs (78) and goats (79). In ewes, 25(OH)D3 and 25(OH)D concentrations are positively associated with the birth weight of single and twin lambs at birth (80). Vitamin D receptors were prominently identified in goat granulosa cells, and vitamin D3 played an important role in regulating the proliferation of granulosa cells during follicular development. Vitamin D receptors increased in parallel with the increase in follicle diameter (Figure 1), (81).

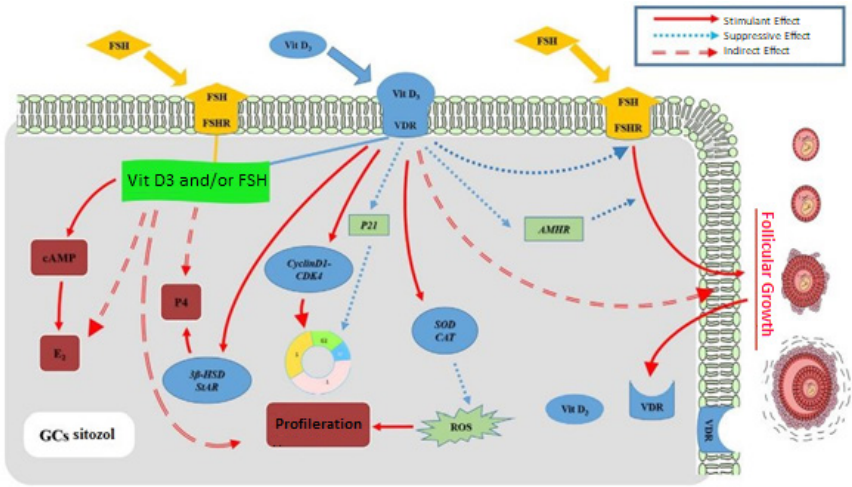

Figure 1: Schematic of the effects of vitamin D_3 and FSH on follicle development (79).

The transport of nutrients and minerals from the mother to the fetus is essential for the establishment and maintenance of pregnancy and the regulation of fetal growth. Calcium-binding proteins, calcium transporters, and vitamin D regulatory pathway components vary on a day-to-day gestational basis in the endometrium and placenta (81). Stenhouse et al. (81) demonstrated that the vitamin D metabolizing enzymes CYP2R1, CYP11A1, CYP24, and CYP27B1 and the vitamin D receptor (VDR) are maternally-conceptual during pregnancy

in lamb. Interestingly, CYP2R1 and CYP27B1 mRNA expression was found to be stable throughout pregnancy. Since CYP2R1 and CYP27B1 are required for the production of active vitamin D, this stable expression suggests an important role for maternal-conceptus vitamin D during pregnancy. High expression of CYP24 mRNA in the endometrium and placenta during early compared to mid and late gestation may be indicative of the high requirements of these tissues to catabolize calciterol, which may not be commonly required during mid and late gestation (82).

L-carnitine is synthesized from the amino acids lysine and methionine. L-carnitine has many functions, such as converting fatty acids into energy, preventing ketosis, transporting ATP from mitochondria to cytosol, increasing the milk ratio, supporting the immune system and thus protecting the body against infections (83). In fat-tailed ewes during the periparturient period, L-carnitine (1 g/50 kg, sc, 7-8 weeks) administration had no effect on serum β-hydroxybutyric acid (BHBA), triglyceride and glucose concentrations, but caused a decrease in serum free fatty acid (NEFA) concentration (84).

Ewes capable of fertilization were found to have high plasma calcium and phosphorus concentrations during the mating period. In contrast, plasma magnesium, sodium and potassium levels were higher in ewes with fertilization (85). In ewes, a significant increase in the amount of Ca in uterine washout was observed on days 10-14 of pregnancy (86). During the peri-implantation period, the Ca level on day 12 of pregnancy was significantly higher than on day 9 of pregnancy. Both calcium and phosphorus are mineral reservoirs for the growing fetus in the amnion during mid and late gestation (82). In some cases, reduced fertility in lamb and cattle is associated with selenium deficiency. Low tocopherol in roughage or the presence in the feed of some vitamin E antagonists may reduce fertility in animals raised or maintained in selenium-deficient areas. Selenium-induced infertility appears to be more pronounced in lambs than in other domestic ruminants (54).

It has been demonstrated that feeding goats selenium derived from sodium selenite and organic selenium in the form of selenium yeast can increase the Se concentration of milk and cheese. The source of selenium had no effect on GPX-1 activity or Se concentration in whole blood and plasma. The efficiency of Se transfer from plasma to milk was greater in goats supplemented with Se yeast than in goats supplemented with selenite, according to these results. The Source of selenium had no effect on milk yield or milk characteristics (87).

4. Conclusion

In light of all the information presented in the literature, it can be concluded that the application of antioxidants, vitamins, and minerals during and outside the breeding season can improve fertility parameters in lambs and goats.

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CHAPTER IV

EFFECT OF ANTIOXIDANTS IN VITRO MATURATION AND *IN VITRO* EMBRYO DEVELOPMENT IN SMALL RUMINANTS

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1. The Role of Antioxidants in the Success of Oocyte Maturation and Embryo Development in Sheep

Sheep, one of the first domesticated animals by human beings, is accepted as a farm animal that provides economic added value in the world with meat, milk and wool efficiency. Due to the increasing population in the world, the demand for products obtained from sheep also have been increased. (1) *In vitro* embryo production technology, which is being used as an important reproductive biotechnology tool today, is one of the important assisted reproductive biotechnologies used in order to increase the number of sheep and the yield characteristics of the offspring and to accelerate the genetic progress. *In vitro* embryo production for sheep, first performed in England in 1986, covers *in vitro* maturation of oocytes, *in vitro* fertilization and *in vitro* embryo culture processes. (2,3,4)

The success of *in vitro* embryo production, cloning, transgenic animal production and other technologies depends on the success of *in vitro* oocyte maturation. Because, *in vitro* oocyte maturation is considered as the most critical step in the production of quality embryos. (5) Therefore, it is very important to

preserve the integrity and quality of the oocyte from the slaughterhouse stage to the laboratory stage and until the end of maturation. Many factors are associated with the quality of the oocyte. One of them is the diameter of the follicle. In fact, it has been shown that a higher rate of meiotic division, fertilization, and embryonic stage access are obtained from the maturation of oocytes obtained from large follicles. (6) The fertilized oocyte is highly affected by the medium content and culture conditions. Therefore, researchers working on *in vitro* embryo production are trying to optimize the conditions of oocyte *in vitro* maturation. (5)

Oocytes must be collected from sheep ovaries before the *in vitro* maturation processes. For this purpose; Immature oocytes are being collected by various methods from small tertiary follicles visible on the surface of the ovary. Obtained oocytes are taken into special solutions and maturation processes are started in the incubator. (2,4) Nuclear and cytoplasmic development of oocytess are adversely affected by some interdependent processes during these processes. One of them is the formation of reactive oxygen species (ROS) originating from oxygen during energy metabolism. Antioxidative defense system elements found in oviduct and follicles fluids in *in vivo* environment protect oocytes and embryos from the negative effects of reactive oxygen species. However, in the *in vitro* environment if the increase in the amount of ROS in the cell exceeds the amount that the cell can tolerate, it causes oxidative stress. Oxidative stress has a critical role in the etiology of detrimetal embryo development, which may cause a break down in cell metabolism and results in increased defective cells [7-10]. This ultimately leads to a decrease in fertilization and pregnancy rates. (4,11) It has been reported that the success rates obtained with *in vitro* embryo production technology in sheep are low. After 70-90% of immature oocytes reach metaphase II, 50-80% of them are fertilized and only 20% to 50% of these reach the blastocyst stage (12). Various antioxidants are used in order to reduce these negative effects created by reactive oxygen species acting on lipid structures in cell membranes. (4,11)

There is a defense system in the cells and tissues in the body that can prevent the formation of free radicals and their oxidative effects. Substances that function in this defense system are called antioxidants. (13,14) Antioxidants according to their functional structures; They are divided into two as “those that prevent the formation of free radicals”, which act by scavenging radicals, and “chain-breaking antioxidants” that end the reactions by forming a balanced product with the radicals. While antioxidants that prevent free radical formation, metal ion binders such as albumin, transferrin, superoxide dismutase, catalase

and glutathione peroxidase are separated within themselves; chain breakers are divided into lipid soluble (alpha tocopherol, ubiquinone, beta carotene) and water soluble (glutathione, cysteine, ascorbate). (14) In addition, there are also substances that act as cofactors for antioxidative enzymes such as selenium and zinc. (15)

Glutathione is one of the non-protein sulfhydryl chemicals, the major free thiol that protects the *in vitro* maturation process from oxidative stress. (16,17) Strong and tight cumulus cells are known to synthesize glutathione (GSH) during the *in vitro* maturation process, and the resulting GSH then accumulates in the ooplasm of oocytes. (18) During the growth and maturation of oocytes in the ovary, glutathione content begins to rise as the oocyte approaches the time of ovulation. (19) After fertilization, GSH, together with oocyte activity, contributes to the process of conversion of sperm and sperm head into the male pronucleus. On the other hand, GSH contributes to many mechanisms such as amino acid transport, protein synthesis, lowering of disulfide level and defense against oxidative damage. Supplementation of antioxidants is required to defend the oocytes/embryo against oxidative damage during *in vitro* maturation, fertilization and embryo development. In various studies, the effect of antioxidant supplementation was investigated and positive data were obtained. (17-20)

In this section, main antioxidants added to culture media and their effects in *in vitro* sheep embryo production will be discussed.

1.1. Vitamin E

Vitamin E is a highly preferred compound among lipid soluble antioxidants, which includes 8 different vitamin forms. (21) One of the chain-breaking antioxidants, α-tocopherol functions especially by protecting the integrity of the lipid structures in cell membranes. (21,22) Since α-tocopherol can act against radicals much faster than poly unsaturated fatty acids, it can provide a very good protection against peroxidation (23). In incubation of sheep oocytes at 20% O_2 level, the addition of 200 μM α-tocopherol to the culture medium has been reported to significantly increase *in vitro* embryo development. IIt also has been reported in the same study that α-tocopherol has no effect on oocyte development at low oxygen levels. (24)

1.2. Vitamin A

Vitamin A has many forms and is a powerful lipid soluble antioxidant. It is responsible for healthy vision, neurological function, healthy skin, formation of strong bones, continuation of gene regulation, facilitating cell differentiation

and supporting immune function. The antioxidant activity of vitamin A occurs in different ways. Peroxyl radicals increase lipid peroxidation of the cell and produce hydrogen peroxides. Vitamin A acts as a chain-breaking antioxidant. (25,26) It is reported that retinol addition to culture media has a positive effect on sheep embryonic development. (27)

1.3. Resveratrol

Resveratrol (3,4',5-trihydroxystilbene) is a subgroup of stilbenes and is a polyphenolic compound found in grapes, wine, peanuts and blueberries. (28,29) Known for its protective effects on the cardiovascular system, resveratrol stands out with its anti-inflammatory, anti-carcinogen and estrogenic effects as well as with its antioxidative properties. (29-31) It has been reported that 0.25 and 0.5 μM doses of resveratrol have positive effects on the expansion of cumulus cells in *in vitro* maturated sheep oocytes and on the blastocyst rate. However, it has been reported that the negative effects of high doses (5 μM) were observed. (32)

1.4. Leptin

Leptin is defined as a protein hormone that consists of 167 amino acids and functions by regulating the energy balance in the body. Leptin regulates lipid synthesis and transport in adipocytes on lipid metabolism. It increases fatty acid uptake and oxidation by inhibiting the activities of regulatory enzymes involved in fatty acid synthesis. In general, it increases the use of oxygen in the tissues and induces the use of lipids as an energy source. Leptin has been reported to have positive effects on reproductive functions and fertility, also has antioxidative effects. (33,34) Positive effects of leptin have been observed in improving sheep oocyte maturation. Also, it has been reported that it has positive effects on the development of sheep secondary follicles cultured *in vitro* and increases the mitochondrial activity of oocytes in the follicles. (35)

1.5. Melatonin

The hormone melatonin is synthesized in the pineal gland depending on the photoperiod and controls the breeding season in sheep. (36,37) Melatonin protects both nuclear DNA and mitochondrial DNA, as well as proteins and lipids. Melatonin provides a wide range of protection, thanks to its unique activity acts as an antioxidant and an indirect antioxidant. Thus, melatonin scavenges reactive species and different forms of free radicals. In addition, by increasing the efficiency of the melatonin electron transport system, it reduces

the generation of free radicals and electron leakage. (36) In order to control the reproduction of sheep, exogenous melatonin applications are performed to improve fertility rate. (37,38) It has been reported that melatonin addition to culture media also improves oocyte and embryo quality. One of the explanations for the positive effects of melatonin in the *in vitro* environment is the reduction in the amount of ROS formed and its antioxidative properties. It has been reported that an increase in growth rates, cleavage rates, and blastocyst rates was observed in groups in which melatonin was applied to oocyte medium. (39)

1.6. L-Ergothioneine

L-ergothioneine is a water-soluble and natural compound and is an antioxidant with radical scavenging and metal chelating properties. (11,40) Also, L-ergothionine inhibits lipid peroxidation by interacting with free hydroxide radicals. Because of these antioxidant properties, L-ergothioneine is also used in freezing gamete cells and in embryo culture media. (11,40,41) It has been reported that L-ergothioneine addition to sheep culture medium increases the development of embryos at the 8-cell embryo and morula stage. (11)

1.7. Green Tea Leaf Extract

Green tea, which is a good source of antioxidants, contains vitamin E, quercetin, kaemferol, myrcetin and rutin. Green tea polyphenols, which are strong antioxidants, reacts with reactive oxygen and nitrogen species and also indirectly show antioxidant activity by triggering the synthesis of intracellular antioxidant enzymes. With these effects, green tea prevents lipid peroxidation and damages that may occur in the DNA structure. The free radical scavenging and iron binding activity of Epigallocatechin gallate in green tea also regulates the work of antioxidant enzymes. (42) It has been reported that the addition of green tea extract to the sheep *in vitro* culture medium contributes significantly to the blastocyst development. (43)

1.8. Vitamin C

Vitamin C, also known as ascorbic acid, is a water-soluble vitamin and is necessary for collagen, carnitine and neurotransmitter biosynthesis. (36,44) Vitamin C acts as a chain-breaking antioxidant in lipid peroxidations and it easily removes reactive oxygen species. Therefore, it provides effective protection against oxidative damage. Vitamin C can act as a co-antioxidant by regenerating vitamin e from α-tocopheroxyl radicals produced by scavenging

lipid-soluble radicals. (36,45) It has been reported that positive effects of vitamin c supplementation on the development of fertilized embryos were observed in *in vitro* culture of clone embryos. (46)

1.9. L-Carnitine

L-carnitine was first isolated from beef in 1905 by Russian scientists, and is a water-soluble semi-essential vitamin-like amino acid. (47-49) L-carnitine takes part in energy production through beta-oxidation by transporting long-chain fatty acids from the inner membrane of the mitochondria. (49,50) L-carnitine has an antioxidative effect by reducing the amount of lipid undergoing peroxidation by transporting lipids into the mitochondria and enabling their use. (48) Acetyl-CoA is produced by the breakdown of carbohydrates, lipids and proteins in the mitochondria. The increase in the level of acetyl-CoA adversely affects the metabolism and creates a toxic effect. L-carnitine eliminates its negative effects by ensuring the transport and detoxification of excess acetyl-CoA accumulated. (51) In a study carried out, although L-carnitine did not have a positive effect on the maturation of sheep oocytes, it has been reported that it has positive contributions on embryonic development at cleavage rate, morula and blastocyst stages. (52)

1.10. Cysteamine

Cysteamine was first used in animals against the negative effects of radiation by preventing the formation of hydroxyl radicals, but after a while, the use of cysteamine for this purpose was discontinued. (53) Cysteamine, which continues to be used for different purposes, also has drawn attention with its antioxidative properties. (53,54) Cysteamine shows its antioxidative properties by stimulating glutathione synthesis and reducing the level of hydrogen peroxide. However, it is reported that high doses decrease glutathione peroxidase activity and cause increases in hydrogen peroxide levels. Various results were obtained in studies in which cysteamine was added to culture medium. It has been reported that the addition of cysteamine to the culture medium increases the intracellular glutathione level and embryo development rates. (55) In another study, cysteamine addition to *in vitro* culture medium in sheep did not have an effect on cleavage rates. (56) However, the addition of cysteamine to the culture medium containing epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1) in different study, presents with a higher cleavage rate, higher morula and blastocyst ratio than the group in the culture medium containing only EGF and IGF-1. (57)

1.11. Royal Jelly and Honeybee Pollen

Royal jelly and honeybee pollen contain a wide variety of natural nutrients such as vitamins, amino-acids, hormones and phenolic compounds. For this reason, these products, which are also used in different sectors and in the treatment of various diseases, have also been the subject to assisted reproductive technologies due to the antioxidant properties they contain. (58,59) It has been reported that honeybee pollen addition to the sheep *in vitro* culture medium increases gene expression, glutathione level and oocyte maturation rate. (59) In a study in which royal jelly was added to the culture medium, it was reported that there was an increase in glutathione level, oocyte maturation was positively affected and fertilization, cleavage and blastocyst rates increased. (60)

1.12. Papaver Rhoeas L. Extract

Papaver rhoeas L. extract, which is also used in the treatment of various diseases, has a beneficial effect on the *in vitro* maturation rate of oocytes when used in culture medium at a concentration of 50.0 µg/mL. (61)

2. Effect of Antioxidants on In Vitro Maturation and In Vitro Embryo Development in Goats

Goat breeding allows the low quality pasture areas, which cannot be used by humans and other animals, to be converted into meat, milk and other products by making use of bushes and heath areas. It is an inevitable fact to benefit from all available resources effectively and efficiently in order to increase the welfare level of human beings. (62) Increasing the number of goats and the yield characteristics per animal is possible to the extent that scientific breeding programs are applicable. The most important yield feature in breeding is the fertility. The ability to obtain a continuous and good fertility from animals largely depends on controlling the factors that affect fertility. (63)

Assisted reproductive techniques in goat breeding may lead to advances in goat breeding as in cattle breeding. The *in vitro* methods used to obtain embryos today may be more advantageous than the in vivo method of washing embryos from donor animals using hormones. *In vitro* embryo production in goats makes it possible to obtain oocytes and embryos from prepubertal and old donors, even from pregnant donors, without the need for the superovulation process used in the *in vivo* method. However, other mammals such as goats, humans and cattle give more variable results in *in vitro* embryo production. (64)

The success of *in vitro* assisted reproductive techniques depends on the success of *in vitro* embryo production. The most basic problem of *in vitro* embryo production is the maturation of oocytes. The more successful the maturation process of an oocyte is, the more successful embryo production will be. Although there have been some developments in the culture of goat oocytes and embryos *in-vitro*, it is not yet at the same level when compared to the success of *in-vivo* production. (65) The development of the oocyte depends on many factors. For example; It has been reported that high estrogen and inhibin A concentrations in a more developed follicle are associated with high embryonic development. (6) It is important that the content of the culture medium of oocytes subjected to maturation processes *in vitro* is well adjusted. (5) Protein and hormone addition to culture medium has positive effects on embryonic development. (66-67) Recent studies show that embryonic development is significantly affected by reactive oxygen species. Undesirable results such as membrane damage, DNA damage and negative effects on protein synthesis occur in cells. In order to increase the success of culture processes, researchers aimed to increase the level of glutathione, which protects cells from the negative effects of reactive oxygen species, in the cell. (65,68-70) The lack of natural antioxidant systems of oocytes in *in vivo* conditions in *in vitro* culture processes encouraged the addition of various antioxidants to culture medium. (5,71)

Studies can be diversified by adding various antioxidants to *in vitro* maturation cultures in goats. Indeed, in a study, it was revealed that resveratrol added to *in vitro* maturation and *in vitro* fertilization media at 0.25 and 0.5 μM doses had positive effects on oocyte maturation and embryo development in goats. It has also been reported that resveratrol supplementation increases the developmental ability of clone embryos. This improvement is due to the generation of beneficial microenvironment in oocytes by increasing intracellular GSH, reducing the level of ROS. (72)

Various compounds were added to the medium to increase the growth potential of mature oocytes *in vitro*. Compounds with antioxidant properties such as β-mercaptoethanol, cysteine, cysteamine, L-carnitine, which increase intracellular glutathione level, were suplemented to *in vitro* maturation medium and also to *in vitro* culture medium and successful results were provided. (72)

On the *in vitro* maturation of oocytes collected from ovaries of 1-6 month-old prepubertal Boer goats brought from slaughterhouse, oocyte grade and β-mercaptoethanol addition to the culture medium positively influenced the progression of prepubertal oocytes to stage of metaphase II, whereas high levels of estradiol *in vitro* has been shown to be inhibitory effect to maturation. (73)

Alpha-lipoic acid, which is a very good antioxidant both lipid and water soluble, is synthesized from octanoic acid in the mitochondria and it is synthesized naturally in plants and animals. (74-77) It has been reported to have positive effects on the developmental competence of oocytes and embryos after goat somatic cell nucleus transfer. (5)

Addition of various antioxidant chemicals such as quercetin, melatonin, royal jelly, C-type Natriuretic Peptide to goat oocyte *in vitro* maturation culture medium increases the rate of embryo division and blastocyst formation, increases GSH content by decreasing the expression of genes that induce apoptosis, and improves the *in vitro* maturation microenvironment by reducing the percentage of apoptosis in blastocysts. has been shown by studies. (64,73,78-80)

Cysteamine is a low molecular weight thiol compound that, when present during development of oocytes and embryos, increases intracytoplasmic oocyte glutathione concentration and improves embryo development rates. (81-83) Cysteamine (100 μM) supplementation has been reported to increase cleveage rate and blastocyst rate. (84)

Zhou et al. (2008) demonstrated that the addition of cysteine at maturation of goat oocyte increases GSH level and blastocyst rate only in the presence of cumulus cells. Addition of cysteine alone is sufficient to improve the maturation rate of cumulus oocytes, while improvement of *in vitro* maturation of bare/non-cumulus oocytes requires supplementation of both cysteine and cysteamine. (85)

In another study, it was reported that a slightly better *in vitro* maturation rate was detected in the presence of the antioxidant ß-mercaptoethanol compared to the base medium (NCS and TCM 199 with 3 mg/ml BSA). These results indicate that the addition of thiol compound to the medium increases the *in vitro* maturation rate and subsequent embryo development *in vitro*. (86)

In a study examining the effects of three different antioxidants on the *in vitro* maturation of goat oocytes, it was found that the addition of L-ascorbic acid to the maturation culture improved but not significantly the cumulus cell expansion of *in vitro* matured goat oocytes, melatonin contributed to nuclear maturation, and the addition of taurine had a significant effect on the nuclear maturation rate. It was concluded that there was no contribution. (87)

Studies with various antioxidants such as IGF-I and cysteamine, crocetin (an active component of the saffron plant), a natural antioxidant anethole (Croton Zehntneri plant) have also shown beneficial effects on embryo development in goats. have demonstrated that it increases the rate of blastocyst formation. (88-90)

Conclusion and Recommendations

Considering all the information mentioned above, the results of the studies show that the addition of antioxidants to embryo cultures is beneficial in general.

More studies are needed to put the antioxidants whose clinical effects on fertility have been studied into practice. As the number of embryos produced *in vitro* produced increase, importance of optimization of *in vitro* embryo production systems and the antioxidant supplementation into *in vitro* maturation and embryo production culture systems will be important. Parallel to the increasing importance of *in vitro* embryo production in cattle, the importance of *in vitro* embryo production in small ruminants is increasing, and studies on this subject are increasing day by day. Because the global warming and pandemic in the world, food shortage that emerged makes it necessary. Therefore, the addition of antioxidant supplements to *in vitro* oocyte maturation and embryo culture systems in small ruminants will continue to be important.

It seems that antioxidants will continue to be studied for a long time from now on. The important thing here is not only to work with compounds known as antioxidants, but also to discover the antioxidant effects of compounds found in nature, plants or another resources. On the other hand, it is also important to have an idea beforehand about the antioxidant to be investigated. Antioxidant's cheapness, unique effects, side effects, and suitability for the area to be investigated should also undergo preliminary research.

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CHAPTER V

THE ROLE OF EXOGENOUS ANTIOXIDANT IN ENHANCING REPRODUCTIVE FUNCTION AND PERFORMANCE IN SMALL RUMINANTS

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1. Introduction

Freezing semen, a biotechnological method, enables the use of semen from males with high breeding value, improves efficiency, and allows them to be transported long distances by crossing international borders. In addition, this method is applied to protect the species in the extinction stage and maintain biological diversity (1). This technology provides many advantages to the livestock industry. However, factors such as the content of extender used in semen freezing, the difference in cryoprotectant, the time and speed of cooling and freezing, and the free radical content damage the spermatozoa during the freezing stage (2). The formation of ice crystals during the freezing process and the biochemical and cellular changes made by these crystals on the spermatozoa after the thawing process cause deterioration in sperm motility and morphology and disruptions in the integrity of the plasma membrane and chromatin (3). All these negativities that occur during the freezing and thawing process of sperm affect the fertilization capacity and the viability of spermatozoa and have an undesirable effect on embryogenesis (4).

1.1. Oxidative stress

Hydrogen peroxide, superoxide ion, peroxyl, and hydroxyl radicals, which are known as reactive oxygen species (ROS) and occur during the reconstitution

and freezing of sperm, cause some physical and chemical changes in the plasma membrane of ram and buck spermatozoa, especially rich in unsaturated fatty acids (5). These peroxides are the most harmful of all metabolic products and are produced continuously by cells in an aerobic environment. Among the different peroxides, hydrogen peroxide is the one that occurs in the highest amounts. This highly reactive molecule can move largely unhindered in different cell compartments once formed and react with many cells (6). Freely dispersible and soluble hydrogen peroxide causes changes in intercellular signal transmission, causing Ca^{2+} homeostasis and cellular dysfunction. In particular, the primary source of peroxides produced during the freezing of ram semen is the active deamination of aromatic amino acid oxidase released from dead and damaged spermatozoa (7). The existing antioxidant systems cannot eliminate these excessive amounts of free radicals during the dilution, equilibration, and freezing stages after semen collection (8).

The ram and buck spermatozoa have a larger surface area than the small size. Besides, the fact that the plasma membrane is rich in unsaturated fatty acids (9), and the low ratio of saturated fatty acids to cholesterol leave the spermatozoa vulnerable to free oxygen radicals in the stage (10). Whenever the ROS production and protection imbalance favours ROS production, thus resulting state is called oxidative stress (11). Even in the case of low oxidative stress, spermatozoa capacitation, hyperactivation, and acrosome reactions are affected. In case of excess, it causes the death of spermatozoa (12).

1.2. Antioxidants

Antioxidants are cell-protective substances added to the sperm extender or applied orally to protect from oxidative stress, strengthen the defence of the spermatozoa, and enable them to show their vitality and fertilization ability. There are many antioxidant substances when evaluated in terms of their molecular and enzymatic structure, mechanism of action, protective effects, and toxic doses (13).

Antioxidants are divided into enzymatic and non-enzymatic, depending on their structure and antioxidant properties. Some antioxidant systems, such as glutathione peroxidase, superoxide dismutase, and catalase in the seminal plasma, prevent damage to spermatozoa by eliminating ROS resulting from lipid peroxidation in the environment (14). These limited amounts of antioxidants that protect cells from free oxygen or nitrogen radicals are known as enzymatic antioxidants. Non-enzymatic antioxidants are divided into hydrophilic and

hydrophobic. Hydrophilic ones are primarily found in the mitochondrial region and cytosol, while hydrophobic ones are found in the structure of lipoproteins and the cell membrane (11). While enzymatic antioxidants reduce the activities of free radicals chemically (6), unlike enzymatic antioxidants, non-enzymatic antioxidants act directly by scavenging free radicals.

Studies have demonstrated that they neutralize free radicals in lipid chains by contributing a hydrogen atom from a phenolic hydroxyl group and transforming the resulting phenolic groups into stable free radicals that do not initiate or expand lipid oxidation (15). Thanks to the complex cooperation among enzymatic and non-enzymatic antioxidants, there is a harmonious protection network for the survival of spermatozoa against reactive species (13). Various methods have been standardized to improve semen quality. However, these methods are being developed with new-generation antioxidants (16). Besides, the buck spermatozoa contain high lipases, which react with semen extenders like milk and egg yolk. Because of this unique composition, buck semen needs more attention during freezing (17).

2. Antioxidant Therapy

2.1. Effects of oral use of antioxidants on semen

The right feeding program directly affects semen quality and fertility in male animals. It has been observed that different fats and fatty acids in the ration, phospholipids, or related minerals that enter the cell membrane structure, improve reproduction in ruminants.

The integrity of the spermatozoa cell membrane is a determinant of motility, sensitivity to cold, and an indicator of spermatozoa viability. Unsaturated fatty acids, sensitive to oxidative damage in spermatozoa cell membranes, act as mediators in various processes, such as cell membrane fluidity and intracellular signalling (18). In particular, the presence of oil seeds rich in long-chain fatty acids, precursors in synthesizing unsaturated fatty acids, improves reproductive performance. For this reason, studies on the oral use of antioxidants have focused on essential fatty acids and trace elements that enter the cell membrane of spermatozoa (19).

When linoleic acid is used as an additive in bucks, it benefits spermatozoa and fertility, suggesting that unsaturated fatty acids can resist bio-hydrogenation in the rumen (20). It has been noted that spermatozoa progressive motility and semen density develop in rams fed with a diet supplemented with sunflower

oil and vitamin C, and this effect is integrated with the spermatozoa lipid layer (21). It has been determined that the trace elements added to the ratio improve spermatozoa quality, libido sexualise, and fertility during spermatogenesis (22). It has been reported that organic copper and zinc added to the ratio of bucks reduce oxidative stress by increasing spermatozoa motility, acrosome integrity, and plasma membrane integrity. These minerals react directly with the cell membrane and preserve the viability of spermatozoa during freezing (16). A similar study conducted on rams revealed that zinc has a positive effect on spermatozoa viability (22).

2.2. Effect of antioxidants on short-term storage of semen

In cases where semen will be used within a short period time or freezing is not possible, short-term storage is preferred. In the short-term storage of semen, spermatozoa are damaged due to the existing aerobic environment, the effect of cold shock, and the rapidly occurring oxidative stress (23). It is reported that the maximum waiting time should be between 6-12 hours for short-term storage of semen (24). Allai et al. (25) indicated that they used skimmed milk powder and argan oil as semen extenders in a study of rams, stated that they stored the semen at 15◦C, and reported that they obtained positive results on spermatozoa progressive motility, abnormal spermatozoa ratio, plasma membrane and chromatin integrity. These positive results were interpreted by the presence of sterol, tocopherol, phenolic structure and coenzyme Q_{10} in the composition of argan oil. In a study examining the effects of short-term storage on Friesland ram semen, it was determined that combinations of vitamin E-phosphate, superoxide dismutase, catalase and glutathione peroxidase at different rates and amounts did not show the antioxidant effect they showed when applied alone (26). In the ram study, in which 2-4 μM astaxanthin was used as an antioxidant, it was shown that astaxanthin had positive effects on spermatozoa viability by preserving the integrity of the spermatozoa plasma membrane in the evaluations made after 72 hours (27). In a study in which antioxidant-added soybean lecithin was used as an extender and diluted semen was evaluated after 6 hours of incubation, it was determined that methionine and cysteamine used did not have a positive effect on the plasma membrane, acrosome, and chromatin integrity and motility, while cysteine improved all examined parameters (28). It has been reported that 2 mg/mL of vitamin B_{12} added to the Tris extender reduces abnormal spermatozoa rate by increasing sperm motility and viability properties in short-term storage at 5°C (29). Allai et al. (30) stated that they obtained similar positive results from

the *Opuntia ficus-indica* they added to Tris and skimmed milk powder semen extender in short-term storage at 5◦C.

2.3. Effect of antioxidants on freezing of semen

Artificial insemination with frozen semen has not found wide application in sheep and goats due to the low pregnancy rates (31). The artificial insemination method is effective in obtaining these undesired pregnancy results and in adverse biochemical events encountered by ram and buck semen during the freezing stage. The reasons, such as sub-lethal changes, including the cholesterol transition seen in the freezing phase of sperm, negatively affecting protein tyrosine phosphorylation, and untimely wasting of a limited energy source, can be counted at the beginning of these complex events. For this reason, studies have aimed to minimize the damage of freezing on spermatozoa (32). In line with this goal, antioxidants obtained from synthetic and natural products were used to eliminate the oxidative stress that occurs during the dilution and freezing of semen (33,34).

It has been reported that when methyl-β-cyclodextrin, an oligosaccharide, is used together with cholesterol, it improves sperm motility and viability, and increases the resistance against osmotic stress. (35,36) Carro et al. (9) in a similar study, it was determined that 10 mM methyl-β-cyclodextrin-cholesterol had positive effects on spermatozoa function and membrane structure. In these studies, it has been claimed that cholesterol provides special biophysical properties that affect membrane fluidity in accordance with the cell structure of ram spermatozoa and therefore play an active role together with methyl-β-cyclodextrin. It has been determined that Entada abyssinica extract is effective in freezing ram semen due to the polyphenol in its structure, and when used at a dose of 375µg/mL, it improves total antioxidant capacity, progressive motility and plasma membrane integrity by eliminating lipid peroxidation (37). In a study in which soybean lecithin was used as a semen extender, it was shown that the added 4-6% rosemary (*Rosmarinus officinalis*) extract had positive effects on spermatozoa progressive and total motility, increased the integrity of the plasma membrane, and decreased the malondialdehyde level (38). In a similar study using the same antioxidant substance, similar results were obtained in the freezing of buck semen (39). In both studies, it was claimed that rosemary extract acts by clearing free radicals and supporting the intracellular antioxidant system of spermatozoa. Thymus vulgaris extract added to Tris extender in the

amount of 4 mL/dL due to its phenolic structure showed a positive effect on spermatozoa (40).

Nanoparticle forms of mint, thyme, and curcumin used to freeze buck semen improved spermatological parameters, chromatin integrity, and free radical scavenging agents in preventing cryodamage and improving the cryotolerance of spermatozoa (41). In a study conducted in Santa Inês rams, it was reported that catalase added to the Tris extender had a positive effect on spermatozoa viability. In contrast, the effect of Trolox was not as expected (42). It has been observed that ascorbic acid, hypotaurine, cysteine, and butylated hydroxytoluene added to Tris extender in Boer goat bucks, have a protective effect on spermatozoa membrane and acrosome integrity, as well as viability. It was also observed that ascorbic acid increased the motility values. It has been claimed that these antioxidants show this protective effect, especially by protecting the integrity of spermatozoa tail and mitochondria (43). It has been determined that resveratrol and quercetin used at doses of 5-20 µg/mL in rams have an effect by preventing damage to mitochondria of spermatozoa (44). Supplementing 50 and 100 µg/mL thymoquinone to Tris extender showed an enhance effect on progressive and total motility, acrosome and plasma membrane integrity. It also reduced DNA damage as well as mitochondrial reactive oxygen species levels ram semen (34).

It has been indicated that iodixanol used in Dorsets increases sperm progressive motility and plasma membrane integrity while decreasing the acrosome and total abnormal spermatozoa rate. It was also determined that the trehalose used in the same study did not have an effect on motility, and cysteamine used at a dose of 5 mM had undesirable effects on spermatozoa viability. In line with the data obtained in this study, it was stated that besides the variety of antioxidants used, their effects vary depending on the dose (45). Aisen et al. (46) reported that 100 mOsm trehalose increased the viability of spermatozoa and that trehalose, a polysaccharide, showed this effect by reacting with the phospholipids in the cell membrane, enabling more accessible and faster replacement of the intercellular cryoprotectant substance and the intracellular fluid during freezing. It has been reported that when 6 mM cysteamine and ergothioneine are added to the semen extender, spermatozoa viability, motility and membrane integrity are improved. It shows these positive effects by reducing lipid peroxidation and contributing to the continuity of the redox cycle on the oocyte, thanks to their thilol structure (47). It has been determined that bioactive peptide at a dose of 40-60 µg/mL added to Tris extender increases spermatozoa motility and viability by inhibiting spermatozoa lipid peroxidase.

It shows this effect by activating the pathways related to cyclic AMP protein kinase activity, which is required for sperm motility (48). It has been shown that *Moringa oleifera* seed extract in 0.5 and 5.0 mg/mL amounts added to Tris extender protects spermatozoa membrane integrity and increases motility due to its antioxidant properties (49). It is said that a 0.5 mM dose of N-Acetylcysteine added to skimmed milk powder semen extender protects ram semen from oxidative stress (50) Önder et al. (51) stated that alpha-lipoic acid had positive effects on spermatozoa after freezing and thawing. However, this effect was determined negatively in long-term storage. Astaxanthin showed a positive effect on spermatozoa by reducing the harmful effects of oxidative stress in the amount of 2-4 μM (52). According to Khalili et al. (53), glycine and cysteine added to the semen extender showed positive results on progressive motility, plasma membrane, axosome integrity, and viability. It has been shown that 0.5-0.75% royal jelly added to Tris extender in bucks (54), 20 mM L-glutamine, and 25 mM L-proline in rams (55) have healing effects on spermatozoa motility, acrosome, chromatin and plasma membrane integrity.

3. Conclusion

Antioxidants have become increasingly important for the protection or management of oxidative stress and can be used as useful tools to protect from oxidative damage. Oral use of antioxidants is effective on spermatogenesis and cell membrane composition. In in-vitro conditions, antioxidants show their effects on spermatozoa depending on the duration of semen cooling and freezing, equilibration time, extender content and antioxidant capacity. If the antioxidants added to the sperm extender are used more than necessary, they have a detrimental effect.

Natural antioxidants are preferred over synthetic ones because they are safer and have less toxicity. In the trials on the combined use of antioxidants, it has been indicated that the individual positive effects are generally more persuasive than combined use. In general, while flattering effects of antioxidant substances on spermatozoa were observed, it has been reported that dose-related adverse consequences may also arise.

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CHAPTER VI

USE OF ANTIOXIDANTS IN VITRO EMBRYO PRODUCTION IN COWS AND HEIFERS

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1. Introduction

Production of bovine embryos in vitro; it consists of the stages of obtaining immature oocytes from ovaries taken from slaughterhouses, in vitro maturation (IVM), fertilization of matured oocytes in vitro (In Vitro Fertilization-IVF) and in vitro culturing (IVC). In order to improve culture media used in in vitro production and to increase blastocyst rates, scientists conduct numerous studies on protein, sugar, antioxidant derivatives, hormones and growth factors, and also try to standardize the O_2 and CO_2 gas systems that embryos need in their development. Substances such as Fetal Calf Serum (FCS), Bovine Serum Albumin (BSA), L-Glutamine, energy substances glucose, sucrose, hormones FSH, LH, Estrogen, which are the main nutrients in the development of cells, are being standardized in in vitro fertilization studies for farm animals is being studied. In the last 10 years, antioxidant substances such as β-Mercaptoethanol (β-ME), cysteamine, cysteine, cystine, resveratrol, which are added to in maturation&culture media at certain rates in order to increase the blastocyst ratios and viability after freezing-thawing of the obtained embryos, are frequently used. In order to ensure that the embryos produced in vitro are of a quality that can be transferred and frozen, studies are continuing to improve the current results by using different chemicals and methods as well as freezing stages. There are some studies showing that antioxidant substances and transfer

methods used in the in vitro production phase of embryos can contribute to the development of genetically superior embryos and the spread of embryo transfer technique. (1,2)

2. IVF Applications in Bovine

The first successful in vitro fertilization in bovine was carried out in 1977 by Iritani and Niwa. Almost five years after this study was published in the global literature, Brackett et al. (1982) reported the birth of the first healthy calf (Virgil) after IVF. Since then, IVF studies on farm animals gained momentum. (1)

2.1. What is In Vitro Fertilization (IVF)?

"In Vitro Fertilization" is the cultivation and development of embryos, which are formed as a result of the fertilization of oocytes (female reproductive cells) outside the body and under suitable laboratory conditions with fresh or frozen spermatozoa, in an environment within vivo conditions for a certain period of time. This process consists of in vitro maturation, in vitro fertilization and finally in vitro cultivation of oocytes. (1)

2.1.1. In Vitro Maturation (IVM)

"In Vitro Maturation" is the process of bringing immature oocytes obtained in mammalian species to metaphase II, where they can be fertilized by providing in vivo conditions under laboratory conditions. (1)

To obtain oocytes to be used for in vitro maturation, various methods are used, including surgery, ultrasound (OPU: Ovum Pick-Up) and follicle aspiration or cutting from ovaries obtained from the slaughterhouse. Today, ovaries obtained from animals in slaughterhouses are used for their cost-efficiency. (3)

Bovine ovaries are brought to the laboratory within 2-3 hours in a thermos with 0.9% NaCl or antibiotic-added phosphate-buffered salt (PBS) solution at 30-35ºC. Immature oocytes are often obtained from follicles with diameters of 2-8 mm on the surface of the ovary by aspiration, puncture or cutting. The reason for this is the high capacity of oocytes obtained from 2-8 mm diameter follicles to reach metaphase II after metaphase I. It is reported that the oocytes to be matured should have at least 3-4 rows of cumulus oophorus cells (COC's) around them, their cytoplasms should be homogeneous and their cell walls should be even. It has also been reported that the maturation media for the maturation of these bovine oocytes should contain chemicals such as mineral substances, Follicle-Stimulating Hormone (FSH), Luteinizing Hormone (LH),

growth factors (Epidermal Growth Factor (EGF), Insulin-Like Growth Factor (IGF)), energy substances, proteins, antibiotics and antioxidants. In addition, researchers have reported mixtures of gas containing 5% CO_2, 5% O_2, 90% N_2, and 90% moisture, temperatures of 37-39ºC, and 22-24 hours of incubation period according to the animal species. (4,5)

2.1.2. In Vitro Fertilization (IVF)

At the end of maturation, the oocytes that are thought to have matured with the expansion of the surrounding COC's are fertilized in in vitro with frozen or fresh spermatozoa. Frozen spermatozoa that can be used for in vitro fertilization should be at least 45-60% motile after thawing. When preparing fresh and frozen spermatozoa for fertilization, washing methods such as Percoll-Gradient, Swim-Up, and Pellet are used, with fertilization media such as IVF-TALP, Tryode or Brackett and Oliphant (BO). (5,6)

The concentration of spermatozoa to be used in fertilization varies according to species and is often adjusted to be 0.4-2x10^6/mL, including bovine. It is often left to incubate in gas mixtures containing 5% CO_2, 5% O_2, 90% N_2, and 90% moisture for 18-24 hours under temperatures of 37-39ºC according to the medium type and the animal species. However, recent research has stated that a fertilization period of 6 hours may be sufficient. (5,6)

2.1.3. In Vitro Culture (IVC)

At the end of fertilization, fertilized oocytes are purified from the spermatozoa mixture and the COC's and then transferred to an appropriate culture media. For culture media, Synthetic Oviduct Fluid (SOF), CR1aa-Charles Rosencrans, KSOM, TCM-199, Menezo-B2, Whittens Medium, Ham's F10 and CZB are often used, with SOF and CR1aa being most commonly preferred. The reason for this is obtaining healthier embryos cultured in these media with higher cryotolerances against freezing processes. The most important point in choosing culture media is being similar to the chemical composition in the oviduct. Researchers have reported that adding oviduct co-culture to media used for this purpose leads to a higher rate of blastocyst production. (6,7,8)

They are often cultured in gas mixtures with 5% CO_2, 5% O_2, 90% N_2, and 90% moisture at temperatures of 37-39ºC for 5-9 days according to the media used. Researchers have reported that in vitro embryos were found to develop later than in vivo embryos, which extended total culture time by 1 day. Culture medium drops often vary between 50-500 µL, but it is emphasized that

there should be 1-10 µL medium per embryo. They have found that the rate of blastocyst production was 18% with an embryo:medium volume ratio of 1:1 and 2.5% with an embryo:medium volume ratio of 1:10. In addition, the percentage of embryos reaching the blastocyst phase may vary based on factors such as the chemical contents of the culture media, the oxygen concentration during incubation and the number of cultured embryos. (7,8)

2.2. In Vitro Maturation and Contents of Culture Media

For in vitro embryo production, the presence of substances required by male and female gamete cells in the medium to provide in vivo conditions is essential for successful in vitro fertilization.

2.2.1. Energy Sources

The energy sources for oocytes, which are glucose, pyruvate, and lactate are added at different rates in culture media. Pyruvate, which can meet the energy needs of the cell to a great extent, is formed by the conversion of glucose as a result of chemical reactions by the COC's. The energy requirements for the nuclear development of bovine oocytes has not been made as clear as in mice. Researchers have reported that COC's prevent the oocyte from reaching metaphase II, particularly in the absence of energy substances. Though it depends on the animal species, a female bovine gamete cell fertilized in vitro shows a developmental block at the 8-16-blastomer phase, that is, cell division cannot continue. Many researchers have reported this to mainly be due to glucose being added to culture media in the early period. However, a study on hamsters has reported inorganic phosphate to have more inhibitory effects compared to glucose for cells at the 2-blastomer stage. Bovine embryos provide their energy needs in division phases before the morula period particularly from pyruvate and lactate, although they need glucose after the morula period. In addition, when used in conjunction with myo-inositol at the in vitro culture phase, citrate is reported to highly support bovine embryo development. (7,8,9)

2.2.2. Protein Sources

Proteins, especially the essential amino acids, which cannot be synthesized by the body, have a key role in embryo development. However, although protein sources such as L-glutamine, fetal calf serum, fetal bovine serum and bovine serum albumin used in in vitro embryo production have been reported to be beneficial at the implantation phase, some researchers have argued that proteins

had no positive effects, particularly at the phase of reaching the blastocyst. In addition, some scientists have stated that serums added to in vitro culture media caused certain changes in the metabolism of the embryo, increasing the accumulation of fatty acids and the number of cytoplasmic oil droplets, consequently reducing the cryotolerances of the cells. (7,8,9)

Released as a result of the metabolism of glutamine, ammonia has a particularly negative effect on the embryo. The most important effect of ammonia is that it is among the main factors that make up the "large calf syndrome". For this reason, some researchers have reported that replacing glutamine culture media every two days during the culture period optimizes the rate of embryo development. However, it has been stated that refreshing the medium eliminates metabolic residues and changes the pH and gas pressure of the environment, eliminating the factors supporting development. (10)

Studies have highlighted that serums being added to media used for in vitro production of bovine embryos may change the ultrastructural structure of the cells, lead to abnormal blastulation formation and unexpected mRNA expressions, and cause large calf syndrome or loss of offspring immediately after birth. (7) Researchers have used inert substances such as Polyvinyl Alcohol (PVA) or Polyvinyl Pyrrolidone (PVP), considering that if serums that are sources of protein are removed from the in vitro culture media, balancing should be done using supplements. This way, embryos can be easily manipulated in a protein-free environment using the synthetic heavy polymer PVA or PVP. Besides, scientists have reported that using PVA instead of BSA in bovine negatively affects the division rates of embryos, the number of cells reaching the blastocyst, and their viability against freezing. (6)

2.2.3. Hormones and Growth Factors

Hormones such as FSH, LH, and 17 β-estradiol are added to in vitro maturation media. In bovine, only FSH receptors are present on granulosa cells in 2-8 mm diameter follicles. LH receptors are found in theca cells. (8) FSH demonstrates a maturation-stimulating effect both by causing the COC's to expand and by leading to a temporary increase in the amount of cyclic adenosine monophosphate (cAMP) in the cumulus-oocyte complexes. FSH added to in vitro maturation media has been reported to support in vitro fertilization and to have positive effects on embryo development, and LH has been proven to support embryo development up to the 4-8-blastomer phase. (7,8) Some researchers have reported that growth factors such as epidermal growth factor

(EGF) and insulin-like growth factor (IGF-1), both commonly used in in vitro embryo production, improve embryo development. Insulin-like growth factor (IGF-1) in particular is known to show a synergistic effect with FSH, supporting oocyte maturation, consequently mitogenesis, production of steroid hormones and protein synthesis. In addition, epidemic growth factor has been proven by many researchers to have a stimulating effect on DNA synthesis and protein and proteoglycan syntheses. (7,8)

2.2.4. Antioxidants and Oxygen Pressure

Until today, many studies have been carried out on oxygen pressure on the female genital tract. It is clearly known that the oxygen pressure in the fallopian tubes and uterus is lower than the atmospheric oxygen pressure. Atmospheric oxygen pressure is routinely used in the in vitro embryo production of mammalian species, but this high pressure leads to the generation of reactive oxygen species (ROS) during embryo culture. The known harmful effects of ROS include DNA damage, lipid peroxidation and oxidative modifications of proteins, and inhibition of fusion of spermatozoa and oocyte. In addition to these known negative effects, ROS can also be characterized physiologically as an important factor of apoptosis in some conditions. ROS can originate directly from male and female gametes or from embryos at various embryonal developmental stages or it can originate entirely from environmental conditions. Among the endogenous sources of ROS, the most important is oxidative phosphorylation. Inhibiting oxidative phosphorylation reduces ROS production and has a positive effect on in vitro embryo development. Oxygen pressure is the most important exogenous factor that increases ROS production. Oxygen pressure in the fallopian tube is only ¼ of the atmospheric oxygen pressure. It has been reported that glutathione (GSH) synthesis from non-protein sulfhydryl compounds and resistance to freezing increase in bovine embryos grown in vitro under low oxygen pressure (5-7%). Various antioxidants such as β-mercaptoethanol, cysteamine, cystine, cysteine, N-acetyl-L-cysteine (NAC) and superoxide dismutase (SOD) are widely used to protect the embryo against oxidative stress in the production of bovine embryos in vitro. Although some scientific studies argue that these positive effects are only valid under certain conditions, antioxidants are known to have positive effects on embryo development. Studies have shown that the positive effects of antioxidants occur only in the presence of 20% O_2 pressure. Similarly, the type of media used and the amount of antioxidants are emphasized. Cysteine, cystine and β mercaptoethanol have a positive effect on TCM-199 medium used

for ripening in the presence of 20% O_2 pressure, while 50 μM cysteamine has a positive effect on TCM-199 medium under 20% O_2 pressure and the same amount of cysteamine 5% It has a positive effect on embryo development in O_2 pressure and SOF environment. Researchers are experimenting with various oxygen pressures under different conditions for in vitro embryo production. For example, 20% O_2 pressure was found to be suitable for culture media with oviduct cells or granulosa cells added, while 5% O_2 pressure was found to be appropriate for media without somatic cells. It is also stated that bovine embryos produced in vitro under 5% O_2 pressure have significantly lower proportions of apoptotic cells compared to those produced under 20% O_2 pressure. (11,12,13)

Antioxidants; it is divided into two subcategories, enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx); non-enzymatic antioxidants can be listed as β-carotene, vitamin C, vitamin E, zinc, selenium (Se), taurine and glutathione (GSH). Thiol compounds with low molecular weight, β-mercaptoethanol and cysteamine, cause an increase in intracellular glutathione synthesis (GSH) by causing cystine to regress to cysteine. Increased intracellular GSH protects the embryo against oxidative stress caused by endogenous or exogenous ROS products and improves cell quality and the number of embryos reaching the blastocyst. If we consider antioxidants as a whole; we can say that endogenous enzymes are at the top and that the highest antioxidant effect is in this group (SOD, CAT, GPx). When we look at the bottom, we can see that there are elements with less antioxidant capacity (carotenoids, flavonoids). It is possible to list the antioxidants with average effect as albumin, transferrin, uric acid, vitamin C, vitamin E and CoQ10. (6,9)

3. Conclusion/Result

In conclusion, considering the cell basis in all animal species, it is an important point that strong antioxidants are needed to cope with oxidative stress and strong immune systems are needed to metabolize them.

Although there is much known about antioxidants, which is still an active research topic, it should not be ignored that many unknown factors may arise.

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CHAPTER VII

THE USE OF ANTIOXIDANTS FOR EMBRYO PRODUCTION *IN VITRO* OF BUFFALOES

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1. Introduction

Domestic water buffaloes which belong to the Ruminant family of Bubalina, are divided into two categories: River Buffaloes and Swamp Buffaloes. Also, there are American Buffalo (Bison bison) and African Buffalo (Syncerus caffe caffer) groups. (1) Buffaloes have existed more than 5000 years in our world, they are important and essential livestock for ecologically disadvantaged farming system. (2) The importance of Italian mediterranean buffaloes has increased in the food industry, their genetic advances have improved in the last 30 years and their first calving has been reduced from 42 months to 36 months. (3) Buffalo milk is especially used in the production of mozzarella cheese and is highly demanded worldwide. (2,4) Although buffaloes reach late sexual maturity, as cows they have estrus year-round, their sexual activities fluctuate depending on months, seasons, day length and the release of melatonin hormone.

2. Reproductive Properties of Buffaloes

Buffaloes are reproductively seasonal polyestrous animals. Their reproductive properties may vary according to the region or continent in which they live. Although the majority of buffaloes are located in tropical and subtropical regions, seasonal anoestrus can be seen due to high temperatures, humidity, and limited food. (5,6,7,8) In the Asian region, the breeding season is from September to February. The fact that the gestation period is 310-320 days compared to cows and they have a long postpartum anoestrus period, this has forced scientists to work harder to increase their production. (9,10,11) Each calf obtained from high-yielding animals increases the genetic progress. Although studies have increased on semen, in vivo and in vitro embryo production in the last 10 years in Turkey, the buffalo reproduction field is still insufficient. For this purpose, although it is tried to obtain offspring by in vivo embryo production and transfer in buffaloes, the anoestrus period of buffaloes is long, affected by the seasons, does not respond to reproductive superovulation practices, there are few ovarian follicles, very rare oocyte and embryo development, it is expensive and embryos cannot be obtained, thus in vitro embryo production testing has begun. (12-15)

3. In Vitro Embryo Production

Mostly, the ovaries of buffaloes slaughtered at the abattoir are used to improve in vitro buffalo embryo production techniques, However, the number of oocytes obtained from buffaloes is quite low compared to cows turning the focus of researchers to obtain more oocytes by using the ovum pick up (OPU) method used in cows. (13)

Although buffaloes are slightly different from cows in terms of reproductivity, the hormones, growth factors, protein sources, energy sources, antibiotics etc. used in the medium in in vitro culture studies are almost the same. The content and environment of the culture medium critically effects the embryo development. (16, 17) TCM 199 with hepes is used in oocyte maturation; Sperm Talp, FerTalp (Tyrode-albumin lactate-pyruvate) are used in fertilization, SOF media is used in in vitro culture. 1 µg/ml estradiol-17, 0.5 µg/ml FSH, 5 µg/ml LH, 0.68 mM l-glutamine, 0.25 mM sodium pyruvate, 10 µg/ml gentamicin, 3 mg/ml BSA, and 10% FBS, into the fertilization medium 6 mg/ml BSA (fatty acid free), 0.2 mM sodium pyruvate, and 20 µg/ml heparin, and into the SOF culture medium 3 mg/ml BSA (fatty acid free), 0.25 mM sodium pyruvate with

0.68 mM l-glutamine , 1% essential and non-essential amino acids, and 50 µg/ml gentamycin sulphate substances are added and embryos are developed in 5% CO2 gas at 38.5°C and in 5% CO2, 5% O2 and 90% N2 gases, at the same temperature.(18)

The content of these medium was not fully sufficient for buffaloes, so embryo division, blastocyst, expanded blastocyst and hatching rates remained low compared to cows. In today's in vitro culture systems, cleavage rates in cows have reached 80-90% and blastocyst rates have reached 30-60%, while blastocyst rates in buffaloes are around 10-25% and cleavage rates are around 40-70%. (19-22) Considering that the first embryo transferred water buffalo was born in 1983 and the first in vitro fertilized embryo transferred buffalo cub was born in 1991, there is much progress to be made regarding the cytoplasmic and nuclear maturation of the oocyte, embryonic metabolites and its environment, culture, and monospermic fertilization conditions of in vivo and especially vitro buffalo embryo production. (23,24)

4. Antioxidants and Oxidative Stress in In Vitro Fertilization

The fat content and structure of the cell membrane and cytoplasm of buffalo semen and oocytes are different from those of other ruminants. Fats, which are organic molecules, play an important role in the maturation of the oocyte and the progression of early embryonic development. (25) Fat droplets, which are abundant in some mammalian species, play a role in communication between spermatozoon and other organelles in the oocyte structure and also provide a dark color characterization of the oocyte cytoplasm. (26,27) The triglyceride ratio in immature oocytes of cattle is estimated to be 58-59 nanograms, while it is thought to be higher in horses and buffaloes. (28)

Reactive oxygen species (ROS), which occurs as a result of the destruction of the high fat ratio in oocytes, becomes stable by electron exchange with proteins, lipids, and carbohydrates in these unstable compounds, and causes oxidative stress by disrupting the plasma membrane integrity and DNA structure of sperm, oocytes, and embryos, and reducing motility. It is known that there is an endogenous antioxidant environment (glutathione (GSH), catalase (CAT), süperoksit dismutaz (SOD)) surrounding the spermatozoa in buffaloes. This biochemical structure strengthens the cells against the stress factors that the sperm may encounter. As a result, the high PUFA (polyunsaturated fatty acid) ratio in the plasma of buffalo semen compared to cattle causes lower motility. Buffalo semen is sensitive to cold shock and cellular adaptation in vitro. For

this reason, it reduces the chance of success in in vivo insemination and in vitro fertilization due to low motility after thawing. (29-32)

In ruminants and other mammalian species, the metabolic activity of the fat in the embryo is specific to each division stage of the embryo. Also, in many mammalian species, fats are energy sources that play a crucial role in early embryonic development. Although fats are effective in the maturation of the oocyte and embryo development, their high content causes the oxidation of fatty acids. For this reason, in recent years, scientists have used antioxidant substances in order to increase the blastocyst rate and pregnancy rate as a result of embryo transfer in in vitro embryo production studies in various mammal species, especially in ruminants such as sheep, goats, cows, camels, and buffaloes. In (IVC) in vitro culture studies, substances such as vitamin C (ascorbic acid), vitamin E (tocopherol), β-mercaptaethanol, vitamin A, vitamin B12, taurine, glutathione were used initially as antioxidant substances in embryo production, following the use of cystine, cysteine, cysteamine, L-carnitine, resveratol, rosmarinic acid, astaxanthin etc. and melatonin hormone (19, 33-40). Some researchers, on the other hand, investigated the effects of oviduct epithelial cells on embryo development in in vitro culture in cattle and buffaloes, with the thesis that the content of oviduct fluid has an antioxidant effect. (41)

We will examine the antioxidant use in in vitro embryo production of buffaloes and how they are effective on embryo division, morula, blastocyst, hatching and post-transfer pregnancy rates, semen and embryo freezing. Saikhun et al. examined the efficiency of heparin, caffeine, calcium ionophore in capacitation in in vitro fertilization of buffalo semen and their combinations with each other, they found the blastocyst rate to be 15% in the heparin group, 12% of caffeine and 9% of calcium ionophore and reported that it was statistically significant at the $p<0.05$ level, in favor of heparin. The same investigators added ascorbic acid and α- tocopherol, which are antioxidant substances, to 250 and 500 μM culture media, found the 250 μM ascorbic acid group buffalo embryos reaching blastocysts at 31% and tocopherol at 31% and while a statistical ratio was not detected among themselves, a statistical significance at the $p<0.05$ level compared to the control group was recorded. They reported the 500 μM of ascorbic acid and tocopherol group used for in vitro development of buffalo embryos resulted in failure. Tocopherol and its derivatives are fat-soluble antioxidants that suppress the oxidation of membrane lipids. It is thought that when the dosages of Vitamin C and Vitamin E, which protect from in vivo and in vitro oxygen radicals, are increased, the positive effects listed are reduced. (42-44)

It has been reported that vitamin B12, which is involved as a coenzyme in different biochemical reactions such as amino acid metabolism and synthesis of methionine, has positive effects on the motility and concentration of human sperm. At the same time, vitamin B12, which plays an active role in spermatogenesis, has been found to contribute to increase motility and membrane integrity after thawing freezed bovine semen. Hussain Ahmed et al. have tried different dosages of vitamin B12 in the range of 1-5 mg/ml in a study on buffalo semen and did not detect a difference in total motility, whereas in groups containing 4-5 mg/ml vitamin B12 in progressive motility, statistical significance level was found to be $p<0.05$ compared to the control group. At the same time, a positive statistical difference was found in the VAP VCL VSL values in the group to which 5 mg/ml was added compared to the control group. Again, a difference was found in the supravital plasma membrane integrity in the group containing 5 mg/ml vitamin B12, and in the group containing 4-5 mg/ml vitamin B12 mitochondrial membrane potential and acrosome integrity was found in the groups compared to the control group. In addition, the results obtained in the group containing 5 mg/ml show that DNA integrity is preserved. When the same investigators added vitamin B12 to the freezing of buffalo semen, they measured the antioxidant enzymes catalase, glutathione peroxidase, superoxide dismutase lipid peroxidase values, and they did not detect any difference between the control and groups containing 1,2,4,5 mg/ml. From these results, we can say that vitamin B12 eliminates the harmful effects of lipid peroxidation and ROS during the freezing of buffalo semen. (29,45-48)

In the last 10 years, melatonin hormone has been used as an antioxidant substance in sperm freezing. Melatonin is found in buffalo seminal plasma and has a protective effect during the freezing of semen. It is reported that melatonin, which is thought to facilitate the capacitation of sperm, increases the quality of semen in the off-season also melatonin applications increase the quality of semen during the season when implanted in Murrah buffaloes. Peripherally produced testicular melatonin in buffaloes and other ruminants plays a role in the viability and function of sperm. Melatonin is also a powerful antioxidant and reduces the effectiveness of free reactive oxygen species to reduce apoptosis and oxidative stress. Melatonin in the systemic circulation or locally produced melatonin plays a role in the functions of sertoli cells, leydig cells and spermatozoa. (5,49,50)

The synthesis of GSH in antioxidant substances is highly dependent on the presence of cysteine in the medium content. Most likely, allene, serine, cysteine are the first precursor amino acid used by mammalian oocytes via the transport system. However, this amino acid is not stable due to the rapid oxidation to

cystine in the extracellular environment. It was observed that the addition of cysteamine to in vitro maturation media significantly supported embryo development, number and quality after fertilization in buffaloes. In the first hour of maturation, cysteine induces the synthesis of GSH before amino acids are oxidized. Cysteamine and beta mercaptoethanol, which are thiol compounds and have low molecular weight, convert cystine into cysteine, allowing it to enter the cell and increase GSH synthesis. Gasporini et al. have added 50 µM cysteamine and 0.3 mM cystine and their combination into the maturation medium of buffalo oocytes in buffalo embryos, they found GSH concentration was higher in thiol-containing groups compared to the control group, and pronuclear development was 71.1%±13.16, 81.4%±7.3 in cystine, cystine+cysteamine groups, respectively and both groups were statistically significant at the $p<0.05$ level compared to control. The same researchers found the highest cleavage rate in buffalo embryos in the cystine+cysteamine group with 78.4% ±5.3. In the same group, they found the highest transferable embryo rate of 30.9% ±5.8, while the highest rate of reaching the blastocyst on the 7th day was 26.9% ±6.8 in the cystine group. When cysteamine and cystine are used together, cleavage is more effective in reaching the transferred embryo than using cysteamine alone. This suggests a synergistic effect between them and a positive effect on embryo development. Thiol compounds show a positive correlation and a presence of a supportive effect between cleavage rates and transferable embryo rates. (51-55)

In the past 10 years, researchers have shown high interest in melatonin, which has a much stronger antioxidant activity than traditional ingredients. Melatonin, which has a very high regulatory effect due to its molecular structure, takes part in electron transfer and eliminates the harmful effects of ROS as in other antioxidants. Melatonin, which supports antioxidant enzymes such as SOD, CAT are produced through the membrane receptors MT1 and MT2. Melatonin displays activity by improving oocyte mitochondrial functions, increasing ATP content, increasing cortical grabulin distribution and intra-oocyte GSH. Researchers have determined that the addition of melatonin to the medium content has a positive effect on buffalo oocytes and fertilization. While melatonin has a positive effect on oocyte maturation in buffaloes and pigs, but it does not have the same effect on cattle. While melatonin in follicular fluid during the acyclic period has less antioxidant capacity, it may be more effective in follicular fluid in buffaloes during the cyclic period. In buffaloes, ROS is high during the acyclic period. Locally, melatonin release affects the function of follicles and oocytes. Considering these effects, it should not be

forgotten that the use of melatonin may have a positive effect on the production of embryos in vivo and in vitro during the buffalo breeding season. (29,56-61) The fact that melatonin reduces the oxidative stress caused by the high ROS content of acyclic buffaloes in the summer months supports our idea. In a study investigating the effectiveness of the season of in vitro embryo production and embryo transfer in buffaloes, although the off-season rate in oocyte retrieval was high during the season (10.0%, 7.6% $p<0.0262$), there was no difference in the rate of blastocyst during and outside the season, but the day 30 of pregnancy. On the day, it was reported that the intra-season pregnancy rate was statistically significant ($p=0.0013$) from outside the season (46.5%, 22.4% respectively). (62) Mohammed et al. investigated the effects of sisteamine and melatonin and the combination of both in the production and freezing of buffalo embryos, and found the rate of cleveage to be statistically important in favor of their antioxidant substances. The rate of blastocyst was found to be important ($p<0.01$) in the group where cysteamine and melatonin were used together compared to the control and cysteamine groups. In a study investigating the effects of antioxidant substances on embryo freezing, thawing and viability, statistically value was found as $p<0.01$ in the group that cysteamine and melatonin were used together. Melatonin was observed to have a synergistic effect with cysteamine, and when they were used individually, there was no statistical difference on embryo development. (63)

Like other antioxidants, L-carnitine, which acts by neutralizing free radicals produced by ROS, protects cellular organelles such as mitochondria against the harmful effects of oxidative stress. L-carnitine, which enters the cytoplasm from the extracellular environment, regulates the rate of acetyl coenzyme A and reduces the rate of apoptosis by promoting cell growth and proliferation. Phongmitr et al. reported that L-carnitine at 0.3 mg/ml increased the nuclear maturation of the oocyte in cattle, and the same dosage had the same effect in buffaloes. (64) Ghanen et al. reported that 1.5 mM carnitine used in bovine embryo cultures increased the blastocyst rate and the number of cells contained in the embryo, reduced apoptosis while Verma et al. used the same dosage in buffalo in vitro embryo culture and reported increase in blastocyst rates and the cryotolerance and quality of embryos. Using more than optimal concentration of L-carnitine can induce harmful effects on cells. When L-carnitine is used at a high rate in embryo development, it binds to intracellular Ca at a higher rate, inhibits intracellular metabolic and enzyme activities of Ca, and eventually changes cellular homeostasis. Compared to other species, the

higher lipid content in buffalo oocyte cytoplasm should be evaluated very well when used in in vitro oocyte maturation and embryo culture. (65-67)

Lianga Y. et al, used low-molecular L-carnitine, which has an important role in normal physiology and can be dissolved in water, at dosages of 0.3-0.6-1.2 mg/ml in the maturation of buffalo oocytes and found the rate of reaching blastocyst 17-22%. They reported statistical significance ($p<0.05$) against the group containing 0.3 mg/ml L-carnitine. When the same investigators examined the viability rate of L-carnitine in frozen oocytes after maturation and embryo development, they could not find a difference between the groups until the morula stage, but they found statistical significance in favor of the groups added 0.6-1.2 mg/ml in blastocyst rates. They measured the intracellular H_2O_2 level in vitrified oocytes and it was found that it decreased H_2O_2 with its antioxidant effect. (68)

5. Conclusion

Recently, although embryo production and transfer studies in buffaloes are increasing, these animals are seasonal, the number of oocytes obtained with OPU is lower than in cattle, the rate of reaching blastocyst is 10-20% worldwide, which shows the inevitable necessity for scientists to further develop the in vitro buffalo embryo conditions and embryo freezing techniques, thus increasing the pregnancy rate after the transfer. Compared to the cattle embryo production market, buffalo embryo production is at the very beginning of the road. However, it attracts scientists because it is a virgin area and species characteristics are durable. (69,70)

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CHAPTER VIII

THE USE OF ANTIOXIDANTS IN IN VIVO EMBRYO PRODUCTION IN BUFFALOES

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1. Introduction

Buffaloes are raised all over the world, mostly in the rural areas of the Asian continent, but they are mostly not seen as an ideal ruminant due to the following reasons: late puberty, estrus signs which cannot be easily observed especially in summer, relatively low pregnancy rates, long periods between calvings and a long postpartum anoestrus. (1-4) When the follicular development was examined, buffaloes were seen to have an average of 19,000 primordial follicles, while their relatives, cows were seen to have 133,000 primordial follicles present at birth and this reveals the critical importance in terms of reproduction. (5,6)

During summer, ovarial inactivity was seen to be higher in buffaloes than in cows and the comparison between seasons was observed that summer was the highest season, while winter was the lowest. The reason for this can be shown as oxidative stress caused by heat or unbalanced nutrition. (7,8)

2. Oxidative Stress

Physiological events in living systems operate in a certain function. When this function is disrupted due to lack of nutrition and nurture that the body needs, it might result in reversible and irreversible damage. The aerobic metabolism is a process that converts carbohydrates, proteins and fats into ATP (Adenosine triphosphate) that is also responsible for the production of prooxidant molecules called reactive oxygen species (ROS). Nevertheless, antioxidants are the bodies

functioning system preventing the production of these compounds which might cause loss of function, disease or cell damage in the organism. (9-12)

3. Antioxidants

Antioxidants, which can be examined in two parts as endogenous and exogenous consist of the following antioxidants: enzymatic (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase), non-enzymatic (melatonin, uric acid, selenium, manganese, copper, zinc, glutathione, albumin etc.), vitamins (vitamin A, E, C, B9) and drugs (mannitol, NSAIDs, local anesthetics, cytokines, barbiturates, etc.). (13,14)

4. Reactive Oxygen Species in Reproductive Systems and Protective Supplements

Gametes and embryos, which need mitochondrial oxidative phosphorylation in order to obtain energy, cause ROS production as a result of these metabolic processes. ROS production developed at physiological levels in the female reproductive system; oogenesis can occur in many stages, including oocyte maturation, folliculogenesis, ovulation, and luteolysis. (15,16) ROS accumulation on oocytes might cause DNA damage, disrupt cell structure, change microtubule function, cause chromosomal scattering and aneuploidy. (17) The cumulus cells surrounding the oocyte produce antioxidants such as superoxide dismutase and protect the oocyte against damage caused by ROS. (18) Increase in the amount of antioxidants in cumulus cells enhances the success of reproductive biotechnological interventions, while increase in ROS values reduces success. (19,20)

Since a high amount of energy is spent during their development process of embryos, especially during genome activation, compaction and hatching, they pass through stages such as oxidative phosphorylation, xanthine oxidase systems and NADPH, thus produce abundant ROS. (21,22) Embryos exposed to high amounts of ROS may result in a decrease in quality, regression of development or cessation. (21) Although it has been reported that ROS occurs less during in vivo embryo production compared to in vitro production, it is still unknown to what extent IVF techniques and conditions contribute to oxidative stress. (23)

Although superovulation studies performed with the help of exogenous hormones during the oestrus cycles of ruminants increase the amount of oocyte and embryo, it has been reported that oxidative stress increases. (24) In addition, a decrease in the number and functions of oocyte mitochondria, and a decrease

in oocyte quality, and an increase in oxidative damage in the uterus and oviduct were observed in this process applied in mammalian species. (25,26)

The addition of antioxidants not only increases the fertilization rate in cattle, but also helps to reduce embryonic deaths in heifer. Studies show that antioxidants help protect against the harmful effects of oxidative stress observed during oocyte maturation, and increase fertilization success by supporting oocyte quality. (27) In addition, antioxidants, which are free radical scavengers, increase uterine contractions and reduce the risk of retention in fetal membranes by supporting involution. (28-32)

Melatonin, selenium, vitamin E, vitamin C, which can be used as food supplements, are antioxidants that improve the quality of gametes and can be used orally. Physiological damages such as disruptions in oogenesis and spermatogenesis, deterioration in ovarian structure and decreased fertilization ability can be seen in the deficiency of vitamin A, which is an antioxidant that has a direct effect on fertility in ruminants. (33)

It has been reported that in cases where vitamin A (β carotene) and E, which are important in terms of physiological systems, are not taken sufficiently with the diet; calm estrus, delayed ovulation, follicular and luteal cysts and early embryonic death occur, besides, phosphorus and manganese have an effect on fertility and pregnancy rates. (34,35)

Selenium and vitamin E supplemented buffaloes have been shown to be effective in their development by supporting reproductive parameters such as the interval between two calves, uterine involution and the time between birth and first heat. (36)

Buffaloes compared to cattle are more affected by air temperatures due to the fact that their bodies are darker and sweat glands are less. Heat stress is the most effective factor on yield characteristics especially in animals living in tropical regions. The hormone melatonin, which has direct effects on the reproductive systems in mammals, is also an antioxidant that has very important roles in providing thermoregulation and improving immunity. It has been observed that the application of melatonin to buffaloes by exogenous methods has healing effects on physiological activities and is beneficial in tolerating heat stress. (37,38)

It has been reported that ascorbic acid polyphosphate (vitamin C) supplementation combined with salts such as sodium bicarbonate, potassium carbonate is beneficial in reducing the heat stress observed in buffaloes. (39,40) In addition, the change in vitamin C density at different times of the estrous

cycle and different types of follicle sizes and developmental stages suggests that this vitamin plays an effective role in the follicular development stage. (41)

Again, in a study conducted in buffaloes, different doses of chromium was added to the diets of animals and the orally ingested element was observed to increase plasma progesterone concentrations and antioxidant status. (42)

5. Conclusion

Injectable uses of antioxidants are more effective than oral uses, as they reach higher concentrations in the blood and spread faster throughout the body. Studies have shown that injectable administration of vitamin E is more effective than oral use because it provides antioxidant activity in a short time. (43,44)

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CHAPTER IX

THE ROLE OF EXOGENOUS ANTIOXIDANTS IN ENHANCING REPRODUCTIVE FUNCTION AND PERFORMANCE IN HORSES

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1. Introduction

Primary sex organs in males are the two testicles, where sperm are produced. The testicular seminiferous tubule and peritubular tissue are composed of germinal epithelium, and this tissue contains the basic cell types: somatic and germinal cells. The spermatogonial stem cells and germ cells, including differentiated cells formed during and after meiosis at dissimilar developmental degrees, are primary and secondary spermatocytes and spermatids, respectively (1).

Leydig cells in testicular tissue play an important role in the formation of spermatocytes and differentiation of sexual organs, secrete testosterone and are surrounded by tubular tissues encircled by capillaries and oxidative stress (OS), which suppresses steroids secreted from the ovaries and testicles. If OS is to be defined, the redox signal should be impaired and there should be control and imbalance in favor of peroxidant species.

Possible targets of oxidative stress include proteins, lipids, nucleic acids, and all cellular components, including sugars, and oxidative stress only occurs when there is an imbalance in the relevant system. This possibly causes semen injury, malformation, and ultimately male sterility. And this causes peroxidative degeneration of the sperm plasma membrane and fragmentation of DNA in the semen at both the mitochondrial and nuclear levels. The formation of OS is a basic etiological factor that causes damage to sperm DNA (2).

The seminal plasma secreted from the male appendages, epididymis, and testicles is effective in many functions of the spermatozoa before fertilization. Ejaculation in stallions consists of nine successive fractions, of which approximately 70% of the semen is ejaculated in the first three, and the content of seminal plasma also differs between these fractions. For, the contents of the add-on sex glands are released in a certain order. During primary urethral contractions, fluids secreted from the ampulla and prostate gland are observed just before the onset of ejaculation. After the cessation of prostate activity, vesicular gland secretions are released. No fluid is released from the bulbourethral glands during emanation or discharge. Bulbourethral fluid is secreted by male germ cells before ejaculation, and then there are secretions from the epididymal and ampullae in the first ejaculate rich in spermatozoa. In the last stage of ejaculation, seminal vesicle fluids are present (3).

2. Can Oxidative Stress Be Prevented by ROS?

The ratio of reactive oxygen species is important in the capacitation, hyperactivation, acrosome reaction, and sperm-oocyte fusion of sperm, which is the male sex cell (4). ROS create a negative environment by negatively affecting the natural antioxidant defense system of the cell, negatively affecting the physiological reactions required for reproduction. High ROS levels may be effective in determining Adenosine Triphosphate, which increases or decreases the motility and energy of spermatozoa (5). OS is often the reason for the onset and/or development of pathologic events including the male and female reproductive system (6), resulting in embryo resorption, as well as problems in repeated conception, intrauterine growth limitation, preeclampsia, fetal demise, and male sterility (7,8). Reactive oxygen species are reactive molecules at different rates and in varying amounts, and they cause cell damage by continuously reducing the electrons of macromolecules such as carbohydrates, lipids, proteins, and nucleic acids (9).

Oxidative stress is effective in many diseases, including fertility and/or sterility processes in men, and is a part of an oxidant-producing system to the antioxidant defense system. DNA damage and lipid peroxidation always stem from OS, which also denatures proteins. ROS are formed as a result of natural enzymatic reactions of intercellular and intracellular communication owing to the metabolism of the normal cell (10).

Lipids are polyunsaturated fatty acids (PUFA) containing more than two carbon-carbon double bonds in the sperm plasma membrane and among the

most effective macromolecules. Lipid peroxidation is defined as a series of chemical reactions occurring after reactive oxygen species attack the PUFA in the cell membrane. ROS attack PUFA in the cell membrane and thus lead to lipid peroxidation, which refers to a series of chemical reactions. Malondialdehyde (MDA), used in various biochemical experiments, is used by spermatozoa to continuously monitor the degree of peroxidative damage and is one of the by-products of lipid peroxidation (11). ROS can easily damage spermatozoa due to the high amount of PUFA in semen. Like all cells, spermatozoa also produce ROS in an aerobic environment, which is a result of normal oxygen metabolism. By neutralizing most of the ROS produced by low molecular and enzymatic scavengers found in spermatozoa, the seminal plasma is neutralized, which is the natural and expected result (12). "The major enzyme systems are the glutathione peroxidase/reductase system (13), superoxide dismutase (14), and catalase (15), and naturally these enzymatic systems are also detected in horse sperm (16,17). In addition to these, other elements of the male sex cell such as vitamin E (18), vitamin C (19), urate (20), albumin, taurine, and hypotaurine may also function as antioxidants. Oxidative stress arises as a result of the instability between ROS production and the scavenging mechanism (21). Valuable predictors for infertility in various animal species may be provided by genetic variation in antioxidant representation, deficiency of SOD in stallion seminal plasma can result in inadequate cryotolerance in spermatozoa, and the ability of stallion sperm to tolerate cooling may be affected by low seminal plasma peroxiredoxin expression (22,23).

The increase and/or decrease in the physiological levels of ROS in the male reproductive system may affect the spermatozoa's capacity, hyperactivation, acrosome reaction, and sperm-oocyte fusion positively or negatively (3). Moreover, ROS levels are known to significantly determine adenosine triphosphate uptake and reduce the motility and viability of spermatozoa. ROS can overwhelm the cell's natural antioxidant defense system and ultimately create an unsuitable environment for the physiological response necessary for normal reproduction (5).

It is quite common to encounter relatively high concentrations of leukocytes in male semen, which makes it even more natural to observe neutrophils as major exogenous source of ROS. While the addition of activated neutrophils to stallion semen may result in decreased sperm motion characteristics (24), contamination with sufficient neutrophils to affect spermatozoal movement in stallions is almost rare (25).

3. What About Stallion Semen?

In stallion semen, as reactive oxygen species increase, lipid peroxidation noticeably increases, but this does not appear to be related to a decrease occurring in sperm motility and/or sperm viability and/or mitochondrial membrane potential (26). With this finding, it is possible to say that the effects of ROS on stallion semen may be mediated by a mechanism unrelated to the lipid peroxidation mechanism, and that sperm motility may be a more sensitive indicator of ROS-related reduction than other experimental endpoints, namely viability, mitochondrial membrane potential, and lipid peroxidation. Other studies report that sperm motility evaluation can be achieved by facilitating lipid peroxidation mechanism and as means for acceptance of lipid peroxidation analysis as a potential clinical test" (25). "Many evaluations have been performed to closely monitor the mechanisms of ROS-induced DNA damage" (27). It is a known and accepted fact that sperm DNA is vulnerable to oxidative damage, which can lead to diminished offspring and perhaps even malformation of pregnancies or various pathological conditions in the offspring (28). The damage caused by oxidation induced by $H_2O_2^-$ in mitochondrial DNA is more than that of nuclear DNA, which makes it the determinant of damage in the cell. It is thought that the increase in the abnormal DNA rate in the spermatozoa will increase the mutagenic load of any conceptus and as a result, it is important in the negative consequences that will occur after fertilization (24). Spermatozoa of stallions are affected by ROS-caused DNA atomization (29).

4. Antioxidants

Natural antioxidants can be classified depending on their activities in various ways such as enzymatic and non-enzymatic antioxidants. Numerous natural antioxidants categorized not only as phytochemicals but also vitamins are relatively smaller organic molecules and have low weights. However, antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, glutathione reductase, peroxiredoxin I-IV, and catalases are in the macromolecule group due to their higher molecular weight. Another aspect of categorization for antioxidants is based on their solubility, briefly whether they are water-soluble or fat-soluble molecules. Enzymatic antioxidants convert oxidized metabolic products into hydrogen peroxide ($H_2O_2^-$) in a multi-step process and then into water that uses cofactors such as zinc, iron, manganese, and copper. However, free radical chain reactions are stopped and terminated by non-enzymatic

antioxidants. Among its non-enzymatic natural antioxidants, we need to count flavonoids, carotenoids, glutathione, plant polyphenols, uric acid, theaflavin, allyl sulfides, curcumin, melatonin, bilirubin, polyamines, along with vitamins E, A, and C (30).

4.1. Enzymatic Antioxidants

While the harmful effects of reactive species can be neutralized by antioxidants and OS in sperm is the ultimate source of DNA damage and lipid peroxidation and denatures proteins. A categorization of scavenging molecules, which are comprised of three enzyme systems: (i) superoxide dismutase (SOD), (ii) catalase (CAT), and the glutathione peroxidase system (GPx) provide protection against the negative effects of ROS in spermatozoa. These are called enzymatic antioxidants (8). It is thought that the spermatozoa of stallions have constant intrinsic SOD, with SOD activity mainly achieved through surface absorption from the seminal plasma to the plasma membrane (31). Glutathione peroxidase has also been classified in spermatozoa or seminal plasma (25), and regard to progress protection against oxidative damage imposed by H_2O_2- (32); however, a major effect of glutathione may be protection against peroxyl radicals of PUFA ($RO_2\cdot$) through switching to relatively inactive hydroxy fatty acids (25). "A structural role of glutathione is also the case, as demonstrated by sulfhydryl oxidation and cross-soldering associated with nuclear condensation and grouping of the midpiece" (32,33) and its display in equine semen (31,34). Stallion semen has catalase activity mainly derived from prostate secretions (16). Various studies have shown that adding catalase to different species of sperm will allow the harmful effects of $H_2O_2^-$ on spermatozoa to be neutralized after in vitro storage (24-27). Likewise, an adequate amount of catalase may be available in oviductal fluids to help sperm protect from oxidative damage (35). "Surprisingly, equine semen with an initial activity of CAT, SOD, and GSH at a higher level did not result in better retention of sperm motility or membrane integrity after refrigerated storage" (25). "Adding CAT to extended equine semen did not improve motility retention after refrigerated storage" (36). "Similarly, the post-thaw quality of equine semen was not improved after the addition of CAT, SOD, GSH, ascorbic acid, or α-tocopherol to semen extender" (31). "In general, spermatozoa have limited endogenous CAT, SOD, and GPx activity. As a result, spermatozoa unlock the extracellular availability of these enzymes against oxidative stress, i.e. from seminal plasma. Such enzymes are available in the seminal plasma apart from other free radical scavengers" (37).

4.2. Non-Enzymatic Antioxidants

As a result of studies conducted for many years, researchers have reported that β-carotene, vitamin C, vitamin E, zinc, taurine, selenium (Se), and glutathione (GSH) are the most common non-enzymatic antioxidants (8). "The lipid radicals formed during lipid peroxidation are neutralized by eight isoforms available in Vitamin E" (38). Vitamin E, one of the active methylated phenolic compounds, contains four tocopherols and tocotrienols, of which active α-tocopherol is the most common biological species (39).

"Since 1920, when vitamin E was discovered, it has been reported to be the most potent membrane-bound antioxidant used by cells to scavenge reactive nitrogen and oxygen species against damage by oxidative stress in cell membrane phospholipids during cellular lipid peroxidation of low-density lipoprotein (LDL) and PUFA" (40). Fat-soluble antioxidants are localized in cell membranes. The antioxidant effects of vitamin C, which is an ascorbate ion, and vitamin E, which affect each other positively, are at the forefront of the defense system (30). Similar to vitamin E, the organic compound, which contains retinol, retinal, retinoic acid and many provitamin A-containing carotenoids such as retinyl palmitate and β-carotene and is soluble in unsaturated fat, is known as vitamin A. Vitamin C (L-ascorbic acid) is a hydrosoluble free radical scavenger that is optically-active and conveys a greatly acidic hydroxyl group. Vitamin C does not act solely as a free radical scavenger but also as antioxidant catalyzing redox reactions and reducing and neutralizing ROS. It primarily functions to scavenge superoxide radical anion, singlet oxygen, and hydrogen peroxide (41). "Carotenoids, a group of phytonutrients produced by some bacteria and fungi, are also known as tetraterpenoids and are produced by plants and algae" (42). Among the carotenoids, lycopene and carotene are known as the best and strongest antioxidants, and they have unsaturated long hydrocarbon alkyl chains and are easily soluble in fat due to these properties (30).

Molecules with antioxidant activity include, but are not fixed to, pyruvate (25), ascorbic acid (43), α-tocopherol (44,45), taurine, hypotaurine (46), butylated hydroxyanisole (BHA) (47), cysteine (48) xanthurenic acid (49,50) carnitine (51), and ergothioneine (52). Most of them have been used as feed supplements or mixed in improving spermatological parameters (36,45,53,54,55). Pyruvate, which is an important substrate for oxidative phosphorylation, is present as the end product of glycolysis. However, if pyruvate is present at fairly high levels, then it may also exhibit antioxidant properties. It has been reported that stallion semen added to a milk-based extender with added pyruvate at 2 mM

concentration, which is kept at refrigerator temperature, provides improvements in sperm motility. Taurine and hypotaurine, which have been reported to neutralize products derived from lipid peroxidation and hydroxyl radicals, maintain redox homeostasis in gametes (56).

Some micronutrients are believed to have a positive effect on reproductive performance, primarily due to their antioxidant properties. Antioxidants can help neutralize ROS or make them gentle or counteract ROS production (57). Antioxidants are a structurally diverse group of small organic molecules and large enzymes that enhance cellular defense and involve complex systems of overlapping activities that work synergistically to counteract the adverse effects of OS resulting from various ROS and reactive nitrogen species (RNS) (30). Superoxides, free radicals, which have a very harmful effect through Fenton, are metabolites formed as a result of many metabolic actions. Under normal conditions, the presence of antioxidants prevents the damaging effects of these free radicals on tissue. Most of these are essential micronutrients, and when these micronutrients are deficient or absent, free radicals cause tissue damage (57). Dietary sources, active forms, areas of action, and mechanisms of action of major antioxidants are as follows. The formation of toxic lipids, reactive proteins, free radical cascades, and nucleic acid damage is known as free radical damage. Transition metals, which are the key components of many antioxidant systems, can change their oxidation states very easily. The reactive species generated in cells include hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), hydroxyl radical ($^{\cdot}OH$), the superoxide anion radical (O_2^{-}), the nitric oxide radical ($NO^{\cdot}$), the lipid peroxyl radical ($LOO^{\cdot}$). Antioxidants reduce ROS, which participate in oxidation reactions that can otherwise form free radicals and damage cellular components such as DNA, proteins, carbohydrates, and lipids (30).

"ROS are by-products of oxygen generated by sperm aerobic metabolism" (58). "Controlled amounts of ROS are essential for semen physiological processes such as capacitation, hyperactivation, acrosome reaction, maintenance of fertilizing ability, and sperm-oocyte interaction" (59,60). "However, when the amount of ROS overwhelms the protective sperm antioxidant capacity or when physiological antioxidant mechanisms are impaired, OS affects sperm physiology through peroxidative damage, which may result in male infertility" (61). "Specifically, OS induces lipid peroxidation of the sperm membrane, which involves a decrease in sperm motility, a reduction of plasma membrane integrity, and an increase in DNA injury, resulting in sperm dysfunction and loss of fertilizing ability" (62).

Many micronutrients, including α-tocopherol (vitamin E), ascorbic acid (vitamin C), retinol (vitamin A), and L-carnitine, are non-enzymatic antioxidants, and other micronutrients, such as zinc, copper, and selenium, form integral parts of enzymatic antioxidants. As these micronutrients are derived from the diet, a nutritional inadequacy in these micronutrients will conduct to a step-down in systemic, and therefore germ cell antioxidant capacity. It is not only micronutrients that are essential for normal antioxidant expression. An insufficiency of macronutrients such as protein will also lead to antioxidant deficiencies, with protein-deficient diets resulting in reduced testicular GSH, catalase, and SOD, with a concurrent increase in semen OS markers and DNA damage. Among the enzymatic antioxidants found in stallion semen, superoxide dismutase, catalase, glutathione reductase, and glutathione S-transferase are found in seminal plasma and semen. In addition, glutathione peroxidase(s) are also found in seminal plasma (GPX5) and semen (GPX4). However, aldehyde dehydrogenase is only found in spermatozoa. Glutathione, melatonin, and ergothioneine, which are non-enzymatic antioxidants found in stallion semen, are found only in spermatozoa, while α-tocopherol (Vitamin E) and Vitamin A/ retinol are found in seminal plasma. Carnitines are present as non-enzymatic antioxidants in both spermatozoa and seminal plasma (63,64). Selenium added to the diet reduces the formation of peroxidase during the formation of glutathione peroxidase as a biologically active antioxidant, both as an intracellular and membrane protector. As a result of the presence of sufficient copper in the feed, superoxide dismutase, which is formed due to the Cu/Zn ratio inside the cell as a biologically active antioxidant, plays a role in the reduction of oxygen, while extracellular ceruloplasmin combines with Cu and Fe oxidizes iron, and at the same time, superoxide dismutase plays a role in reducing the oxygen formed outside the cell. The conditions that apply to copper are (Cu), which acts as a biologically active antioxidant in the extracellular environment, and metallothionein acts in Zn and binds to metal ions. Manganese ion, on the other hand, acts as Mn superoxide dismutase in the intracellular environment and helps to reduce the oxygen formed. While iron reduces peroxides with the help of catalase in the intracellular environment, it shows its effect by combining with Fe with the help of transferrin in the extracellular environment. Cobalt ion is found in vitamin B_{12} as a biologically active antioxidant. Vitamin E acts on the membrane by blocking peroxidation as α-tocopherol, while Vitamin A plays a role in maintaining cell integrity as retinol in the extracellular environment. As a result of the sufficient presence of β-carotene in the feed, it acts as singlet oxygen

in the membrane, and with the help of retinol, it functions in maintaining the integrity of the cell in the extracellular environment. Glucose, on the other hand, exerts a radical scavenger effect as ascorbate in the extracellular environment. As a result of the presence of sulfur-containing amino acids in the feed, they support the regeneration of glutathione peroxidase together with the glutathione antioxidant effect in the intracellular environment (57).

In case of insufficient carnitine in the diet (feed) of stallions, results such as sperm lipid peroxidation, oxidative DNA damage, and decreased fertility may occur. In the absence of peroxyredoxin/thioredoxin antioxidants genetically, the cooling tolerance of stallion semen decreases (63).

There are two reports of dietary supplementation of antioxidants in the stallion and subsequent effects on semen quality or storage. Adding 3000 IU of d-alpha-tocopherol daily for 14 weeks to the diet of stallions with low post-thaw motility (<35% progressively motile sperm) did not improve the motility parameters of sperm after freezing and thawing compared to untreated control stallions. However, there were significant improvements in the maintenance of sperm motility in cooled semen stored for 48 hours compared with controls. In a separate study, the dietary addition of antioxidants (tocopherol 300 mg/day; ascorbic acid 300 mg/day; l-carnitine 4000 mg/day; folic acid 12 mg/day) did not improve motility parameters of sperm stored for 24 hours. There was, however, a small, but significant, improvement in sperm morphological parameters in stallions fed with the antioxidant diet. These studies suggest that there may be some benefit to the dietary addition of antioxidants in the stallion, although the effects are not clear-cut (25). It has been reported that the addition of L-carnitine to the feed of stallions causes less oxidative damage to DNA and improves pregnancy rates per cycle during the breeding season (65).

It has been reported that adding different antioxidants to feeds and/or adding them to semen extenders for in vitro studies improves semen quality in fresh and chilled stallion semen. During the breeding season, the testes of stallions do not degenerate compared to other species. Since there is no need for fresh semen for artificial insemination (AI), ejaculates are usually preserved by freezing. There are not many studies on the quality of chilled semen during the mating season. However, there are freezing studies to evaluate semen quality when it is not the breeding season. During the mating season, stallion semen is valuable because it is a monetary gain. Therefore, it is not easy to collect semen for research, and it causes the breeders (stallion owners) to be negatively affected economically. Therefore, it is easier to obtain semen after

the season (66,67). Researchers (67) collected in-season and off-season semen from warm-blooded stallions and examined the volume, concentration, motility, and morphological parameters in these ejaculates and spermatological features in frozen-thawed semen. Surprisingly, the freezing results of ejaculates obtained in autumn were much better, although in the spring (ie during the season) the ejaculate quality of the stallions was good. In a similar study, it was determined that the best morpho-functional and motility characteristics in the semen of breeding stallions were in the spring, which is the normal breeding season (68). Another research result (69) revealed that the motility of semen in frozen stallion semen was improved by nitric oxide. The researchers also underline the crucial role of nitric oxide in the process of modulating capacitation-related various variables in stallion semen. Another study (70) was conducted on infertile and subfertile stallions in and out of the breeding season. This study also focused on semen DNA damage and lipid and protein oxidation in semen with a TUNEL test in stallions. Interestingly, it was the breeding season when protein oxidation was found to increase in stallion semen, associated with sperm motility and viability. Protein and lipid oxidation levels had a correlation during the non-breeding season in both semen and seminal plasma in subfertile stallions. This indicates the importance of protein and lipid oxidation as markers during the non-breeding season in identifying subfertile male equine.

In a study evaluating the effects on semen quality of stallions as a result of the administration of exogenous antioxidants as a food supplement, such antioxidant supplementation is reported to affect the ability of spermatozoa to counteract the effects of OS (21,71). In one study (72), the authors first investigated the effects of dietary supplementation with Lepidium meyenii (Maca) on fresh and chilled stallion sperm properties. As a result, they reported for the first time the effects of Lepidium meyenii (Maca), a natural antioxidant as a feed additive, on the properties of fresh and chilled stallion semen. In conclusion, the present paper shows that dietary supplementation with Maca increases sperm production and stabilizes semen quality during cold storage. Tafuri et al. (2021) (73) have lately suggested that high levels of glucosinolate and macadamia are available in the Maca plant and that these compounds may account for their antioxidant activity. It is believed that this tuber had antioxidant activity and reproductive features because of its glucosinolate and macamides metabolites (74).

Researchers working on coenzyme Q10 (75) report that coenzyme Q10 is associated with α-tocopherol, effectively protecting sperm membrane integrity and functionality. Adding α-tocopherol to the extender, researchers also state

that this substance has positive effects on stallion semen parameters (76). Other researchers added α-tocopherol and coenzyme Q10 to the extender and diluted stallion semen and kept them at refrigerator temperature (77). As a result of the analysis, coenzyme Q10 and α-tocopherol are shown to be effective in cooling processes of stallion semen, increasing total mobility levels, and reducing lipid peroxidation.

Sperm motility and viability as well as the oxidation state of semen and extender were evaluated by a group of researchers (77), with and without additives in the extender, to find out antioxidant features. For this purpose, the protective and antioxidant effects of lactoferrin and caseinate milk proteins added to the equine semen cooling extender on sperm parameters, nitrite, and $H_2O_2^-$ concentrations were investigated. It was observed that lactoferrin supplemented as an antioxidant to a modified caseinate extender failed to provide benefits for membrane integrity and sperm motility and chilled semen. However, it was also suggested that caseinate may protect stallion sperm similar to milk in the process of cooling.

Nitric oxide provides spermatozoa motility and capacitation of semen in frozen stallion semen. Lipid and protein peroxidation in the seminal plasma and semen of stallions is a marker for the identification of subfertile stallions during the mating season. Exogenous antioxidants added to the feed of stallions reduce the negative effects of the semen by undergoing oxidative stress during the freezing of stallion semen. It has been reported in studies that the semen of stallions added to their feeds with Lepidium meyenii (Maca) suffers less oxidative damage and increases the quality of their semen. Glucosinolates and macamides are contained in Lepidium meyenii, and there are reports that these substances increase fertility-related properties. Although Coenzyme Q10 and α-tocopherol have been reported to have positive effects on stallion sperm parameters, it has also been reported that they affect the increase of total motility by maintaining the integrity of the sperm plasma membrane and keeping lipid peroxidation at minimum levels in chilled stallion semen (10).

5. Conclusion:

Reactive oxygen and nitrogen species that cause infertility in all species cause oxidative damage to macromolecules. In order to protect themselves against oxidative damage, tissues express genes encoding intracellular and extracellular antioxidant enzymes and/or endogenous antioxidants and try to protect themselves through this mechanism. Oxidants themselves possess some crucial

cellular functions and their elimination is not possible. The antioxidant defense system plays an important role in maintaining normal physiological functions and processes, keeping the intracellular and extracellular physiological balance stable, fighting disease-causing factors, and improving the immune system. Exogenous antioxidants in the feed and thus ingested have a critical role in leading a healthy and long life. However, the external administration of large amounts of exogenous antioxidants may impair the way the defense system of the endogenous antioxidant activates. In addition, studies on the short and/or long-term storage of semen from both stallion and other species with enzymatic and/or non-enzymatic antioxidants added to semen extenders are continuing. In conclusion, further research is recommended to thoroughly understand the significance of antioxidants in sperm cryopreservation.

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CHAPTER X

IMPROVING THE REPRODUCTIVE PERFORMANCE THROUGH ANTIOXIDANTS ADDED TO EXTENDERS IN FREEZING BULL SEMEN USED IN ARTIFICIAL INSEMINATION FOR CATTLE

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1. Introduction

Artificial insemination instead of natural mating is a widely used modern technique for reproduction among the livestock. Over 100 million cattle are artificially inseminated worldwide (1). Artificial insemination has certain advantages such as preventing infectious genital diseases that can be transmitted by semen, creating a semen bank and inseminating numerous female animals. However, antioxidants play substantial roles in preserving the spermatological properties of semen kept outside and then frozen (2).

There must be a balance between the free radicals' formation rate and the rate at which they are neutralized by antioxidants. Thus, the cell is protected

from the negative effects of free radicals (3). If this balance is disturbed in favor of free radicals and they are neutralized more slowly than they are produced, the amount of free radicals boosts in the cell (4).

The increase of free radicals in the cell and their negative effect on cell functions is called oxidative stress. Antioxidants are reported to protect spermatozoa from abnormal spermatozoa that produce free oxygen radicals, prevent premature sperm maturation through DNA breaks, reduce the effect of cold on spermatozoa and increase the success of reproductive techniques by supporting spermatozoa (5,6).

Although seminal plasma contains enzymatic and non-enzymatic antioxidants, its protective effect against oxidative stress is considerably weakened by diluting semen with a pre-freezing extender. The adverse effects of ROS (Reactive Oxygen Species) on spermatozoa reduce sperm viability and motility and prevent fertilization. In conclusion, numerous recent researches on using exogenous antioxidants to maintain ROS balance and protect spermatozoa from oxidative damage are present (5,6).

In this section, important antioxidants added to the extender to eliminate the negative effects of oxidative stress in cryopreservation of bull spermatozoa and their effects have been addressed.

2. Antioxidants added to diluents in freezing bull semen

2.1. Resveratrol

Resveratrol is a widely used natural plant polyphenol, antioxidant, and therapeutic agent (7). An extra 50 μM of resveratrol added to the freezing extender for buffalo bull semen reduced capacitance-like changes and increased in vitro fertility by reducing oxidative stress while maintaining sperm membrane integrity (8). Adding 50 and 100 μM resveratrol to the buffalo sperm extender improved post-thaw sperm quality parameters, antioxidant status, and fertilization potential, and also prevented DNA fragmentation and lipid peroxidation (9).

2.2. Glutathione

Besides minimizing and preventing intracellular damage caused by ROS, glutathione also plays material roles in mitochondrial function, stabilization of the cellular membrane and reduction of oxidative stress in mammalian spermatozoa (10, 11). Glutathione addition to the bull semen extender at 0.5 mM improved post-thaw sperm motility, viable sperm count, and acrosome integrity (12).

2.3. Quercetin

Quercetin is a suitable flavonoid that scavenges reactive nitrogen species and ROS (13). 200 μM of quercetin added to the extender to buffalo bull spermatozoa had beneficial effects on the membrane, acrosome and DNA integrity while increasing post-thaw motility (14). Although adding 25 $\mu g.mL^{-1}$ quercetin to the semen extender positively affected the DNA integrity of bull spermatozoa, it did not improve the forward motility rate (15).

2.4. Iodixanol

Iodixanol indicates antioxidant properties depending on the amount of free radical formation in an extender or medium. Adding 1.25%, 2.5%, and 5% (v/v) iodixanol to buffalo semen during cryopreservation minimized antioxidant consumption, prevented membrane lipid peroxidation, and protected spermatozoa (16). Although the addition of 2.5% iodixanol to the sperm extender did not affect IVF and artificial insemination success, it preserved the forward motility, and viability of frozen bull spermatozoa as well as protected the spermatozoa's acrosome integrity with the plasma membrane (17).

Adding iodixanol to bull semen freezing extender at high concentration (10%) preserved the plasma membrane (18). During cryopreservation of bull spermatozoa, iodixanol was reported to act probably by altering the ice crystal structure (19).

2.5. Cysteamine

Cysteamine protects cells from oxidative stress by stimulating glutathione production in spermatozoa (20). Adding 2.5 or 7.5 mM of cysteamine to freezing and thawing solutions for bull semen was determined to reduce DNA damage and malondialdehyde (MDA) content of spermatozoa, as well as the activated antioxidant enzymes (21).

In another study, low cysteamine concentrations (1 and 2 mM) did not provide a sufficient protective effect in buffalo semen cryopreservation, while high cysteamine concentrations (5 mM) caused harmful effects (22).

2.6. L-arginine

The purpose of the L-arginine addition to the semen extender is to make use of its potential to regulate superoxide and hydrogen peroxide levels (23). The addition of 1 mM L-arginine to the extender protected buffalo spermatozoa from lipid peroxidation and increased motility and viability (24). For frozen-thawed

bull spermatozoa, 1 mM L-arginine added to the in vitro capacitation medium led to proteome redundancy (25).

2.7. Catalase

Catalase is a sensitive enzyme that can reduce hydrogen peroxide (H_2O_2) intoxication in cells by splitting H_2O_2 into water and oxygen molecules (26). In a study, adding different concentrations of catalase to the semen extender during freezing improved the post-cryopreservation bull spermatozoa quality, but did not affect IVF rate nor embryo development (27).

2.8. Cysteine

Cysteine is an intracellular glutathione precursor and contains a thiol group that can penetrate the spermatozoa's plasma membrane. It also functions as an antioxidant (28). Adding 2.0 mM cysteine during cryopreservation of buffalo spermatozoa was reported to increase spermatozoa motility and in vivo fertility as well as activate the antioxidant system (29).

3. Conclusion

In this section, antioxidants added to semen extenders and their importance in increasing reproductive performance in artificial insemination through frozen semen, one of the assisted reproductive techniques in cattle breeding, have been mentioned.

The amount of antioxidant added to bull semen extenders varies depending on the type of extender used, freezing and storage technique, and in vitro stress factors occurring in spermatozoa. More numbers of research is needed to determine the ROS effects of these antioxidants on spermatozoa, and the fertility results to be obtained with artificial insemination through semen frozen with ideal antioxidants will have substantial contributions to the development of animal husbandry.

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CHAPTER XI

INCREASING REPRODUCTIVE PERFORMANCE FOR DOGS AND CATS THROUGH ANTIOXIDANTS ADDED TO SEMEN EXTENDERS DURING SEMEN FREEZING PROCESS

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1. Introduction

Developing assisted reproductive technologies is substantial for the continuity of reproduction of domestic and wild animals. In the preservation of genetic material in male dogs and cats, semen cryopreservation is the most widely used technique and remains popular today. In particular, artificial insemination, one of the assisted reproductive techniques, is an essential alternative option for preserving endangered animal species. The production of reactive oxygen species (ROS) at high concentrations bring detrimental effects on spermatozoa in both in vitro and in vivo systems. Furthermore, cellular and DNA damage, as well as decreased fertility rates, are

observed during semen freezing process (1). Antioxidants can help neutralize ROS by making them harmless, slowing down or ceasing ROS generation. For this reason, numerous studies have been conducted against the harmful effects of ROS through food supplementation or by adding various antioxidants to the in vitro environment (2-6).

In this section, it has been tried to give information about the effects of antioxidants added to semen extenders during the freezing of spermatozoa, the gamete cells of male cats and dogs, on spermatological parameters and fertility rates.

2. Antioxidants added to semen extenders in male dogs and their effects

2.1. Curcumin

Curcumin is a polyphenol component found in turmeric. The effects of curcumin, an antioxidant, on the post-thaw motility of frozen canine spermatozoa were investigated. The antioxidant effect of curcumin was most successfully obtained by adding 2.50 mM to the extender in sperm freezing, and it was determined that it activated the post-thaw kinematic parameters, DNA integrity, and oxidative defense system (7).

2.2. Metformin

Metformin was used in reproductive biology due to its antioxidant and therapeutic features. Using metformin, a molecule that contributes to metabolic activities, improves the quality of canine spermatozoa frozen and thawed during cryopreservation process. In particular, metformin was added to the extender before and during freezing process, post-thaw spermatozoa were observed to keep membrane integrity or acrosomal reaction unaltered. The post-thaw motility and oxidative defense values were preserved by adding 50 μM metformin to the semen extender in the cryopreservation of dog sperm. In addition, it has been suggested to achieve these beneficial effects of metformin on canine sperm parameters by activating the 50-adenosine monophosphate-activated protein kinase (AMPK) pathway (1).

2.3. Lecithin

Soy lecithin, as an antioxidant, provides higher vitality and motility after cryopreservation by protecting sperm from oxidative stress (8). The effects of different vitrification techniques and different concentrations of sucrose and soy lecithin were investigated for cryopreservation of dog semen. The results showed

that the vitrification medium containing 1% soy lecithin and 0.25 M sucrose had the highest viability, total motility, and normal morphology of spermatozoa (9).

2.4. Superoxide dismutase, Catalase, Glutathione peroxidase

The effects of SOD, CAT, and GPx addition to glucose tris-citrate extender on oxidative stress status and canine spermatozoon parameters were investigated on sperm stored at 4 °C for 10 days. In dogs divided into two groups as fertile and subfertile, it was observed that the addition of SOD, CAT, and GPx to the extender preserved the sperm quality of semen from both fertile and subfertile male dogs during 10 days of storage at 4 °C (5).

2.5. Myoinositol

Myoinositol is an active form of inositol belonging to the vitamin B complex. It displays antioxidant properties. It is also used in the treatment of male infertility due to its beneficial effects on sperm quality. Furthermore, myoinositol is present in seminiferous tubule fluid and participates in sperm maturation, capacitation, motility, mitochondrial function, viability, and acrosomal reaction (10). It indicated beneficial effects on sperm motility, kinematic parameters, and membrane integrity when added at 1 mg/mL to semen extender for male dogs.

The presence of myoinositol in the semen extender during cryopreservation enhances sperm viability, hyper-activation, and kinematic parameters, thereby increasing fertilization ability (11,12). In addition, the addition of myoinositol significantly decreases the expression of proapoptotic and mitochondrial ROS modulators by increasing the expression of protamine and anti-apoptotic genes in sperm. It has been reported that myoinositol neutralizes free radicals developing in cryopreservation of canine semen and that the myoinositol addition to semen extender can provide protection against oxidative stress with an increase in sperm motility and improve the post-freeze-thaw spermatozoa quality (10).

2.6. Astaxanthin

Astaxanthin is a red carotenoid used for its anti-cancer, antioxidant, anti-aging effects by its role in delaying or preventing degenerative conditions. Astaxanthin indicates the highest antioxidant activity among the carotenoids, and its direct addition to canine semen extenders during semen freezing process has a significant protective effect on sperm quality.

During canine semen cryopreservation, the addition of astaxanthin at a dose of 1 μM to the semen extender led to a significant increase in post-thaw

spermatozoa characteristics (viability, kinematic parameters, plasma membrane, chromatin and acrosomal integrity, mitochondrial activity) (13).

2.7. Quercetin

Quercetin is a flavonoid having anti-bacterial, anti-carcinogenic, anti-inflammatory, and antioxidant activity features (14). In canine semen cryopreservation, quercetin addition to semen extender at a dose of 5 µg/mL was determined to have beneficial effects on the post-freezing and thawing kinematic sperm parameters and on fertility preservation.

In addition, during fertility assessment, an artificial insemination technique was applied to female dogs but the effects of quercetin on sperm viability, chromatin integrity, plasma membrane and acrosome integrity, oxidative stress levels, and mitochondrial activity were not evaluated in the current study (15).

2.8. Resveratrol

Resveratrol is a powerful antioxidant from a polyphenol group found in plants. Adding 200 µM of resveratrol to the semen extender during the canine semen cryopreservation mollified the deleterious effects of cryopreservation. That effect was attributed to the resveratrol's antioxidant activity and supported potential male fertility by protecting the sperm's chromatin integrity (16).

2.9. Iodixanol

Iodixanol, used as a contrast agent, has been recently used in sperm cryopreservation. In dogs, 1.5% iodixanol not only preserved post thaw sperm motility but also reduced oxidative stress. It also reduced deficient protamine levels and the relative expression of proapoptotic and mitochondrial ROS modulator genes in sperm. When a dog semen supplemented with iodixanol, was incubated in a capacitation medium, sperm viability and acrosome integrity values were found to be significantly higher than those of the control group (17).

2.10. Spermine

The spermine protein, which has protective effects on sperm cells, is a polyamine found in seminal plasma. This protein is specific to human and rat seminal plasma. The addition of 5.0 mM spermine protein to the canine sperm cryopreservation medium prevented apoptosis-like changes and cryo-capacitation of canine sperm by preserving membrane integrity and sperm motility, as well as regulating oxidative stress (18).

1.11. Kinetin

By adding 50 µM of kinetin, which has immune and antioxidant properties, to the extender in freezing canine semen, post-thaw sperm kinematics, membrane and acrosome integrity, and significant protection of mitochondrial activity from oxidative stress were ensured (19).

1.12. Melatonin

Melatonin and its metabolites exert both direct and indirect antioxidant effects on treated cells. The use of melatonin, a potent antioxidant and free radical scavenger, in dogs is within the 0.1 - 0.25 mM range.

By reducing oxidative stress, melatonin decreases ROS levels, plasma membrane lipid peroxidation, DNA fragmentation, and apoptosis-like changes, while significantly preserving post-thaw sperm motility, membrane and acrosome integrity, and mitochondrial activity (20).

1.13. Dihydroxyphenylglycol (DHPG)

3,4-Dihydroxyphenylglycol (DHPG) isolated from olive oil waste is a phenol with strong antioxidant properties. The addition of 50 µg/ml DHPG to Tris basic semen extender used in freezing dog semen preserved the kinematic parameters of frozen-thawed sperm significantly but also reduced DNA damage (21).

3. Antioxidants added to semen extenders in male cats and their effects

Potential endogenous sources of ROS are immature spermatozoa and leukocytes. Endogenous antioxidants such as SOD, CAT and GPx protected spermatozoa against the harmful effects caused by ROS within in vivo conditions.

3.1. Superoxide dismutase, Catalase

The effects of SOD and CAT, which are antioxidants added to the semen extender, on motility, viability, and acrosomal integrity of frozen and thawed male cat semen were investigated and no difference was found between extenders with and without antioxidants (22).

3.2. Other antioxidants

Exogenous antioxidants were also referred in the studies. It was found that frozen-thawed cat spermatozoa, in Tris-egg yolk extender supplemented with cysteine or vitamin E, improved membrane and DNA integrity and motility (23).

In male cats, the addition of cysteine or Trolox (a water-soluble vitamin E analog) to the Tris yolk semen extender was observed to improve motility, spermatozoa membrane, and DNA integrity, but not to affect acrosome integrity (24).

4. Conclusion

In this review, antioxidants added to semen extenders in dogs and cats and their importance have been discussed. The amount of antioxidants added to semen extenders in male dogs and cats varies depending on the type of extender used, freezing technique, and in vitro stress factors forming in spermatozoa. The evaluation of fertility results in future scientific studies in this field will be decisive for antioxidants use.

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CHAPTER XII

ADVANTAGES OF USING ANTIOXIDANTS FOR IN VITRO EMBRYO PRODUCTION IN FELINES

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1. Introduction

The rapid worldwide decline in wild animal species is a worrying problem. At present, according to the IUCN's (The International Union for Conservation of Nature) Red List of Endangered Species, most of the 35 extant wild cat species except for the domestic cat (*felis catus*) are classified as endangered, threatened, or vulnerable. (1,2) This is mainly due to illegal hunting and the degradation of the natural environment in which they live. Conservation strategies such as habitat conservation, habitat relocation, and the use of reproductive biotechnology-derived methods are essential for the protection and continuity of genetic diversity. In vitro reproductive research programs are widely employed to build a genome resource bank, produce offspring, and reintroduce endangered species. Although natural breeding is the primary method of choice to sustain endangered wildlife populations, it cannot be opted for several reasons, including the aggressiveness of male cats, behavioral incompatibility, and infertility. (3,4)

2. Reproductive Properties of Feline

Domestic cats are polyestrous animals on seasonal basis. Their sexual activities occur in the spring months when the day times start to lengthen. Unlike many mammalian species, ovulation is in the form of provoked ovulation. However, under a natural photoperiod, they are likely to undergo the heat period throughout the year if there is no deficiency in ovarian reserves. Follicular enlargement can often be recognized by sudden and obvious signs of sexual behavior; e.g., behaviors such as rubbing, lordosis, attracting the male cats around, and tail lifting are among the indicators of follicular growth. Unlike domestic cats, many feral cat species rarely indicate overt signs of sexuality. However, the primary estrous behaviors of Asiatic lions (*Panthera leo persica*) appear as rolling and vocalizing. In domestic cats, vaginal cytology and ultrasonography are used to detect heat (estrus), track the estrus duration, and monitor ovarian activity. Yet, these methods cannot be used in wild felines without anesthesia. (3)

The feline estrous cycle is variable, ranging from reflex LH release by vaginal stimuli during mating (stimulated ovulation) to endogenous LH surges (spontaneous ovulation). Therefore, in cats, variations are observed between individuals and between estrous cycles within the same females, with as yet unknown triggering mechanisms. Spontaneous ovulation occurs in 60% of domestic cats. In general, estrogen positive feedback is virtually a key regulator to induce a gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) surge leading to spontaneous ovulation, whereas induced ovulations require mating stimuli to induce ovulation through brain control. (3,6) As a result of all this variability, assisted reproductive technologies (ART), namely artificial insemination (AI), in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), somatic cell nuclear transfer (SCNT) and induced pluripotency stem cell (IPSC) technologies are being used to protect endangered species. These differences in reproductive endocrinology and physiology in felines have also reduced the success of these assisted reproductive techniques but led to long-term progress. (3,7)

3. Assisted Reproductive Technologies

In vitro embryo production technology (IVEP) is an extremely important biotechnological method that can help to safeguard the future of endangered cat species (Anatolian leopard, panther, lion, white tiger, etc.). Since the 1970s, domestic cats have been used as a model to understand the basic reproductive

biology of wild cats, and important steps have been taken in parallel with wild cat studies. (8,9) In vitro fertilization (IVF) in felines was first attempted with oocytes obtained from the ovaries of neutered (ovariohysterectomy) domestic felis, and in the first embryo transfer, offsprings (kitten) were obtained in 1979 through in vivo embryo production method. (9,10) The first successful IVF in wild cats was conducted in leopards in 1989, tigers and pumas in 1990, and cheetahs in 1992. (8,11-14) Assisted reproductive techniques, which have been studied recently on domestic cats, include intracytoplasmic sperm injection, embryo cloning, and cryopreservation of oocyte/embryos. The success of in vitro maturation/in vitro fertilization/in vitro culture (IVM/IVF/IVC) stages in domestic cats is directly proportional to the quality of the obtained oocytes. (15) Domestic cats are used extensively for in vitro fertilization and in vitro embryo culture studies, as reproduction and kittening problems occur in domestic cats much less than in wild cats. (9,16) Oocytes obtained from cat ovaries, especially after ovariohysterectomy operation, are used for developing mediums, increasing blastocyst transport rates, obtaining quality blastocysts, embryo freezing and obtaining offspring after transfer. To briefly summarize the criteria considered in these steps; oocyte collection, the quality of the cumulus cell layers, and the homogeneity of the cytoplasm with intact corona radiata can be listed. (15) In the case of wild felines, the use of IVEP may involve collecting the ovaries from females following autopsy or ovariectomy for medical reasons, followed by transporting the explanted gonads from the collection site to the laboratories. From this point of view, numerous studies have been conducted to establish ideal conditions during the transportation of domestic cat ovaries. These scientific studies indicated that pre-antral follicle morphology was preserved in cat ovaries transported at a low temperature of 4 °C and within 24 hours, and meiotic and cytoplasmic development of oocytes taken from antral follicles was sufficient. Some studies note that keeping the transport temperature the same and extending the storage period to 48 hours, thus extending this period for over 24 hours significantly reduces the IVEP efficacy. There are also scientific studies in which oocytes from ovaries stored at 4 °C for a maximum of 24 hours reached the blastocyst stage after in vitro fertilization. (2,17) Kittens born alive after transferring embryos obtained from oocytes collected from ovaries stored at 4 °C for 24-28 hours were also reported (2). The temporary storage of ovaries sometimes makes it possible to recover oocytes from the ovaries of endangered cats that died suddenly in the research field or to obtain oocytes from ovaries after ovariohysterectomy for medical reasons. (18) In a study analyzing the

impact of storing domestic cat ovaries at 4 °C for up to 96 hours on the oxidative status and developmental competence of oocytes after in vitro maturation (IVM) and in vitro fertilization (IVF), it was determined that extending the storage period beyond 24 hours causes a progressive increase in reactive oxygen species (ROS) associated with high lipid peroxidation of oocytes after IVM and reduces their ability to develop into the blastocyst stage. (19)

4. Antioxidants and Oxidative Stress

Oxidative stress is known to adversely affect oocyte quality, fertilization, and embryo development. Under normal physiological conditions, the oocyte has both an enzymatic and non-enzymatic antioxidant defense mechanism that inhibits excessive free radical formation. However, several factors may interfere with oocyte redox balance, leading to increased ROS production and/or decreased intracellular antioxidant concentrations. During the in vitro production stages, chemical substances called antioxidants are employed to minimize the effects of reactive oxygen derivatives. There are two main groups of antioxidants: enzymatic (superoxide dismutase-SOD, catalase-CAT, glutathione-s-transferase, glutathione peroxidase-GPx, ascorbate oxidase, and polyphenol oxidase) and non-enzymatic (total reduced glutathione, vitamins A, E, C, carotenoids). Many of these antioxidants are present in the female reproductive system and play a key role in both oocyte maturation and embryo development. (7,15) In addition, taurine and hypotaurine, the main components of oviduct fluid, and free amino acids function as molecular scavengers that prevent the accumulation of toxic substances derived from hydrogen peroxide and chloride reactions. Various antioxidants such as beta-mercaptoethanol, cysteamine, vitamin E, taurine, hypotaurine, allopurinol and superoxide dismutase, and resveratrol have been successfully used for in vitro production of bovine embryos. (20,21) Free radical scavengers are also known by their contribution to the in vitro embryo culture of mice, hamsters, pigs, and rabbits. (15) Resveratrol (*Resv; 3,4,5-trihydroxy-trans-stilbene*), a phytoalexin produced by plants, has many biological activities, including antioxidant activity. Scientific studies have revealed that resveratrol mainly exhibited its antioxidant effect by scavenging ROS and enhancing the glutathione content (GSH) in oocytes during in vitro production. (2)

Current studies concerning in vitro oocyte maturation or embryo development on cats provided lower scores than the studies conducted on other species. Only 40%-60% of feline oocytes cultured in vitro can reach metaphase II (MII). Similarly, although 60-95% of cat embryos produced in vitro can

pass into the morula, quite a few of them manage to reach the blastocyst stage. (15) Cocchia et al. evaluated the effects of SOD addition to IVM medium and GPx addition to IVF medium on in vitro development and embryo production to improve the feline oocytes development and reported that SOD and CAT in IVM medium were not effective in improved oocyte maturation, the cleavage and blastocyst rates were higher in the experimental groups and were statistically significant while there was no significant difference between control and experimental groups. They also observed higher rates of cleavage and blastocyst accession in the experimental groups with GPx added to IVF compared to the control group and reported that adding antioxidants to the IVM and IVF medium had a beneficial effect on the feline oocytes' access to the blastocyst. (16) In another study of Cocchia et al. in which they investigated the effects of SOD (4-8C, 3–72 h) addition to ovarian transport medium in cats on the viability of cumulus cells, apoptosis, and in vitro embryo production, they found that the number of viable COCs incubated with SOD was higher than the number of COCs incubated without SOD after 24, 48, and 72 hours of incubation, and reported that the process reduced cellular apoptosis, increased COC viability and IVEP production. (22) Likewise, Luu et al. observed that adding different doses of relaxin to the IVM medium after storage of the feline ovary at 4 °C for 24 hours had no effect on nuclear maturation and GSH concentration, whereas the blastocyst formation rate of oocytes matured with 10 ng/ml relaxin (16. 0%) was higher than that of the group in which relaxin was not used (5.9%), and a significant decrease occurred in cleavage and blastocyst formation rate of the group in which 40 ng/ml was added after fertilization compared to the group in which 10 ng/ml was added. Therefore, they reported that adding a low concentration (10 ng/ml) of relaxin to IVM medium improved the blastocyst development rate of feline oocytes yielded from ovaries stored at 4 °C for one day but did not improve meiotic and cytoplasmic maturation of oocytes. (18)

5. Conslusion/Result

Considering the prospective scientific studies to be conducted in line with the above-mentioned information, it would be possible to create fully productive and optimal IVEP conditions for cat species by eliminating developmental blocks through the modification of culture media, gas atmosphere, culture temperature, ion sources, carbohydrates, protein, and the use of chemical substances such as antioxidant supplements.

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CHAPTER XIII

INFLUENCING FACTORS IN *IN VITRO* MATURATION OF CANINE OOCYTES: AN ANTIOXIDANT ENIGMA

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1. Introduction

Assisted reproductive technologies (ARTs) are widely used to improve reproductive performance among humans and animals. ARTs are employed particularly in veterinary medicine to improve animal genetics, save endangered animals and produce offspring in cases of male or female infertility. Known ARTs include artificial insemination (AI), embryo production (IVEP), embryo transfer (ET), intracytoplasmic sperm injection (ICSI), gamete/embryo sexing, gamete/embryo cryopreservation, gamete/embryo micromanipulation, somatic cell nuclear transfer (SCNT), genome resource banking (GRB) and within IVEP context, in vitro maturation (IVM), in vitro fertilization (IVF) and embryo in vitro culture (IVC). (1,4)

However, using ARTs in canines could not fully achieve improving fertilization due to their species-specific reproductive characteristics. That they ovulate intermittently once or twice a year due to being monoestrous, have anatomical difficulties in reproducing, ovulate at an immature phase compared to other species, need 48-72 hours to complete post-ovulation nuclear maturation, have canine oocytes reaching Metaphase II (maturation) with lower rates of meiotic maturation, the factors negatively affecting meiotic maturation

(donor age, inadequate in vitro culture medium) are likely to adversely affect the ability of dog oocytes to maintain meiosis. The in vitro medium allows not only the production and growth but also the increase in the concentration of substances that may harm the gametes, such as reactive oxygen species (ROS). ROS produced at high concentrations becomes harmful under in vitro and in vivo state. Here, scientific studies have indicated that using the antioxidants can help neutralize and counteract ROS. (1,4)

2. Assisted Reproductive Technologies

2.1. Artificial Insemination (AI)

In 1784, Spallanzani was the first to perform successful artificial insemination in a dog. In 1922, Ivanoff experimented with on dogs, foxes, rabbits, domestic livestock and poultry. Polge et al. revolutionized the worldwide transport of frozen semen through artificial insemination. Today, artificial insemination is being regularly practiced in animals such as cattle, goats, sheep, pigs, turkeys, chickens and rabbits. Each year, over 100 million cattle, 40 million pigs, 3.3 million sheep and 0.5 million goats are inseminated worldwide through artificial insemination. (4)

2.2. In vitro embryo production (IVEP)

By the second half of 1980s, IVF had become a purely in vitro method called IVEP, which comprised three procedures, which are namely oocyte IVM, IVF and embryo IVC. IVEP systems perform very well in cattle, but there is always field for improvement. Approximately, 90% of oocytes yielded by various methods reach metaphase II (MII) and 80% are fertilized in vitro. Approximately 50% of fertilized oocytes develop into blastocysts at the IVC stage. Embryos are well developed in sheep IVEP, but success rates are lower than in cattle. So far, the IVF technique has not been well-established in horses. The failure of IVF in horses is believed to be due to insufficient capacitation of stallion spermatozoa. In pigs, IVM, IVF and IVEP procedures often lead to low rates of embryonic development. For pigs, the biggest obstacle during IVF is polyspermy. As for dogs, IVF failure is due to the longest phase of this species, anestrus and ovulation of oocytes in metaphase I. Under normal conditions, it takes 2-3 days for these oocytes to reach MII in the oviduct. In cats, on the other hand, oocytes ovulate in the MII state and thus, in vitro fertilization is more efficient in this species than in dogs. (1-3,5)

2.3. Embryo Transfer (ET)

The first successful ET was achieved in rabbits in 1890. The first successful ET in farm animals was reported in sheep, pigs and cattle in the early 1950s. In 1972, researchers noted the birth of viable mouse pups produced from transferring frozen embryos. By using ET or Multiple Ovulation and Embryo Transfer (MOET) techniques, livestock improvement can proceed more rapidly through expanding elite herds, acquiring genetic gain and preserving rare genetic stocks. (2,3)

3. Oxidative Stress and Antioxidants

Oxidative stress may increase in cases of elevated levels of Reactive Oxygen Species (ROS) and inadequate antioxidant defense. There is no adverse effect on the production of ROS at specific rates under normal conditions, but the increase in production levels can be harmful. For example, physiological levels of ROS have a key role in capacitation, hyper-activation, acrosome reaction and sperm-oocyte fusion. However, high ROS levels can reduce sperm motility and viability and even impair the cell's natural antioxidant defense system, rendering physiological reproductive functions inadequate. In such a case, one can mention neuroendocrine stress. The cell will try to reduce the amount of stress to acceptable limits and if the stress level does not decrease, chronic, harmful changes will occur within the cell. Accordingly, ROSes, which are unstable with highly reactive molecules, initiate the pathological process by triggering a chain reaction that will result in cell damage. Oxidative stress is responsible for DNA damage, denaturation of proteins and lipid peroxidation. The cell is capable of counteracting the harmful effects of reactive species through antioxidants. Antioxidants comprise two subcategories, which are enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) while non-enzymatic antioxidants can be listed as β-carotene, vitamin C, vitamin E, zinc (Zn), selenium (Se), taurine (Tau) and glutathione (GSH). (2,3,5)

3.1. Enzymatic Antioxidants

SOD, CAT and GPx seek to neutralize excess ROS and thus minimize cell damage. These antioxidants have a center with different valences to balance the molecules during the transfer of electrons through the detoxification process. SOD catalyzes the dismutation reaction of the superoxide anion to H_2O_2. There

are three isoenzymes for this, which are SOD 1, SOD 2, and SOD 3. SOD 1 (cytoplasmic) and SOD 3 (extracellular) contain copper (Cu) and zinc (Zn) as cofactors, while SOD 2 (mitochondrial) contains manganese (Mn). As the CAT concentration increases, it eliminates H_2O_2 and acts in different cells, especially peroxisomes, endoplasmic reticulum, mitochondria, and cytosol. GPx catalyzes the reduction of H_2O_2 and non-organic hydroperoxides. (1,5)

3.2. Non-Enzymatic Antioxidants

Non-enzymatic antioxidants include vitamin C (ascorbic acid), vitamin E (α-tocopherol), GSH, Zn, taurine, selenium, and carotenoids. Vitamin C is an antioxidant that catalyzes redox reactions, reducing and neutralizing ROS. Vitamin E has eight isoforms that counteract the radicals derived from the lipid peroxidation process. The reaction catalyzed by this vitamin E leads to the formation of α-tocopheroxyl, which can be reduced again and react with antioxidants such as vitamin C, ubiquinol or retinol. GSH, a tripeptide, is the main non-enzymatic antioxidant present in oocytes and embryos. Taurine and hypotaurine products help maintain redox homeostasis in gametes by neutralizing the products of lipid peroxidation and hydroxyl radicals. In addition, elements such as Se, Cu and Zn are essential for some antioxidant enzyme activities. Melatonin is an effective antioxidant as well. Unlike other antioxidants, they cannot undergo redox reactions. Once oxidized, they cannot return to the reduced state because they form stable end products soon after the response. Melatonin improves the transport chain of electrons and reduces mitochondrial DNA damage. (1,5)

4. Biochemical Factors in Oocyte Maturation In Vitro

4.1. Culture Medium

The culture medium used for in vitro maturation (IVM) is known to influence both maturation and the subsequent embryonic process. Culture media can be classified as simple or complex depending on their content. Simple media comprise buffered salt solutions with energy sources such as pyruvate, lactate and glucose. The complex culture medium additionally contains a mixture of amino acids, vitamins and other molecules. The most commonly employed medium for canine IVM is the simple medium known as Modified Krebs Ringer. (2,3)

4.2. Hormones

For the maturation of canine oocytes, it is observed that some preliminary preparation processes are necessary to stimulate the resumption of pre-ovulation

meiosis and maturation. Oocytes collected from superovulated female dogs are likely to have higher proportions of oocytes reaching the MII stage. On the other hand, it is known that FSH/LH (Follicle Stimulating Hormone / Luteinizing Hormone) combinations added to culture media are capable of improving GVBD (Germinal Vesicle Break Down) rates, but do not contribute positively to the progression of meiotic division. (2,3)

4.3. Proteins

Heat-inactivated serum or serum albumin, frequently used in culture media, can support the viability of canine oocytes in vitro when used at rates ranging from 5 to 20%. However, it does not have a positive effect on maturation development. Although some scientific studies argue the opposite of this case, it can be concluded that the use of bovine serum albumin leads to the inactivation of toxic metabolites of free oxygen radicals. (2,3)

4.4. Antioxidants

Exogenous antioxidant supplementation is known by its contribution to the prevention of oxidative damage caused by high oxygen tension to which cells are exposed during the in vitro culture process. To make an evaluation by considering all antioxidants, one can say that endogenous enzymes are at the top and the highest antioxidant effect is present in this group (SOD, CAT, GPx). When looking at the bottom, particular elements with less antioxidant capacity (carotenoids, flavonoids) can be noticed. Antioxidants with an average effect can be listed as albumin, transferrin, uric acid, vitamin C, vitamin E and CoQ10. (1-3)

5. Conclusion/Result

In conclusion, antioxidants are chemicals that play a very important role in the protection of reproductive cells against oxidative stress.

Although there are numerous scientific studies on antioxidants to date, we think that more data can be obtained and their functions can be clearly understood with scientific studies on different animal species.

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CHAPTER XIV

THE EFFECT OF FLAXSEED ON THE OXIDATIVE DAMAGE AND ANTIOXIDANT PARAMETERS IN DIABETES

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1. Introduction

Fats are among the most important organic substances required for human health and nutrition. Fats are of great importance as they are a source of energy, contain fat-soluble vitamins, form lipoproteins, and contribute to metabolic activities. (1) Fats are of animal and vegetable origin and are abundant in nature. The fats in the macro element group are taken into the human body via natural or processed products. The human body can synthesize fats with the help of some special enzymes and they are utilized in metabolic events. (2)

The basic structural components of fats are fatty acids. The physical, chemical, and physiological character of fats depending on the type and amount of fatty acids as the main factors. (3) The physical, chemical and nutritional roles of fatty acids are determined by the number of carbon atoms in the molecule, the degree of saturation, the number of carbon atoms, the number of double bonds between the carbon atoms, and the position of hydrogens attached to carbon atoms. (4) Making a general classification, fatty acids can be grouped under two, Saturated and Unsaturated Fatty Acids.

2. Saturated Fatty Acids

Saturated Fatty Acids have no double bonds or other functional groups in the connecting chains of fatty acids. In general terms, the term "Saturated" is used in relation to hydrogen and means that other carbons other than the carbon in the carboxylic acid [-COOH] group have bonded with as much hydrogen as possible. Saturated fatty acids form flat chains, therefore they are in a compact form and enable living things to intensively store chemical energy.

Calories taken with saturated fatty acids cause fat accumulation and weight gain in the human body. To eliminate the risk factors that cause cardiovascular diseases, it has been stated that the consumption of saturated fatty acids should be reduced and the amount of saturated fat that should be ingested should be less than 7% of the total energy intake. (5) Saturated fatty acids prevent the clearance of low-density cholesterol (LDL, Bad Cholesterol) lipoprotein in the blood. It has been reported that saturated fatty acids increase the blood fat rate and LDL levels and increase the tendency to diabetes. (6) The best plant sources of saturated fatty acids are coconut oil and palm oil. Palmitic acid and stearic acid are the most abundant unsaturated fatty acids in vegetable oils. Saturated fatty acids possess no physiological activity. However, almost all lipids contain saturated fatty acids.

3. Unsaturated Fatty Acids

Unsaturated fatty acids contain at least one double bond in their chain structure. Unsaturated fatty acids are divided into two groups, monounsaturated and polyunsaturated fatty acids. Those with one double bond on the chain are called monounsaturated fatty acids, whereas those with more than one double bond are called polyunsaturated fatty acids. Monounsaturated fatty acids can be synthesized in the human body. Monounsaturated fatty acids include oleic acid (Omega-9), gadoleic acid, palmitoleic acid, and erucic acid. Examples of polyunsaturated fatty acids include linoleic acid (LA) (Omega-6), α-linolenic acid (ALA) (Omega-3), γ-linolenic acid (GLA), arachidonic acid (AA), docosahexaenoic acid (DHA).

Although the effects of monounsaturated fatty acids on LDL are neutral, they have an increasing effect on the high-density lipoprotein (HDL cholesterol, good cholesterol). As monounsaturated fatty acids play a role in improving cardiovascular disease risk factors, their consumption should be increased and added to the diet. However, despite these positive effects, it has been reported stated that the consumption levels of monounsaturated fatty acids should not

exceed 20% of the total energy intake. (5) The most important monounsaturated fatty acid is oleic acid and it is mostly found in olive oil and rapeseed oil.

Polyunsaturated fatty acids cannot be synthesized by the human body and they are fatty acids that must be taken externally. The most important plant sources for these fatty acids are safflower and sunflower for Omega-6 and flaxseed for Omega-3. Omega-3 deficiency is observed in societies that do not use oil. In omega-3 deficiency, growth slows down, skin crusts are observed, and acne formation and hair loss are seen. The symptoms of this deficiency disappear in the consumption of oils with a high ratio of polyunsaturated fatty acids in the diet. Since similar symptoms are also seen in vitamins, poly-double-linked unsaturated fatty acids were used to be called vitamin F. (7) However, this term is no longer used and these acids are called essential fatty acids. Fatty acids that carry two or more double bonds cannot be synthesized in the human body and must be externally taken in the diet and are called "essential fatty acids". Omega-3 and Omega-6 are known as essential fatty acids. Essential fatty acids are involved in the structure of cell membrane phospholipids. Essential fatty acids constitute an important component of cell membranes and affect membrane fluidity, the behavior of membrane chain enzymes, and receptors. (8)

Omega 3 (α-linolenic acid), Omega 6 (linoleic acid), and Omega 9 (oleic acid) omega-fatty acids have important functions in the treatment of obesity, supporting the immune system, and preventing cholesterol-related and cardiovascular diseases. Lack of omega fatty acids in the human body leads to some dermatological problems such as dry skin, also diabetic conditions, and mental disorders such as growth retardation and learning difficulties. (9,10) Omega fatty acids play a role in the constructive and reparative functions of the body's physiological and biochemical activities and are effective in the development of healthy tissues. (11)

With the development of the food industry, nutritional habits in humans have also changed and, accordingly, the fatty acid ratios taken from the foods have also changed. Consumption of omega-6 fatty acids increased whereas consumption of omega-3 fatty acids decreased. This caused an imbalance of the Omega-6/Omega-3 ratio and moved it away from the required 1:1 ratio. Although the Omega-6/Omega-3 ratio should be kept low, this ratio is reported as 1:1 or 2:1. (12)

It has been reported that Omega-3 and Omega-6 fatty acids play an important role in the synthesis of metabolites with hormonal activity (Prostaglandin, Thromboxane, and Leukotriene). (13) It has been reported that the presence

of high levels of Omega-3 and Omega-6 fatty acids in the diet strengthens the immune system and prevents high blood pressure, and some mental disorders. (14) Also, Omega-3 and Omega-6 fatty acids are used by many cosmetic and pharmaceutical companies as they provide a smooth skin structure and reduce the burning sensation against diaper rash in babies. (15)

Omega-3 and Omega-6 fatty acids have been also reported to prevent miscarriage during pregnancy, prevent premature birth, and increase birth weight in babies. It has been known that the need for Omega-3 in the last trimester of pregnancy increases due to the intense nervous system and vascular development in the fetus. (16) High levels of Omega-3 and Omega-6 fatty acids in the diet have a very low effect on directly treating diseases, but they affect preventing the emergence of diseases and reduce symptoms such as inflammation and pain that occur during the diseases. (17)

In recent years, with the increase in the trend of living a healthy life, the awareness of healthy nutrition, and the increase in the risks of diseases, consumers have started to prefer natural methods rather than synthetic and chemical drugs. There are many clinical trial results reporting that a diet based on herbal products can reduce the risk of chronic diseases, especially obesity and cancer. One of the most important plants that have been emphasized in this regard in recent years is flax.

4. The importance of flax for health

Flax (*Linum usitatissimum* L.) is classified in the *Lineaceae* family and is a herbaceous plant with high fiber content. Plant height and branching characteristics differ in oily and fibrous varieties. (18) In oily plant types, the stem branches from the lower side and has many branches, while in fibrous types, the stem branches from the top and in few numbers. There are 250 different species of flax plant in the world and 42 of these species are found in Anatolia. Of the flax species found in Turkey, 26 are endemic. Therefore, the high biodiversity of flax in Turkey presents an important potential for flax breeding studies. It has been proven by many archaeological and genetic studies that flax cultivation has been carried out for 8000 years and still exerts economic value. The earliest known sources show that flax cultivation was carried out in Egypt. As the Egyptians used flax fibers for mummification, priests wore clothes made of flax fibers as a symbol of purity. (19) The Phoenicians marketed Egyptian flax to Mediterranean countries. The Romans used flax to make rafts. Flax production has increased in the world in recent years. As the usage

areas of flax expanded, the production volume increased. However, flax is still at the bottom of the list in terms of oil crops. According to the FAO data, approximately 2.5 million tons of flaxseed have been produced in the world every year since 2006. The countries that produce flax the most are Canada, China, Russia, America, and India. (20) Cooking oil production in the world is mainly obtained from olive, soybean, sunflower, cotton seed, palm seed, and rapeseed. The fact that majority of soybean production is in Africa, the palm oil industry is located in certain countries, and the resources such as olive oil are only in Mediterranean countries. Due to the fact that not every oil plant grows everywhere, some countries become absolute producers while forcing others to be permanent importers. Decreases in production in some years cause oil and oilseed prices to fluctuate. Also, the demand for biodiesel that has emerged in recent years caused an extra consumption of cooking oils and an increase in oil prices. In such cases, importer countries may have difficulties. As one of the countries that cannot meet its own oil needs, Turkey imports oil seeds and vegetable oil. Therefore, countries like Turkey try to find alternative oil sources that can be grown on their lands. Flax is considered an alternative oil source and it can grow naturally in Turkey, it can be used both as oil and fiber, and it is a valuable plant in terms of health.

Since the beginning of civilization, in addition to using plants as a source of food, many plants have also been used for therapeutic purposes. The Latin name (*Linum usitatissimum* L.) of the flax means “very beneficial”. Its cultivation dates back to 6000 BC and has been accepted as one of the most medicinal plants. (21) The oil ratio in flaxseed is 35-45% and the seed comprises 35% shell, 28-30% protein, 6% minerals, and ash. (18) With all these features, flaxseed is a natural source of phytochemicals such as flavonoids, lignans, and phenolic acids, as well as being rich in α-linolenic acid and quality proteins. (22) Flaxseed has a high protein content and the main amino acids in its content are albumin, globulin, arginine, and glutamine. Glutamine strengthens the immune system and arginine plays a protective role against heart diseases. (23) Flaxseed contains small amounts of water-soluble vitamins, and high levels of vitamin E, one of the fat-soluble vitamins. The highest form of vitamin E in flaxseed is gamma-tocopherol, which regulates sodium excretion from the body, protects the cell structure from oxidation, and contributes to reducing the risk of Alzheimer’s disease (Table 1). The mineral content in flaxseed is rich in magnesium and is equivalent to the magnesium content in boiled eggs. (24)

Table 1. Vitamin and Mineral Content of Flaxseed (25)

Vitamins	Flaxseed (100 g)	Minerals	Flaxseed (100 g)
Vitamin E (mg)		Potassium (mg)	831
Alpha-tocopherol	7.00	Phosphorus (mg)	622
Delta-tocopherol	10.00	Magnesium (mg)	431
Gamma-tocopherol	552.00	Calcium (mg)	236
Vitamin C (mg)	0.50	Sodium (mg)	27
Thiamine (mg)	0.53	Iron (mg)	5
Niacin (mg)	3.21	Zinc (mg)	4
Folic Acid (mcg)	11.00	Manganese (mg)	3
Biotin (mcg)	6.00	Copper (mg)	1
Riboflavin (mg)	0.23		
Pyridoxine (mg)	0.61		
Pantothenic acid (mg)	0.57		

There are various studies on the supportive effect of flaxseed in the diet in various areas, research on its effect on health began in the late 20th century, so there is little information on how to consume flaxseed and on its health benefits. (26) Research results on flax in the diet have rapidly increased. Today, it is known which diseases flax can treat or prevent. The health effects of many bioactive substances in flaxseed are known and they are becoming more and more prominent in the treatment of diseases. In the treatment of diseases, flaxseed has an important place not only in pharmacological treatment but also in the diet.

Dietary supplements containing ground flaxseed have health benefits. Cardiovascular diseases and cancer are the most common research areas to study the effect of flaxseed. Also, the effect of flaxseed on gastrointestinal health and diabetes has been investigated. More research is required to obtain definitive results of its effects on human health. The positive preliminary data on this subject encourages further studies. (26)

The main bioactive compounds in flaxseed include α-linolenic acid (ALA), lignans, and fiber. There are four forms of flaxseed available for human consumption. These include flaxseed, ground flaxseed, linseed oil, and partially defatted flaxseed meal. (27) A new commercially available form is flax milk (Pizzey Materials Inc., Manitoba, Canada). An alternative to milk like almond milk, flax milk is finely ground flaxseed mixed with filtered water and other minor compounds. Flax milk is high in ALA and is an excellent

alternative to cow's milk as it contains no cholesterol or lactose. It is suitable for those who are allergic to soy, hazelnut, and gluten, and is more beneficial to health than almond milk. (28) However, flaxseed has several properties that can adversely affect the flavor profile and therefore raise concerns about its consumption. The acid content in flaxseed increases as a result of oxidation and bitterness occurs. The high amount of ALA in its structure makes the flaxseed susceptible to oxidation, and as a result, this oxidation causes the flaxseed to become rancid, causes off-flavors and a musty aroma. Secoisolariciresinol diglucoside (SDG) in flaxseed provides antioxidant effects, therefore, it is of great value in limiting the oxidation process. (28) The foods containing the highest levels of flaxseeds are baked goods. (29) While using flaxseed in these bakery products, various sweeteners (such as cinnamon, banana, chocolate, and grape) are added to mask the negative flavor of flaxseed. (30, 31) The processing, storage temperature, and storage time of flaxseed, as well as the form of flaxseed (flaxseed, ground flaxseed, linseed oil), affects the stability of the product. (32) Grinding or crushing flaxseed is a necessary process for ALA and SDG to become bioavailable. (33) It has been reported that ALA in oil or ground form has greater bioavailability for the body. (33) Adding ground flaxseed to baked goods ensures that ALA and SDG are well preserved intact. (32) Cold storage and shorter storage time are more effective for maintaining the bioavailability (ALA, SDG) of flax oil. (32)

Concerns have recently been raised regarding the presence of certain components found in flaxseed. (34) The presence of especially protease inhibitors, phytic acids, linatin and cyanogenic glycosides in flaxseed is effective in the formation of these concerns. However, no harmful effects of these components have been reported in human studies. Concentrations of these components ingested with flaxseed in the diet may be below the level to induce any biological conditions of concern. (34) However, it is necessary to take this negative case into account. By employing effective methods such as plant bio-cultivation and/or food processing, this can be achieved by reducing the levels of components that are thought to have negative consequences for human health. (26)

Omega-3 (w-3) fatty acids play a crucial role in normal growth and development. It also has anti-inflammatory, anti-thrombotic, vasodilator, and anti-atherogenic properties and it beneficially modulates lipid metabolism. (35) Today, fish oil, which is rich in omega-3, and flaxseed oil, which is rich in ALA, are often used as nutritional supplements. For vegans, flaxseed oil and flaxseed

are one of the main sources of omega-3 fatty acids. Also, ALA is an essential fatty acid that must be included in the diet. (36)

Type 2 diabetes (T2D) is a complex endocrine metabolic disorder associated with various complications. There is substantial evidence that oxidative stress and inflammation are major factors for the development of T2D. (37) The risk of liver dysfunction is higher in T2D. Oxidative stress in diabetes develops due to the oxidation of glucose and excessive glycation of proteins, including hemoglobin, leading to the production of reactive oxygen species (ROS). (38) The primary defense against oxidative stress is regulated by antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) (Table 2, Figure 1). The enzymatic activity of these antioxidants is reduced in the diabetic state. Overexpression of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant-protein-1 (MCP-1) also play a role in the pathogenesis of T2D. In diabetes, excessive glycation of proteins including hemoglobin and albumin leads to the formation of advanced glycation end products (AGEs). It has been argued that AGEs are involved in various pathological processes in diabetes. (39) AGEs can induce inflammatory cytokines that stimulate oxidative stress and higher levels of inflammatory responses in various tissues. (39)

Table 2. Endogenous Antioxidants (40)

Non-enzymes	
Albumin	Binds Cu and Hem groups and removes HOCl from the medium
Ceruloplasmin	Binds Cu ions and enables reoxydation of Cu using H_2O_2
Transferrin	Binds iron ions in ferric forms (Fe^{3+})
Lactoferrin	Binds iron ions in ferric forms (Fe^{3+}) at low pH
Haptoglobin	Binds hemoglobin
Hemopexin	Binds Hem Group
Bilirubin	Clears peroxyl radicals
Glucose	Scavenges hydroxyl radicals (OH•)
Urate	Clears radicals and binds metals
Melatonin	Clears hydroxyl radicals (OH•)
Mucus	Clears hydroxyl radicals (OH•)
Those in enzymatic structure	
Superoxide dismutase (SOD)	Clears/removes superoxide radicals (O^{2-}) $O_2- + 2H^+ \rightarrow H_2O_2 + O_2$
Catalase (CAT)	Removes Hydrogen peroxide from the medium if it is at a high concentration $2H_2O_2 + O_2 \rightarrow 2H_2O + O_2$
Glutathione peroxidase (GPx)	Hydrogen removes from the medium if it is at a low concentration H_2O_2+2GSH → GSSG+$2H_2O$
Cytochrome oxidase	Prevents active oxygen from releasing into the medium during the reduction of oxygen to water, thus inhibiting the formation of ROS (H_2O_2, OH•, O^{2-})

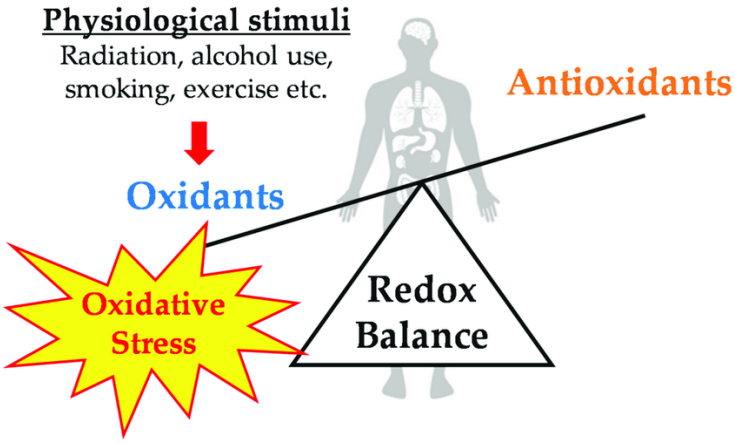

Figure 1. Oxidative Balance

It has been suggested that diet plays an important role in preventing T2D and that diet and dietary intervention may be beneficial in the management of diabetes. In this context, various nutrients including omega-3 fatty acids were investigated for their potential health benefits for diabetes. ALA, EPA, and DHA have been shown to have inhibitory effects on oxidative stress production. (41- 43) As is the case in fish oil (44), it has been observed that ALA from flaxseed oil (45) and EPA-DHA can suppress the production of TNF-α, IL-6, IL-16. Considering these findings, it is thought that increased oxidative stress and inflammatory formations can be corrected by the consumption of omega-3 fatty acids. The effects of flaxseed oil and fish oil diets on oxidative stress and inflammatory changes in the liver of streptozotocin-nicotinamide (STZ-NIC) rats were evaluated.

To the best of our knowledge, the effects of dietary flaxseed oil and fish oil on the hepatic gene expression of antioxidant enzymes, inflammatory markers, and the extent of protein glycation in STZ-NIC-induced diabetic rats have been demonstrated for the first time. (36)

Various epidemiological and experimental studies have been carried out using fish oil and its beneficial effects on diabetes have been shown. (46) However, there are few studies investigating the effects of dietary flaxseed oil on hepatic complications in STZ-NIC-induced T2D. The STZ-NIC rat model is characterized by conditions similar to T2D in humans and is essential for investigating the short- and long-term effects of drugs and natural compounds on diabetic complications. (47,48) Higher blood sugar levels are usually caused

by the excessive glycation of abundant proteins such as hemoglobin, albumin, and collagen. Glycated versions of these proteins are commonly associated with diabetes and its vascular complications. (49) Also, glycated hemoglobin levels serve as a pathological indicator of the progression of diabetes mellitus. (50) Persistent glycation in the sequences leads to the formation of compounds including carboxymethylicin (CML) and carboxyethylicin (CEL), which are well characterized as AGEs. Also, AGEs act as major proinflammatory agents through interactions with receptors for enhanced glycation end products (RAGE) and by inducing the expression of inflammatory cytokines, adhesion molecules, and matrix metalloproteinases (MMPs). (51) It has been reported that blood sugar levels were marginally lowered in rats receiving a flaxseed oil diet and a fish oil diet. (36) This is similar to previous studies showing partial control of hyperglycemia in STZ-induced diabetic rats with fish oil. (52)

However, both dietary flaxseed oil and fish oil were able to reduce the glycoprotein levels of diabetic rats, preventing further increases in oxidative stress and inflammation. (36) The flaxseed oil diet also reduced glycosylated albumin levels in rats. (36) The effect of SOD-1, GPx-1, CAT, PON-1, HO-1 on mRNA expression in the liver of rats induced with flaxseed oil or fish oil STZ-NIC rats and their effects on TNF-α, IL-6, MCP-1, INF-cand, NF-JB p65 subunits were examined. (36)

The TBARS level is a widely recognized marker of oxidative damage and antioxidant status. It is now clearly known that hyperglycemia diabetes is responsible for increased ROS production, which leads to disruption of natural antioxidant defenses, oxidative damage to membranes, and increased susceptibility to lipid peroxidation. There are also reactive nitrogen species (RNS) produced which feature radicals including NO, and NO_2. The production of lipid peroxides by ROS has been hypothesized to be one of the primary causes of diabetes-induced damage. It has been shown that w-3 fatty acids in combination with vitamins E and C can reduce lipid peroxidation and prevent oxidative damage. (53) Similarly, it has been reported that dietary flaxseed oil or fish oil significantly reduced hepatic NO concentrations in addition to hepatic TBARS and plasma TBARS levels in diabetic rats. (36) SOD also scavenges free superoxide radicals in the toxicity associated with ROS, thus protecting the tissue against ROS. CAT and GPx catalyze the decomposition of HO to HO and O_2 and protect the tissue from oxidative damage. (54) Jangale et al. (36) stated that a flaxseed oil diet increased both hepatic SOD and hepatic CAT activity while fish oil increased CAT hepatic activity. The results of this study

showed that the flaxseed oil diet significantly up-regulated SOD, CAT, and GPx transcripts, while the fish oil diet was affected by regulating CAT and PON-1. (36) HO-1 is both an inducible isoform of the oxygenase system and an enzyme that is stimulated by oxidative and inflammatory attacks. (36)

It has been suggested that mRNA expression of HO-1 is a sensitive marker of cellular oxidative stress, and it has been shown that over-expression of HO-1 in the liver of diabetic rats can be reduced by antioxidant supplementation. (55) Also in the current study, diabetic control mice showed increased hepatic expression of HO-1. (36) In the study, it has been reported that a fish oil diet decreased HO-1 levels in rats, however, this decrease was statistically not significant. (36)

Inflammation is closely related to the effect of ROS. It was first suggested in 1997 that diabetes is an inflammatory disease state. (56) Previous studies have shown that inflammation in various tissues such as the liver, muscle, adipose tissue, and islets can reveal insulin resistance in T2D. (57) High expression of TNF-α interferes with insulin signaling pathways and is associated with an insulin-resistant state in T2D patients. (58) IL-6 is a pleiotropic cytokine that plays a major role in vascular inflammation, and over-expression of IL-6 is associated with lipid peroxidation and inflammation. (59) MCP-1 is a proinflammatory chemokine and plasma levels of MCP-1 are significantly higher in patients with T2D. Over-expression of MCP-1 contributes to the development of insulin resistance in diabetes. (60)

INF plays a role in inducing the inflammatory response under diabetic conditions. (61) NF-JB is a proinflammatory nuclear transcriptional factor known to have a main role in regulating the expression of genes encoding proinflammatory cytokines. A vicious circle is established in which increased oxidative stress due to NF-JB, particularly increased ROS levels, and elevated TNF-induced levels of NF-JB activity, increase the expression of proinflammatory cytokines. This cycle can effectively destroy tissue parenchyma and impair its functions. Therefore, proinflammatory cytokines are suitable targets for controlling tissue damage. The mechanism where W-3 fatty acids alter gene expression results in the down-regulation of transcription factors such as NF-JB. (62) In a similar vein, both flaxseed and fish oil diets have been shown to cause significant down-regulation of TNF-α, IL-6, MCP-1, and INF transcripts. (36) Also, histopathological parameters revealed less degeneration in hepatocytes of rats receiving flaxseed oil and fish oil diet compared to diabetic control rats, which was associated with decreased expression of inflammatory genes and

oxidative stress in the liver. (36) FDA recommends 1-1.5 g/day of ALA intake. When ALA (flax oil) and EPA-DHA (fish oil) were calculated using the formula by Paget and Barnes (1964), the recommended human equivalent dose was 880 mg ALA and 300 mg EPA. As a result, it has been reported flaxseed oil and fish oil diets significantly decreased blood glucose, hepatic TBARS, plasma TBARS, and HbA1c levels. These diets also decreased hepatic inflammatory biomarkers and significantly reduced hepatic expression of inflammatory biomarkers. It was also determined that both diets increased hepatic CAT gene expression. (36)

However, dietary flaxseed oil and fish oil have shown some different effects. The flaxseed oil diet was found to be more effective in increasing the hepatic activities of SOD, CAT, and hepatic gene expression of SOD and GPx. (36) It was also found to be better at reducing hepatic NO concentrations. (36) However, the fish oil diet was found to be more effective in decreasing serum albumin glycation levels, decreasing hepatic HO-1 expression, and increasing PON-1 expression. (36) Omega-3 diets have proven beneficial in protecting against liver tissue injury and potential complications of STZ-NIC-induced diabetic rats. (36)

Metabolites, glutathione reductase (GR), glutathione-S-transferase (GST), glucose 6-phosphate dehydrogenase (G6PD), which underlie or are involved in the pathophysiology of the overproduction of ROS and glyco-lipo-oxidation products (AGES/ALES) in the initiation and progression of diabetic complications and endogenous antioxidants such as 6-phosphogluconate dehydrogenase (6PGD). (63-66) However, many antioxidant systems are deactivated by glucose autooxidation, overproduction of mitochondrial ROS, over-oxidation of enzymes, and the sorbitol pathway activated during diabetes. (67-70) On the contrary, some studies have reported that G6PD was over-expressed in the heart or hepatocytes taken from genetically diabetic or STZ-diabetic animals. (71, 72) Also, overproduction of G6PD has been shown in adipocytes in case of high glucose. (71)

Recent *in vitro* studies showed that pentose phosphate pathway (PPP) metabolites accumulate significantly during chronic glucose treatment due to prolonged inhibition of 6PGD, resulting in impaired pancreatic β-cell function. (73) In another previous study, it was shown that the activity of G6PD as well as 6PGD increased or did not change depending on the diversity of tissues such as brain, aorta, heart, liver, or kidney in STZ-diabetic rats. (65-67) Other previous studies showed decreased tissue GST activity in STZ-diabetic rats.

(74) Furthermore, it was suggested that an increased GST activity shown in STZ-diabetic rats was associated with the prevention of STZ and cells against the harmful effects of STZ. (75,76) Pharmacological interventions, including ethnopharmacology, are important in the management of diabetic complications. (77)

Studies showed that administration of flaxseed to diabetic rats or patients results in a reduction in blood sugar, triglycerides, very-low-density lipoproteins, and total cholesterol, and inhibits carbonyl compound production and carbonyl-protein adduct formation. (36, 78, 79) Flaxseed was shown to exhibit biological activities such as stimulating insulin secretion from the pancreas, increasing glucose utilization, and inhibiting the inflammatory course. (80, 81) Also, flaxseed is islet regenerative (80) and helps prevent diabetic complications by normalizing antioxidant status. (82)

In these studies, the importance of the antidiabetic properties of flaxseed was revealed, however, its regulatory effect on PPP and glutathione-dependent enzymatic activities remains unknown. Therefore, the effects of flaxseed supplementation on G6PD, 6PGD, GR, and GST activity in the brain, lung, pancreas, and lens of STZ-diabetic rats were investigated. (83) Intracellular redox homeostasis, determined by the action and signaling of oxidants and reducers, has been shown to play an important role in regulating cell survival and death. NADPH is a cofactor for NADPH-dependent oxidases (NO cethetase) and helps scavenge ROS. (66, 84, 85) Up-regulation of tissue PPP in diabetic animals may be due to oxidative stress related to a less-effective GSH. (63, 69, 86) Therefore, the NADPH-producing enzyme, G6PDH, has an important role in overcoming oxidative damage in diabetic tissues. (63, 66) Also, the effect of 6PGD regulated in the pancreas and lungs of untreated diabetic rats was shown. Accordingly, reverse 6PGD activity was decreased in the lens of untreated diabetic rats and remained unchanged in the brain. (83)

Previous studies have reported that antioxidant supplementation with vitamins is very important in cellular protection against glucose toxicity in diabetic animal models. (77, 87, 88) Also, it was shown that using natural essential fatty acids and different polyphenolic compounds with antidiabetogenic properties as supplements in animals and cells (89, 90) exerting their effects by protecting insulin-secreting cells or inhibiting the metabolic pathway that produces excessive sorbitol, and reducing the of AGES/ALES production or AGES-protein interactions. The researchers showed that up-regulation of G6PDH as a compensatory response to the pro-oxidant property of diabetic hyperglycemia

was prevented by flaxseed supplementation in the brain, pancreas, and lens of diabetic rats, but not in the lungs. (83) Flaxseed treatment has also been shown to be effective in regulating the activity of 6PGD in the diabetic group. (83) GR reduces glutathione disulfide (GSSG) to thiol and forms GSH. Changes in the GR activity can affect the cellular defense system and cause oxidative stress in cells. (92) Therefore, it can be argued that decreased-GR activity may contribute to the impaired thiol-disulfide redox state in cells by reducing the GSH/GSSG ratio, which has been widely discussed as an underrecognized mechanism of β-cell failure and diabetic complications. (80) It has been reported that GR activity significantly increased in the lungs and pancreas of non-diabetic treated rats compared to that in the control group. (83) The increased GR activity in the lungs and pancreas of rats who did not receive diabetes treatment can be evaluated as an adaptive response to increased oxidized glutathione (GSSG) production and GSH consumption in diabetes. (93) Flaxseed treatment has been shown to provide a beneficial effect of flaxseed as a regenerative islet or liver regenerative by improving PPP and glutathione-dependent enzyme activities in diabetic animals, especially with the regulation of GR activity. (80, 82) However, substantially unchanged GR activity was determined in the brain and eye lens of diabetic untreated rats. (83)

It was determined that while STZ injection caused a significant decrease in brain and pancreatic GST activity, flaxseed application preserved the GST activity. (83) It was determined that the effect of diabetes on GST varied depending on the organ types and the duration of diabetes. (94) Numerous studies have reported a significant decrease in GST activity in liver, kidney, brain, and pancreas tissues in 8-12-week-old diabetic rats. (95, 96) The significant decrease in pancreatic GST activity in untreated diabetics can be associated with the selectivity of the diabetogenic agent to the pancreas. This leads to oxidative damage through NADPH depletion. (97) As a contrary result, it was determined that GST activity in the lens of the eye and lungs was not affected in the case of diabetic hyperglycemia. (83)

GR reduces GSSG to the thiol form GSH. Changes in the GR activity can affect the cellular defense system and cause oxidative stress in the cell. (92) Therefore, it was thought that reduced GR activity may contribute to the impaired thiol-disulfide redox state in cells by reducing the GSH/GSSG ratio, which has been widely discussed as beta cell failure and poorly understood mechanisms of diabetic complications. (80) Significantly increased GR activity was determined in the lungs and pancreas of diabetic untreated rats compared to

control rats. The increased GR activities determined in the lungs and pancreas of untreated diabetic rats may reflect the adaptive response to increased GSSG production and GSH consumption in diabetes. (93) Flaxseed treatment partially but significantly improved the observed abnormalities in GR activity in diabetic animals, which is consistent with previous studies showing a beneficial effect of flaxseed as an islet or liver regenerative by improving PPP and glutathione-dependent enzyme activities. (80, 82) However, it was shown that significantly unchanged GR activity in the brain and eye lens of diabetic untreated rats probably had a sufficient capacity to protect the brain and lens of the eye against oxidative toxicity caused by STZ diabetes. (83)

It was thought that, while G6PD increased dramatically, diabetes leads to a significant reduction in eye lens 6PGD activity, a defensive response to NADPH depletion. Accordingly, it was shown to inhibit diabetic cataractogenesis or markers with antioxidant and aldose reductase suppressive activity. (70, 90, 91) There have been no studies showing the effect of flaxseed on the polyol pathway, but flaxseed consists of PUFAs and essential fatty acids, including linolenic and α-linolenic acids (82), which can affect eye lens aldolase reductase, sorbitol accumulation or cataract formation is seen under hyperglycemic conditions. (89, 90, 98-100) On the other hand, flaxseed has been identified as a beneficial antioxidant nutrient thanks to its high lignan content (82), contributing to the reduction of carbonylation or glyco-lipo-oxidation. (82, 101) Glycation or glyco-lipo-oxidation of enzymes can alter their activities and functions. (36, 78, 79, 102) Therefore, the results showing the protective activity of flaxseed against diabetes-induced degradation on PPP and glutathione-dependent enzymes may be related to the reduction of excessive carbonylation or glyco-lipo-oxidation. The curative effect of flaxseed on oxidative stress perception mechanisms has been reported to regulate antioxidant defense in diabetes by increasing some antioxidant genes and attenuating protein glycation. (36, 78, 79) Dietary consumption of some natural antioxidants (bioflavonoids, vitamin E, punicic acid, quercetin) was found to be associated with a reduced incidence of cataracts in humans and animals, however, the medical use of these compounds as therapeutic or nutritional in both cases has been limited to preventing or delaying cataracts. Therefore, it could not be implemented due to various challenges. (103)

Previous studies have reported that flaxseed intake reduces glucose and HbA1c and improves insulin sensitivity in diabetic patients. (78, 104) Enzyme levels such as AST and ALT are largely used by STZ to evaluate liver

damage. Diabetes caused by STZ caused a significant increase in ALT, AST and triglyceride levels, indicating significant hepatocellular damage. However, a significant improvement in AST, ALT, and triglyceride levels were observed with flaxseed treatment, indicating better utilization of glucose by hepatic tissue and the lipid-regulating effect of flaxseed as previously stated in hepatically injured animal models. (36, 79, 82) Flaxseed treatment also improved the body weight of diabetic animals and promoted overall improvements in glycemic and lipemic control with flaxseed. (83)

5. Conclusion

In the published literature, new findings were obtained about experimental diabetes-mediated disruptions in antioxidant defense systems, indicating that the ongoing oxidative stress in diabetes may be tissue-specific. To the best of our knowledge, these have been the first studies to determine the effect of flaxseed on tissue G6PD, 6PGD, GR, and GST in a diabetic animal model. In these studies, the protective effects of flaxseed (*Linum usitatissimum* L.) supplementation have been reported against oxidative damage in tissue G6PD, 6PGD, GR, and GST in addition to blood glucose, plasma triglyceride, ALT, and AST levels in diabetic rats.

In conclusion, there is evidence that the intervention of flaxseed can have a significant effect on the prevention of diabetic complications by improving oxidative modification PPP and glutathione-dependent enzyme activities in various tissues. Future studies should aim to examine the effects of flaxseed on the aldose reductase and polyol pathway and the efficacy of flaxseed on the possible inhibition of this pathway for the treatment of diabetes and to prevent or treat cataracts and other complications.

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CHAPTER XV

PROTECTIVE EFFECTS OF ANTIOXIDANTS AGAINST MALE REPRODUCTIVE SYSTEM TOXICITY

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1. Introduction

Developing technology in recent years, increasing environmental pollution, exposure to pesticides, alcohol consumption, cigarette smoking, and X-ray and ultraviolet (UV) radiation cause oxidative stress by changing the free radical levels and antioxidant activities, which are normally in balance in the organism, in favor of free radicals (1,2). Oxidative stress leads to various diseases such as infertility, atherosclerosis, heart diseases-myocardial infarction, cancer, diabetes, acute renal injury, bronchitis, liver diseases, rheumatoid arthritis, and emphysema in humans and animals (3). For this reason, it is important to consume exogenous antioxidants that prevent the harmful effects of free radicals.

Studies have shown that antioxidants have protective effects on many systems, including the reproductive system. This review will focus on the protective effects of antioxidants on the male reproductive system.

2. Mechanisms of Action of Antioxidants

1. Antioxidant compounds act through 6 different mechanisms:
2. They can reduce the local oxygen concentration by reacting with oxygen or replacing oxygen.
3. They can prevent radical formation by binding catalytic metal ions.
4. They can remove reactive oxygen species such as superoxide and hydrogen peroxide.

5. They can scavenge hydroxyl, alkoxyl, and peroxyl radicals.
6. They can exert their effects by converting peroxides to nonradical products such as alcohol. For example; glutathione peroxidase is an antioxidant that scavenges peroxides through this pathway.
7. They have an oxidant chain-breaking ability. They can react with free radicals that cause chain formation and prevent the release of hydrogen ions from fatty acid chains.
8. They can suppress or scavenge singular oxygen, which can form peroxide by affecting membrane lipids (4).

3. Classification of Antioxidants

Antioxidants are classified into two main groups: endogenous and exogenous antioxidants. Endogenous and exogenous antioxidants maintain provide oxidant/antioxidant balance through neutralize free radicals, and prevent the organism from the harmful effects of free radicals (5).

3.1. Endogenous Antioxidants

Endogenous antioxidants are classified into two subgroups as enzymatic and nonenzymatic antioxidants (6).

3.1.1. Enzymatic Antioxidants

Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (Glutathione-S-Transferase, GST), and heme oxygenase (HO) are enzymatic antioxidants (5)

3.1.1.1. Superoxide Dismutase (SOD)

It is an enzymatic antioxidant that forms the first line of defense against reactive oxygen species (5,6). SOD catalyzes the conversion of the superoxide radical (O_2^-), to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). Then hydrogen peroxide is removed by CAT in lysosomes or GPx in mitochondria (7).

There are three forms of SOD in humans. Superoxide dismutase (Cu/Zn SOD) (32 kDa) containing copper (Cu) and zinc (Zn) in the cytosol, superoxide dismutase (Mn-SOD) containing manganese (Mn) (96 kDa) in mitochondria, and extracellular superoxide dismutase (EC SOD) (135 kDa) in extracellular fluids is found (5,7).

The physiological function of the enzyme protects cells that catabolize oxygen against the harmful effects of superoxide radicals. Thus, it inhibits lipid peroxidation. Mn-SOD also has anti-tumoral activity. It shows this effect by suppressing the increase in the number of cancer cells and their growth (8). SOD is also involved in the intracellular killing of phagocytized bacteria. Therefore, SOD is crucial for granulocyte function. There is a higher amount of SOD in lymphocytes than in granulocytes (9).

3.1.1.2. Catalase (CAT)

Catalase is an enzyme found in plant and animal cells as well as aerobic bacteria. This enzyme is found mostly in the peroxisome and less in mitochondria and endoplasmic reticulum. CAT is one of the enzymes with the highest enzyme activity. While SOD converts the superoxide radical to H_2O_2, CAT also provides the conversion of H_2O_2 to H_2O and O_2 (10). Catalase, which is a hemoprotein containing four heme groups, has different tissue activities. The highest activity is detected in the liver and kidney, while the lowest activity is observed in the supporting tissue (11).

3.1.1.3. Glutathione Peroxidase (GPx)

GPX located in the cytosol is a metalloenzyme with four selenium atoms (5,8). GPx catalyzes the breakdown of H_2O_2 and lipid hydroperoxides (ROOH) using glutathione (GSH) as an electron source. In this way, it protects membrane lipids and hemoglobin against oxidative stress (12). While GPx converts reduced GSH to oxidized GSH, it also converts H_2O_2 to H_2O. While hydroperoxides are reduced by enzyme activity, glutathione is also oxidized (8).

The reduction in GPx activity leads to cell damage, which is particularly evident in selenium deficiency. Because selenium is an integral part of this enzyme. It protects the membranes against oxidative stress by reducing the phospholipid hydroperoxides (PL-OOH) in the membrane structure to alcohols, especially in cases of vitamin E (Vit E) deficiency (8,13).

3.1.1.4. Glutathione Reductase (GR)

Glutathione reductase is a flavoprotein enzyme containing flavin adenine dinucleotide (FAD). GR is converted back to reduced GSH by transferring an electron from NADPH to the disulfide bonds of oxidized GSH. NADPH, the most important source of which is the hexose monophosphate (pentose phosphate) pathway, is necessary to prevent free radical damage (6).

3.1.1.5. Glutathione Transferase (GST)

Glutathione transferase, which is dimeric, is mainly found in the cytosol and has many isoenzymes. GSTs, which have important roles in the biotransformation of foreign substances, catalyze the conjugation of various endogenous and exogenous compounds through glutathione (14).

3.1.1.6. Heme Oxygenase (HO)

Heme protein is involved in the structure of hemoproteins, which are composed of iron and protoporphyrin-IX complex and have very important functions in living cells (15).

Heme oxygenase (HO), one of the endogenous antioxidants, plays an important role in the regulation of intracellular heme levels. It is diffused within the endoplasmic reticulum. HO converts heme to biliverdin, carbon monoxide (CO), and free iron (Fe-II) by binding to the carbon of the heme molecule using NADPH and molecular oxygen, causing the oxidative breakdown of the porphyrin ring of heme. Biliverdin is converted to bilirubin through biliverdin reductase (16). Bilirubin acts as an antioxidant by preventing the peroxidation of cell membrane lipids. CO also has an anti-apoptotic effect as bilirubin. In addition, it causes relaxation in smooth muscles and vasodilation in vessels by activating guanylate cyclase (17,18).

HO has three isoforms, HO-1, HO -2, and HO-3. While HO-1 and HO-2 are detected in mammalian cells, HO-3 is specific only to rats (15).

3.1.2. Nonenzymatic Antioxidants

Nonenzymatic antioxidants are glutathione, melatonin, albumin, bilirubin, ceruloplasmin, transferrin, uric acid, coenzyme Q10, selenium, and α-lipoic acid (2).

3.1.2.1. Glutathione (GSH)

Glutathione, a water-soluble antioxidant and reducing agent, is a tripeptide that can be synthesized from glutamate, cysteine, and glycine, which are found at high levels in many tissues, especially the liver (12). GSH can be found mostly in the cytoplasm, but also in the mitochondria, nucleus, peroxisomes, and endoplasmic reticulum (19).

GPx is a substrate or co-substrate of enzymes such as GR and GST. GSH detoxifies lipid peroxides and H_2O_2 by its catalytic effect or removes singlet

oxygen (O_2^-) and OH. In addition, GSH provides amino acid transport from the plasma membrane (5).

3.1.2.2. Melatonin

Melatonin, which is produced in the pineal glands, plays an important role in cell renewal, boost the immune system, regulation of sleep rhythm and body temperature, as well as having a very strong antioxidant effect (20).

Melatonin shows its antioxidant effect by eliminating the hydroxyl radical (OH). Since melatonin has a lipophilic feature, it can reach all organelles and nuclei of the cell, and moreover, it shows a wide range of antioxidants by easily crossing the blood-brain and blood-testis barrier. The decrease in melatonin production with aging is thought to be important in the pathogenesis of aging and aging-related diseases (21). Melatonin strengthens cellular membranes and thus may assist the cell membrane in resisting oxidative damage (22).

3.1.2.3. Albumin

The myeloperoxidase enzyme released from neutrophils and monocytes catalyzes the formation of hypochlorous acid (HOCl), a strong oxidant compound. Albümin prevents the alteration of the α-antiproteazın at the primary biological target of HOCl by eliminating HOCl oxidants (23).

3.1.2.4. Bilirubin

Bilirubin acts as an antioxidant, inhibiting lipid peroxidation as well as scavenging superoxide and hydroxyl radicals (24).

3.1.2.5. Ceruloplasmin

The ferrous ion (Fe^{+2}), causes oxidative stress by catalyzing the conversion of H_2O_2 to OH^- by the Fenton reaction (25).

3.1.2.6. Transferrin

Transferrin binds circulating free iron and acts as an antioxidant by preventing the Fenton reaction (25).

3.1.2.7. Uric Acid

HOCl is an antioxidant molecule that acts by scavenging reactive oxygen species such as OH^-, O_2^- and lipid peroxyl (LOO) and by binding and neutralizing iron and copper ions (26).

3.1.2.8. Coenzyme Q10 (CoQ10)

Coenzyme Q10 (ubiquinone), which is synthesized endogenously in all tissues, is a fat-soluble vitamin-like 1,4-benzoquinone compound (27). CoQ10, which has an important role in energy production in the cell, also shows a very strong antioxidant activity (28). CoQ10 interacts with oxygen-based radicals and singlet oxygen and shows its antioxidant effect by preventing the initiation of lipid peroxidation and biomolecular damage (29).

3.1.2.9. Selenium (Se)

Selenium (Se), which is a very important essential mineral for humans and animals, participates in the structure of many enzymes as a cofactor. It plays a role in antioxidant enzyme defense, regulation of the immune system, thyroid hormone mechanism (30,31), reduction of heavy metal toxicity, cell growth, and physiological events such as selenocysteine, coenzyme Q, glutathione peroxidase (GPx), and thioredoxin reductase synthesis (32,33). Selenium exerts its antioxidant effect by increasing GPx activity and suppressing ROS formation (34).

3.1.2.10. Alpha Lipoic Acid (ALA)

Alpha lipoic acid, called 1,2-dithiolane-3-pentanoic acid, is a molecule that can be synthesized naturally in the liver (35). ALA is both water and fat soluble and is synthesized in mitochondria from octanoic acid and a sulfur source (36). ALA, which is rapidly absorbed from the small intestine, is reduced to dihydrolipoic acid (DHLA) by ADH, dihydrolipoyl dehydrogenase, and GR in the liver (37). Both the oxidized form and the reduced form (DHLA) of ALA show antioxidant activity (38).

ALA acts as an antioxidant by scavenging hydroxyl hypochlorous acid, peroxynitrite anion, and singlet oxygen. DHLA also exerts an antioxidant effect by scavenging superoxide and peroxyl radicals (39).

3.2. Exogenous Antioxidants

Vitamin E (α-Tocopherol, Vit E), Vitamin A (β-carotene, Vit A), Vitamin C (ascorbic acid, Vit C), and Vitamin B9 (folic acid, FA) are vitamin-derived antioxidants taken exogenous (2).

3.2.1. Vitamin E (Alpha Tokoferol, Vit E)

Vitamin E is a fat-soluble vitamin containing tocopherol derivatives and has a high antioxidant potential. It naturally exists in various forms such as alpha,

beta, gamma, delta, eta, and zeta. Alpha-tocopherol has the highest antioxidant activity (40). The antioxidant capacity of Vitamin E is strong and very wide. It performs its antioxidant effect by using all mechanisms such as the destruction of oxidant radicals, breaking the chain, and strengthening the endogenous defense systems (2).

3.2.2. Vitamin A (Vit A, Beta-Carotene)

Beta-carotene is the metabolic precursor of vitamin A and is involved in the LDL structure. It acts as an antioxidant by protecting LDL against oxidation. It shows this effect by suppressing the harmful effects of stimulants such as flavins and porphyrins, suppressing singlet oxygen, and scavenging peroxyl radicals (41, 42).

3.2.3. Vitamin C (Vit C, Ascorbic Acid)

Vitamin C is a water-soluble and antioxidant vitamin. It protects the cell membrane and DNA against oxidant damage by neutralizing superoxide and hydroxyl radicals. In addition, it prevents the inactivation of antiproteases by oxidant substances (8). Vit C also shows an oxidant behavior by converting Fe^{+3} to Fe^{+2}, which increases lipid peroxidation (2).

3.2.4. Vitamin B9 (Folic Acid, FA)

Vitamin B9, a water-soluble vitamin, has a very strong antioxidant effect by scavenging reactive oxygen radicals (43). In addition, it is necessary for DNA synthesis, the production of red blood cells and normal fertility in men and women (44).

4. Experimental Studies Showing the Protective Effects of Antioxidants Against Male Reproductive System Toxicity

4.1. Experimental Studies Related to Melatonin

Low melatonin level in the seminal plasma has been observed in infertile patients with poor sperm motility, leukocytospermia, varicocele, and non-obstructive azoospermia, and has been associated with oxidative stress in the male reproductive system (45). Intraperitoneal injection of melatonin in rats with varicocele has been shown to reduce oxidative stress in the testis (46).

Moradi et al. (2021) determined that injection of bleomycin (0.5 mg/kg), etoposide (5 mg/kg), and cisplatin (1 mg/kg) (BES) in male rats significantly reduced testicular weight, body weight, serum testosterone level, sperm count,

sperm viability, sperm motility, and testicular total antioxidant capacity. In addition, it has been suggested that it significantly increases abnormal spermatozoa levels (%), testicular MDA and NO levels. In addition, in histological examinations, they found that it decreased seminiferous tubule height, seminiferous tubule diameter, and immunohistochemically the level of anti-apoptotic Bcl-2 gene expression, and increased apoptotic p53 and TNF-α expression levels. On the histopathological examination, they determined that BES injection also caused necrosis and atrophy in the seminiferous tubules as well as loss of interstitial connective tissue. Against BES-induced testicular toxicity, 10 mg/kg and 20 mg/kg doses of melatonin injection have been shown to significantly reduce the toxic effects of BES, and it has been established that melatonin has protective effects on the male reproductive system (47).

In studies conducted in rats and mice, melatonin supplementation (20 mg/kg, i.p.) decreased significantly testicular damage and apoptotic cell number in the testis that had been increased by the heat stress (39 and 42 °C). Thus, it was concluded that melatonin treatment may be beneficial to protecting the testis from damage caused by heat stress and to supporting male reproductive health (48, 49).

In another study, bisphenol S administration (75 mg/kg, by gavage) to hamsters for 28 days decreased testicular weight, body weight, serum testosterone level, sperm count and viability, seminiferous tubule height and diameter, immunopositive PCNA cell count, testicular SOD and CAT activities, and levels of testicular HO-1, connexin-43 and Bcl-2 expression, as well as to the contrary, increased level of testicular MDA and caspase-3 expression. In addition, it was observed atrophied Leydig cells, regressed seminiferous tubules, vacuole formations in the seminiferous tubule epithelium, and giant germ cells in histopathological examination. In this study, it was shown that melatonin administration (10 mg/kg, by i.p.) significantly reduced the testicular damage induced by Bisphenol S, and the protective effects of melatonin were demonstrated (50).

4.2. Experimental Studies Related to CoQ10

Ayengin et al. (2020) found that CoQ10 administration in rats with testicular ischemia/reperfusion model improved histologically damaged testicular structure (degeneration and necrosis in germ cells, edema in the interstitial area, and dilatation in blood vessels), increased the decreased seminiferous epithelium height, and decreased the increased serum IL-1, IL-2 and MDA levels, levels

of testicular MDA and immunohistochemically caspase-3 and caspase-8 expressions. In the presented study, it was suggested that CoQ10 administration has a protective effect against testicular ischemia/reperfusion by suppressing oxidative stress, reducing pro-inflammatory cytokine levels, and inhibiting the caspase-3 and caspase-8 apoptosis pathway, and it can be used as a supportive treatment in the testicular ischemia/reperfusion injury (51).

In a study in rats, It has been determined that CoQ10 supplementation at a dose of 10 mg/kg (i.p.) increased the decreased plasma testosterone and FSH levels, testicular GSH concentration, testicular SOD, CAT, GPx, and GR activities, and anti-apoptotic Bcl-2 level with 20 mg/kg lead acetate administration intraperitoneally for 7 days. It was also determined that CoQ10 treatment decreased the increased lipid peroxidation, testicular TNF-α and IL-1β levels, and levels of apoptotic Bax and caspase-3 expression with lead acetate application. On the other hand, degeneration of spermatogenic cells, separation of spermatogenic epithelium cells from the basement membrane and the appearance of vacuoles in the seminiferous tubule epithelium were detected in the histopathological examination of the testis of rats injected PbAc. It was determined that CoQ10 supplementation repaired histopathological abnormalities and preserved normal testicular histological structures. The present study demonstrated that CoQ10 may act as a natural therapeutic agent to protect against lead acetate-induced reproductive impairments (52).

Delkhosh et al. (2021) determined that heat stress at 43 °C for 20 minutes every other day for 8 weeks in rats decreased the level of anti-apoptotic Bcl-2 expression, testicular SOD, CAT, and CPx activities, and serum testosterone level, as well as increased testicular MDA level, relative mRNA expressions of caspase-3 and Bax genes and proinflammatory cytokine activations (IL-1β and TNF-α). It was determined that when rats were treated with CoQ10 (10 mg/kg, by i.p.) before heat stress application, the harmful effects of heat stress on the mentioned parameters were reduced. In the presented study, it was revealed that CoQ10 could suppress degenerative effects following testicular hyperthermia with its anti-apoptotic, anti-inflammation, and antioxidative effects (53).

Tsao et al. (2021), it was observed that CoQ10 administration (10 mg/kg, by oral gavage, for 8 weeks) in an experimental chronic kidney mouse model increased testicular CAT and GPx activities, serum testosterone level, seminiferous tubule epithelium height, and sperm count and motility. In the presented study, it has been shown that CoQ10 improves testicular dysfunction

and low sperm quality due to its antioxidant effect against chronic kidney disease that causes infertility in men (54).

In another study conducted in rats, BPA administration at a dose of 100 mg/kg (i.p.) by oral gavage for 14 days decreased serum testosterone level, sperm viability, testicular GSH activity, and histomorphometrically stage VII-VIII seminiferous tubule diameter and epithelium height, and increased testicular MDA level, head and neck piece abnormal sperm ratios, TUNEL positive cells (apoptotic cells) per seminiferous tubule, and tunnel positive tubules (%). It was determined that when rats were treated with CoQ10 (10 mg/kg, by gavage for 14 days) before BPA treatment, the harmful effects of BPA on the mentioned parameters were reduced. In the presented study, it was suggested that CoQ10 supplementation has a protective effect against induced-BPA testicular toxicity in rats (55).

4.3. Experimental Studies Related to Selenium

Recent studies have shown that selenium deficiency may be associated with infertility, aging, cancer, insulin resistance, diabetes, cardiovascular and neurodegenerative diseases, increased mortality risk, and immune system diseases (56,57).

Khoshbakht et al. (2021) determined that serum FSH, LH, and testosterone levels, seminiferous tubule diameter and epithelium height, and sperm count were significantly decreased, and abnormal sperm count and immunohistochemically caspase-3 expression increased in rats exposed to electromagnetic field (2100 MHz) for 2 hours daily for 16 days. It was determined that when rats were treated with selenium (0.2 mg/kg, for 16 days) after exposure to the electromagnetic field, the harmful effects of the electromagnetic field on the mentioned parameters were reduced. In the presented study, it has been shown that the harmful effects of electromagnetic radiation on the male reproductive system can be prevented by the use of selenium (58).

In a different study in rats, it determined that the selenium treatment (0.8 mg/kg) before irradiation by cobalt-60 gamma-ray source decreased histopathological changes such as spermatogenic arrest in the testis and epididymis, atrophy of the seminiferous tubules, thickening of the basal lamina, Leydig cell hyperplasia, edema, reduction of epididymal sperm density and epididymal vacuolation (59).

Owumi et al. (2020) determined that selenium administration (0.125 and 0.250 mg/kg) against induced-diclofenac (10 mg/kg, by gavage, for 14

days) toxicity in rat testes increased the decreased serum LH and testosterone levels, testicular and epididymal SOD, CAT, GSH, GST and GPx activities, sperm count and motility, as well as decreased the increased MPO activity and testicular and epididymal NO, LPO and RNOS levels. In addition, it was determined that selenium co-administration (0.250 mg/kg) significantly reduced interstitial congestion in the testis and the presence of immature germ cells in the epididymis lumen caused by diclofenac in the histopathological examination. In the presented study, it was suggested that selenium co-administration against diclofenac-induced reproductive toxicity improves testicular and epididymal functions by suppressing nitrosative and oxidative stress (60).

Kaur et al. (2020) reported that BPA administration (1 mg/kg, by gavage) in mice decreased antioxidant enzyme activities (SOD, CAT, redox ratio), and increased the expressions of oxidative stress-activated kinases (c-Jun NH_2-terminal kinase, extracellular signal-regulated kinase, and p38) and the expression of proapoptotic genes (caspase-3, caspase-8, and caspase-9). However, they determined that selenium supplementation (0.05 ppm, by gavage) increased antioxidant enzyme activities, and increased the activation of oxidative stress-activated kinases and pro-apoptotic markers. Thus, it demonstrated that Se ameliorated the negative effects of BPA on mouse testis by its antioxidant effect (61).

In another study in mice, it was determined that cadmium chloride administration (0.35 mg/kg, i.p., for 30 days) decreased testicular SOD, CAT, GR, and GST activities and serum testosterone level, and increased DNA damage in testis. In addition, histopathological examination revealed that Cd administration caused thickened basement membrane and few layers of spermatogenic cells without sperm. In addition, it was determined that selenium supplementation repaired histopathological abnormalities and DNA damage in the testis by increasing the enzymatic antioxidant activities (62).

4.4. Experimental Studies Related to ALA

The positive effects of ALA have been observed in various pathological conditions such as infertility, diabetes, atherosclerosis, neuron degeneration, and multiple sclerosis due to its antioxidant effects (63).

Tohamy et al. (2022) determined that silver nanoparticle injection (AgNPs, 50 mg/kg, by i.p.) in rats decreased testicular GSH, GR, and GPx activities, serum testosterone level, testicular antioxidant thioredoxin-1 (Tnx-1), TGF-1β and anti-apoptotic Bcl-2 expression levels, as well as increased testicular MDA

level and apoptotic Bax expression level. In addition, it has been determined that ALA supplementation (100 mg/kg, by gavage) is beneficial in reducing the harmful effects of AgNPs on testicles due to the antioxidant, anti-inflammatory, and anti-apoptotic effects of ALA (64).

In another study, it reported that testicular SOD, CAT, and GPx activities, serum testosterone level, anti-apoptotic Bcl-2 and steroidogenic gene expressions (StAR and 3β –HSD) decreased, and testicular MDA level, apoptotic Bax and caspase-3 expressions, and protein levels of endoplasmic stress markers [(glucose-regulated protein 78 (GRP78) and CCAAT/enhancer binding protein homologous protein (CHOP)]) increased in chicken testis expose to heat stress (32 °C) for 14 days. It was determined that ALA dietary supplement (500 mg/kg diet) in chicken expose to heat stress reduced the harmful effects of heat stress on the mentioned parameters. In the presented study, it was revealed that ALA can reduce heat-induced testicular damage and ameliorate reproductive performance in chickens under heat stress (65).

Elbakary et al. (2020) determined that polychlorinated biphenyl (PCB) treatment at a dose of 5 mg/kg by gavage for 30 days in albino rats decreased the serum testosterone level, as well as caused large spaces between the seminiferous tubules, partial or complete separation of the seminiferous tubule epithelium from the basement membrane and irregularly shaped the seminiferous tubule structure. Besides, in electron microscopic examination, vacuolation in Sertoli cell cytoplasm and dilatation in rER, cytoplasmic vacuoles and shrinkage in primary spermatocytes, and vacuoles and destruction of mitochondria in Leydig cells were observed. It was determined that when rats were treated with ALA (25 mg/kg, by gavage) before PCB treatment, the harmful effects of PCB on the mentioned parameters were reduced by the antioxidant effect of ALA. Thus, in this study, it demonstrated that ALA has a protective effect against PCB-induced testicular toxicity (66).

Shaygannia et al. (2018) determined that ALA supplementation in rats with varicocele increased the decreased sperm concentration and motility, and decreased the increased lipid peroxidation and DNA damage. In the presented study, it was suggested that ALA has suitable potential for the treatment of infertile men with varicocele (67).

Gules and Eren (2016) found that PCB treatment (5 mg/kg, by gavage, for 30 days) in rats increased TUNEL positive cell count and total oxidant capacity in serum and testis, and also caused histological changes such as vacuolization in the tubule epithelium and immature germ cells in the tubule lumen. It was

determined that when rats were treated with ALA (25 mg/kg, by gavage, for 30 days) before PCB treatment, the harmful effects of PCB on the mentioned parameters decreased. In the presented study, it suggested that ALA has a protective effect against PCB-induced testicular toxicity (68).

4.5. Experimental Studies Related to Vit E (α-Tokoferol)

Qari et al. (2021) determined that body weight, serum testosterone level, and CAT activity decreased in rats exposed to heat stress (41 °C) for 1 hour daily for 14 days. On the contrary, it was founded that Vit E supplementation (100 mg/kg, by gavage) before heat stress increased body weight, serum testosterone level, and serum CAT activity. However, it was found that increased serum MDA level and GPx, SOD, and GSH activities, and seminiferous tubule diameter in heat stress did not affect by Vit E treatment. Thus, in the presented study, it was demonstrated that Vit E can partially protect against male gonadal harm caused by heat stress, and long-term exposure of the body to heat stress is harmful to male fertility and should be avoided (69).

Ojo et al. (2021) determined that Vit E supplementation in rats with diabetes causing mitochondria-mediated apoptosis significantly decreased immunohistochemically cytochrome c, caspase-3, and caspase-9 levels, and also increased serum testosterone level. In this study, it was revealed that Vit E provides protection against induced-diabetes testicular damage by its free radical scavenging and can be used as a pharmacotherapeutic agent (70).

In a study conducted in rats, it was determined that cadmium administration (2 mg/kg, by i.p., for 28 days) decreased the anti-apoptotic Bcl-2, mitochondrial membrane fusion 1 (Mfn1), and Mfn2 expression levels in the testis, and increased the testicular Bax and caspase-9 expressions. It was determined that Vit E injection (100 IU/kg, i.p., for 28 days) against induced-cadmium testicular toxicity increased the Bcl-2, Mfn1, and Mfn2 expressions in testicles and decreased testicular Bax and caspase-9 expressions. Thus, in the presented study, it was demonstrated that the protective effect of Vit E against cadmium, which causes apoptosis through the mitochondrial route (71).

Babaei et al. (2020) found that Vit E administration in rats infected with Candida albicans increased the reduced sperm motility, number and vitality, testis and seminiferous tubule volumes, Leydig and Sertoli cell counts, plasma LH and testosterone levels, plasma and testicular total antioxidant capacity, and decreased the increased plasma and testicular MDA levels. In the presented study, it has been clearly shown that Vit E is effective in reducing the harmful

effects of Candida albicans infection on male fertility and can be used as a therapeutic agent (72).

In another study conducted in rats, it was determined that doxorubicin injection (3 mg/kg, i.p.) increased the immunohistochemically apoptotic index in the testis and epididymis, sperm and testicular total oxidant capacities, and decreased sperm viability, motility and number. In addition, it was founded that a very intense degeneration, irregularity and vacuolization in the seminiferous tubule epithelium, a decline in germinal cells, and immature germinal epithelium cells in the tubule lumen were observed in histopathological examination. Thus, it was suggested that Vit E supplementation (50 mg/kg) to drinking water has a protective effect against induced-doxorubicin testicular toxicity (73).

4.6. Experimental Studies Related to Vit A (β-Carotene)

Yokota et al. (2019) found that long-term (for 7 weeks) dietary intake of Vit A (1,000 IU/g) reduced body and testis weights, sperm count, viability and motility, and seminiferous tubule area, and increased abnormal sperm rate and serum testosterone level. In addition, they detected vacuolization in the seminiferous tubule epithelium and the immature germ cells in the lumen in the histopathological examination (74).

Arena et al. (2018) determined that cyclophosphamide injection at a dose of 100 mg/kg (i.p.) once a week for 5 weeks significantly reduced seminal vesicle and ventral prostate weights, daily sperm production and testicular sperm count, testicular SOD, GPx and CAT activities and epididymal CAT activity, as well as increased epididymal LPO level. However, it was determined that β-carotene supplementation (10 mg/kg) reduced the testicular damage induced by cyclophosphamide by its antioxidant effect (75).

In another study in rats, it was determined that β-carotene supplementation against induced-favism testicular damage increased the decreased body and testis weights and serum testosterone level, and decreased the increased serum FSH and LH levels. In addition, it was shown that β-carotene supplementation ameliorated damage at the basal membrane and epithelium of the seminiferous tubule (76).

4.7. Experimental Studies Related to Vit C (Ascorbic Acid)

Pal et al. (2022) determined that sodium fluoride exposure (15 mg/kg, by gavage, for 30 days) decreased testicular weight, sperm count, motility and viability, testicular and epididymal SOD and CAT activities, and also increased

testicular and epididymal MDA levels. In addition, they detected testicular DNA fragmentation by comet assay, seminiferous tubule damage, and immature germ cells in the seminiferous tubule lumen. In addition, it was shown that Vit C (200 mg/kg) and Vit E (400 mg/kg) supplementation against induced-sodium fluoride testicular toxicity reduced reproductive damages by their antioxidant effects (77).

Rauf et al. (2021) determined that Vit C and E nanoparticle supplementations (50 mg/kg and 100 mg/kg, gavage) against reproductive toxicity induced by cisplatin (3 mg/kg, by i.p.) significantly increased the reduced body, testis, prostate, and seminal vesicle weights, serum testosterone level and seminiferous tubule area and epithelium height. In the presented study, it was suggested that Vit C and E nanoparticle supplementations in male patients underlying chemotherapy were beneficial in reducing cisplatin-induced reproductive toxicity (78).

In another study, it was determined that Vit C supplementation (100 mg/kg, i.p., for 7 days) against the testicular damage in rats induced by dexamethasone (DEX, 7 mg/kg, by i.p., for 7 days) increased the reduced spermatid count, sperm motility, daily sperm production, and serum testosterone level, as well as decreased the increased testicular MDA level. In addition, it was determined that Vit C supplementation improved testicular damage such as apoptosis of Leydig cells, irregular and vacuolated seminiferous tubules, and less compact arrangement of spermatogenic cell occurred by DEX treatment. Thus, it has been demonstrated that Vit C, a powerful antioxidant, can ameliorate all the harmful effects that occurred by DEX treatment on the reproductive system (79).

Shen et al. (2018) found that di-(2-ethylhexyl) phthalate (DEHP) administration (500 mg/kg) to immature male rats decreased protein expressions associated with the blood-testicular barrier (BTB) such as connexin43 (Cx43), zonula occludens-1 (ZO-1) and occludin by Western blot and immunofluorescence analyzes, and damaged seminiferous tubules in histological examination. It was determined that Vit C (100 mg/kg) and Vit E (200 mg/kg) supplementation against the testicular damage in rats induced by DEHP treatment increased the reduced protein expressions associated with the blood testicular barrier, healed damaged seminiferous tubules. In the presented study, it was revealed that Vit C and Vit E supplementation can prevent spermatogenesis dysfunction, BTB deterioration, and oxidative stress caused by DEHP (80).

Artıran et al. (2017) determined that gentamicin injection (5 mg/kg, by i.p., for 14 days) in rats caused histopathological changes such as degeneration of the

seminiferous tubule epithelium, congestion, and edema in the interstitial area, thinning of the basement membrane and tubular atrophy. In the presented study, it was suggested that vitamin C (200 mg/kg, i.p.) given with gentamicin daily for 14 days reduced the testicular damage caused by gentamicin.

4.8. Experimental Studies Related to FA

Rad et al. (2021) showed that FA supplementation (1 mg/kg, by i.p.) against testicular damage induced by 3,4-methylenedioxymethamphetamine (MDMA) administration (10 mg/kg, i.p.) increased the reduced testicular weight, seminiferous tubule diameter, sperm concentration, motility and viability, testicular CAT and SOD activities and serum FSH levels, as well as reduced the increased testicular MDA level and serum LH and testosterone. In the presented study, it was demonstrated that FA could reduce the negative effect of MDMA on fertility in adult male rats (82).

Gules et al. (2019) determined that FA supplementation (20 mg/kg, by gavage, for 14 days) against testicular damage induced by BPA (50 mg/kg/day, by gavage, for 14 days) in adult rats increased the decreased stage VII-VIII seminiferous tubule epithelium height, serum testosterone level, and sperm viability, and decreased the increased TUNEL (+) cell count (apoptotic cell) and abnormal sperm rate. In the presented study, it was revealed that the toxic effects of BPA on the testis can be minimized by FA treatment (83).

Sakr et al. (2018) showed that FA supplementation (1.1 mg/kg, by gavage, for 14 days) against testicular damage induced by methomyl treatment (1 mg/kg, by gavage, for 14 days) ameliorated the testicular damage such as vacuolization, and desquamation in the seminiferous tubule epithelium, increased the decreased serum testosterone level, total antioxidant capacity, sperm count, motility and vitality and seminiferous tubule epithelium height, and decreased the increased testicular MDA level, abnormal sperm ratio, and caspase-3 immunoreactivity. The present study suggested that FA supplementation may have a beneficial effect in preventing or reducing the detrimental effects of methomyl exposure in albino rats on testicular structure and function (84).

Fakouri et al. (2017) showed that folic acid supplementation (2, 5, and 10 mg/kg, by gavage, for 7 days) in rats with testicular ischemia/reperfusion model improved histologically the testicular structure (tubular degeneration), increased the decreased sperm viability and motility, as well as testicular SOD and GPx activities, decreased the increased testicular MDA level. In the presented study, it demonstrated that FA can be used as an important therapeutic agent in testicular ischemia/reperfusion injury (85).

5. Conclusion

Toxic substances such as PCB, cadmium chloride, DEHP, lead acetate, and Bisphenol A and S, exposure to heat stress and electromagnetic radiation, the testicular ischemia/reperfusion injury, chronic kidney disease, varicocele, diabetes, Candida albicans infection, and drugs such as doxorubicin, cyclophosphamide cisplatin, dexamethasone, gentamicin, MDMA, methomyl, and diclofenac cause structural and functional damages on the male reproductive system. In studies conducted animals and men, it was determined that antioxidant supplementation reduced the harmful effects of toxic substances, various diseases, and some drugs on the male reproductive system by their antioxidant, anti-apoptotic and anti-inflammatory effects. Thus, it was suggested that antioxidants have protective effects against on the male reproductive system toxicity (45-85).

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CHAPTER XVI

PSYCHOLOGICAL AND OXIDATIVE STRESS EFFECTS DURING THE COVID-19

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1. Psychological Stress

In the modern world, stress is believed to be a part of daily life and stress is considered to be responsible for many acute or chronic health disorders. Stress, which has many definitions, has different meanings to different people under different circumstances. According to the first and broadest definition made by Hans Selye, "stress is the body's non-specific response to any demand." In the behavioral sciences, stress is expressed as "perception of threat, resulting anxiety, emotional tension, and difficulty adjusting". In terms of neuroendocrinology, stress can be defined as any stimulus that triggers cortisol release (1).

While mild stress can be very beneficial as it can increase task and cognitive performance, continuous and high-intensity stress leads to anxiety and depression (2). Accordingly, it is possible to define stress as "a physical, chemical or emotional factor that causes bodily harm and usually leads to illness".

On the other hand, if we focus on stress as an "emotional factor", the relationship between stress and anxiety, depression and cognitive dysfunction will come to mind. If we focus on stress as a physiological stressor, this time we are reminded of hypertension and other cardiovascular conditions. Studies show that stress is a trigger for the co-occurrence of physiological or emotional states.

Several attractive theories have been proposed to address the biochemical basis of these observations, one of which is the oxidative stress theory (3).

2. Oxidative Stress and COVID-19

Oxidative stress can be caused by physical(head cold, effort, anxiety, insomnia), Chemicals wastes (heavy metals, industrial chemicals), viral (hepatitis viruses, HIV/AIDS, SARS, MERS viruses) or microbial origin(Mycobacteriumtuberculosis, Strepylobacter and Salmonella). These, in turn, result in cellular damages in the functions of nucleic acids lipid peroxidation, and cell death(4,5). With yhe understanding that oxidative stress is the leading cause of both biological(bacteria and virüs) and toxic chemical-based diseases, an increase in the interested of the society towards oxidative stress has been observed. Although the long-term effevts of the disease are not known today, the lack of a definitively approved treatment is still one of the factors that trigger stress. The COVID-19 virus, which is a single-stranded positive polarity, enveloped RNA type seen in all countries of the World today, is considered the seventy identifies coronavirus(CoV) that can infect humans, and the oxidative stress it creates is too great to be ignored. While traditionally thought oxidants exert their effects through direct toxic effects on target cells, recent studies have also suggested a contributing role of oxidants in gene induction(6). On 31 January 2020, WHO declared 2019-nCoV to be a public health international emergency (PHEIC). The main clinical symptoms in infected patients are fever, dry cough, fatigue, myalgia, and shortness of breath. As the severity of the disease increases, respiratory symptoms become more pronounced(7-9). The main organs involved in the disease are the lungs. The disease starts as “silent hypoxemia” in the early period and then progresses to severe acute respiratory failure syndrome (ARFS) with the release of proinflammatory cytokines triggered by the virüs. Proxidative damage to lipids and macromolecules in cell membranes due to oxidative stress directly on many organs, especially the lung, in COVID-19 infected patients is significant. Indirecly, reactive oxygen metabolites (superoxide anion(O2-), hydrogen peroxide (H_2O_2), hydroxyl radical(OH-), singlet oxygen(O_2), nitric acid(NO), peroxyl radical (ROO), hydroperoxyl radical (HO_2), alkoxyl radical(RO) induced changes in cellular membranes and components can alters physiology and possibly results in pathology(10-14).

RNA viruses disrupt the intracellular oxidative balance by increasing the production of reactive oxygen and nitrogen species (RONS) by various

mechanisms and accelerating the consumption of antioxidants. Studies Show that cytokine dysregulation, also called "cytokine storm" causes a rapid influx of pathogenic CoVs, infection of infammatory cells, followed by an increase in the production, cytokine expression and release of reactive oxygen species that lead to acute lung injury, contributing to the disease (9,13,15). In addition, it is stated that local oxidative stress may play a rol in maintaining the local pulmonary inflammatory response through gene induction in alveolar macrophages isolated from patients with acute respiratory distress syndrome (ARDS) (16).

Studies also Show that the infection causes a rapid flow of inflammatory cells. Ingeneral, RNA viruses are effective in reducing the levels of antioxidant molecules such as ascorbic acid, carotenoids and reduced glutathione, as well as in the emergence of changes in the body's antioxidant defense system, which also affects enzymes such as superoxide dismutase and catalase (17,18). Local and systemic inflammatory response is associated with the production of ROS such as superoxide anions, hydrogen peroxide and peoxynitrite(19,20). In addition, the presence of 0xidative compounds such as reactive oxygen species along with inflammatory and immune response signaling plays important roles in the pathogenic mechanism of cell damage caused by CoVs through oxidative stress(21,22). However, the processes leading to the oxidative stress and inflammation cycle in Covid-19 have not yet been clarified.

3. Psychological Stress in the COVID-19 Process

Various different measures taken to control the COVID-19 epidemic and reduce the spread of the disease have changed the ordinary course of human life. Cancellation of face-to-face education activities at all levels from kindergarten to university in the field of education at the beginning of the process, limiting the purchase need to certain time periods only to meet the basic needs, additional measures regarding commuting, use of masks covering a large part of the face, acting in accordance with social distance rules. efforts, quarantine and isolation have greatly changed people's lives and have also affected them psychologically (23).

Several early studies on the psychological effects of COVID-19 have reported anxiety, depression, stress (24), and post-traumatic stress disorder (25). Studies have shown that stressors are strong risk factors for various health problems such as depression, anxiety and insomnia (26).

1130 people from 28 cities of Turkey participated in a study examining the general public in Turkey in order to determine the psychological impact, anxiety,

depression and stress levels in the first stage of the COVID-19 epidemic. The data obtained showed that 52.7% of the participants rated the psychological impact of the epidemic as moderate or severe, while 18.6% showed moderate to severe depressive symptoms, 26.5% had moderate to severe anxiety symptoms, and 7.9% showed moderate to severe stress levels (27).

The ongoing pandemic caused by the severe acute respiratory syndrome coronavirus is a major stressor that greatly impacts environmental sustainability. The current pandemic process and many different studies conducted around the world have reported stressors associated with COVD-19. Moreover, different pre-existing environmental stressors and other stressors related to coronavirus disease-2019 (COVID-19) induce oxidative stress generation, exacerbating the effects of viral disease (28).

In a Swiss study examining the impact of COVID-19 on mental health and involving more than 10,000 participants, it was reported that 46.9% of participants showed an increased level of stress compared to 40% during partial quarantine (29).

In a study aiming to determine the increasing psychological stress and related risk factors in parent-child relationships during the Covid 19 process, it was reported that there was a significant increase in the psychological stress of 252 adolescents and 217 parents in 731 couples consisting of 1221 adolescents aged 10-17 and their parents. Risk factors for increased psychological stress included sociodemographic and emotional regulation (eg, parental rejection of emotional responses, limited access to emotion regulation strategies in adolescents) (30,31).

In a study investigating the psychological impact of the COVID-19 pandemic in the general adult population (N = 3055) as well as the levels of anxiety, stress and depression in the early stages of the epidemic in Spain, the extent to which various variables were related to the mental health of the participants was investigated. The results showed that approximately 36% of the participants experienced moderate to severe psychological effects, while 25% showed mild to severe anxiety, 41% reported depressive symptoms, and 41% felt stressed 32).

Anxiety is an aversive emotional state in which the emotion is intensified. The nature of the fear experienced is disproportionate to the nature of the threat (33). This emotion includes behavioral, expressive and physiological characteristics such as avoidance of behavior. Anxiety is a normal emotional response to a threat or potential threat. When this emotion is inappropriate,

excessive, and persistent, it is classified as pathological (34). It plays a role in a number of psychiatric disorders such as anxiety, depression, panic attacks, phobias, generalized anxiety disorder, obsessive-compulsive disorder and post-traumatic stress disorder (35).

Patients with confirmed or compatible symptoms of COVID-19 may fear the consequences of the illness, and certain symptoms, such as fever or shortness of breath, may exacerbate mental distress and anxiety. In addition, the unpredictability of the current crisis and widespread misinformation make the whole situation more stressful (36).

A study by Brooks et al (37) reviewed the psychological impact of quarantine using three electronic databases. In the study, 24 of 3166 articles were included in the review. Most of the studies reviewed reported negative psychological effects such as symptoms of post-traumatic stress, confusion, and anger. Stressors included longer quarantine time, fears of infection, frustration, boredom, inadequate supplies, insufficient information, financial loss, and stigma.

In China, people have experienced an increase in psychological problems such as anxiety, stress and depression, as well as boredom, loneliness and anger when they are indoors (38).

Recent research highlights the psychological distress associated with the pandemic, while also identifying the detrimental emotional consequences of school closures for the most vulnerable social groups (39).

Bol (40) and Cullinane and Montacute (2020) found that families with low cultural capital were less confident in supporting their children's learning during quarantine, and as a result, they experienced intense psychological anxiety and depression related to the necessities of homeschooling (41).

In the cross-sectional study conducted by applying an online survey to 3,040 participants aged 18-30, 90% of the respondents stated that they washed their hands more frequently since the epidemic and 50% wore protective gloves. What are your feelings about the coronavirus?" 38% of the respondents answered "concerned". A Survey of Attitudes, Anxiety Status, and Protective Behaviors of the University Students During the more COVID-19-related stress was reported among 742 co-parent in the USA, predicting greater co-parent and family dissonance (42).

Fear is directly related to the rapid and invisible rate of transmission of COVID-19, as well as morbidity and mortality rates (43) Increasing fears and misunderstandings about COVID-19 can lead to mood disorder.

Due to the sudden nature of the epidemic and the contagious power of the coronavirus, people may show psychological and stress-related reactions. Some bans and measures taken against the coronavirus epidemic, such as social isolation, quarantine, travel restrictions, avoidance of contact. It is claimed that these measures affect people's social life, emotional state and psychological well-being (44).

Even for virus-free households, it acts as a major stressor of the pandemic, especially in terms of chronic anxiety and economic hardship. Such effects may be exacerbated by self-isolation policies that can increase social isolation and relationship difficulties, loneliness and social isolation exacerbate the burden of stress and often have detrimental effects on mental, cardiovascular and immune health (45)

Older adults, who are most at risk for severe symptoms of COVID-19, are also highly susceptible to isolation (46).

Distance threatens to exacerbate feelings of loneliness and can cause negative health problems in the long run. People subject to quarantine or self-isolation are at risk for confusion and anger (37), emotional tendencies that can be explosive when multiple household members endure them for weeks or months at a time.

Stress is an inevitable part of human life and is experienced even before birth. Some degree of stress may be considered normal and even necessary for survival and regular psychological development during childhood or adolescence. However, prolonged exposure to stress can become harmful and strongly affect mental health and increase the risk of developing psychiatric disorders (47,48).

4. Relationship of Oxidative Stress and Psychological Stress

Oxidative stress is a cellular or physiological condition of high concentrations of reactive oxygen species that cause molecular damage to vital structures and functions. Various factors have been reported to affect susceptibility to oxidative stress by influencing antioxidant status or free oxygen radical formation. There are various studies that review the effect of psychological stress, which is one of these factors, on the development of oxidative stress. It has been reported that Oxidative stress and various psychological disorders, which have started to attract attention in behavioral medicine, are associated with their prognosis. Oxidative stress increases imbalances in general. It is assumed that oxidative stress resulting from dysfunction of oxidative and antioxidative systems may

cause various disorders and diseases such as psychiatric diseases such as anxiety, neuroticism (N) and various adjustment disorders (48). It has been suggested that psychological stress is associated with increased oxidant production and oxidative damage, and therefore, long-term exposure to psychological stressors may play a role in various diseases and increase the risk of disease (49).

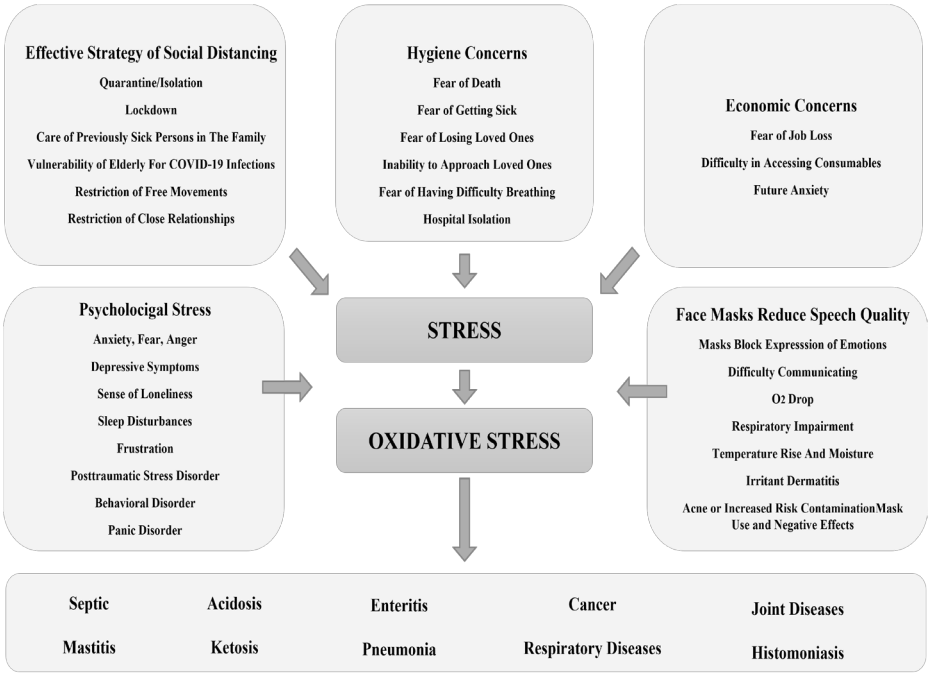

Figure 1. Relationship of oxidative stress and psychological stress

While it has been suggested that oxidative damage may play an important role in mediating the health risks associated with oxidative stress, psychological stressors such as anxiety, depression, adjustment difficulties and various psychological conditions have also been found to be directly related to oxidative stress (50). Although psychosocial stress (PS) and oxidative stress (OS) have different mechanisms that affect health, both cause a physiological imbalance that can later lead to a disease state (51,52).

Oxidative stress is an imbalance between cellular production of reactive oxygen species and opposing antioxidant mechanisms. The brain, with its high oxygen consumption and a lipid-rich environment, is considered highly susceptible to oxidative stress or redox imbalances. Therefore, it is not surprising

that oxidative stress plays a role in a variety of mental disorders, including depression, anxiety disorders, schizophrenia, and bipolar disorder (53).

The brain's vulnerability to oxidative damage is consistent with the theory that anxiety can be directly generated by Oxidative stress (OS). Antioxidants may constitute a potential treatment when OS is the causative factor in anxiety. Additionally, since OS is associated with anxiety disorders, a mixture of antioxidants and anxiolytics may also be a useful treatment for patients with anxiety (54,55).

Anxiety is one of the most common forms of psychological distress (56). In particular, phobic anxiety is an important category within the broad dimension of psychological distress due to its early age of onset and chronic and persistent nature (56,57).

Phobic anxiety was found to be associated with cardiovascular disease (58,59) and higher oxidative stress and inflammation (60). assumed to have adverse biological consequences. The potential relationships between phobic anxiety and oxidative stress are of particular interest, as oxidative damage is assumed not only to be a biological consequence of chronic psychological distress, but also to play an important role in the development of psychopathology, including anxiety (61).

Oxidative DNA damage has been observed in patients with major depressive disorder (60) and bipolar disorder (62). Furthermore, such evidence is not limited to psychiatric clinical populations, finding more signs of oxidative DNA damage among people with higher levels of occupational psychological stress and depression in their more general sample of participants.

Some researchers have hypothesized the etiological role of oxidative stress in the development of symptoms of psychological distress and psychiatric disorders (63).

In cross-sectional studies with humans, higher levels of oxidative stress have been associated with depression (63), anxiety disorders, and obsessive-compulsive disorders (64).

Recently, there has been an exponential increase in knowledge regarding the effect of traumatic stress on oxidative components and redox state homeostasis. Most of the evidence indicates that there is an imbalance of pro-/anti-oxidative mechanisms under conditions of traumatic stress, leading to a systemic oxidative dysregulation accompanied by toxic oxidation byproducts. (65).

Data from a review that aims to highlight the scientifically determined psychological stress and biological stress occurring in various populations with

different social norms show that the COVID-19 pandemic can affect patients, healthcare workers, the general population, and vulnerable populations such as women, children, the elderly, traders and migrant workers. proven to cause unstable psychological conditions such as anxiety, depression, post-traumatic stress disorders. In addition to psychological stress, COVID-19 infection has been shown to induce biological stress by promoting oxidative stress and cortisol secretion, which negatively affects the survival of patients (Htike, Mansor and Firdous, 2013). Another study reported that exposure to chronic stress promotes oxidative stress (66).

Data from a study reviewing the relationships between oxidative stress and psychological disorders revealed a wide spectrum of degenerative syndromes and psychiatric disorders associated with oxidative stress. The multidimensional information described in the study supports the role of oxidative stress in various psychiatric disorders. The literature also provides a detailed table and information with concrete references that oxidative stress is or may be associated with different psychological disorders (67).

Unlike minor daily stress, severe life stress (SLS) is defined as a serious psychosocial event with the potential to affect psychological trauma. Recent developments and numerous studies have sought to understand how the central nervous system (CNS) responds to SLS, revealing that this response includes various morphological and neurochemical modifications, among which oxidative stress is almost always observed (67-69).

Emotional well-being is central to normal health and well-being. Persistent psychological stress often impairs emotional health and triggers the onset of neuropsychiatric disorders. It has been suggested that psychological stress adversely affects the normal functioning of the immune system, which contributes to the pathophysiology of some neuropsychiatric conditions. A summarized discussion of the evidence linking the impact of psychological stress on the immune system in a review is presented. Accordingly, examining the interaction between the immune system and the central nervous system may reveal molecular targets that are critical for the development of possible therapeutic and preventive measures. In the study, it was also stated that mental stress potentially affects immune function, and a special emphasis was placed on oxidative stress mechanisms that lead to the activation of multiple cascades that lead to the emergence of psychiatric symptoms (68).

The correlation between affective disorders and the nearly ubiquitous pathological oxidative stress can be multifactorially identified as an important

mechanism of central nervous system disorder. It remains an interesting question whether the obvious changes in the oxidative balance of affective disorders are part of the constitutive mechanism or a side effect. However, it is now clear that oxidative stress is a component of these disorders that are characterized by different aspects in a disease-related manner. Still, there is much debate regarding the importance of oxidative stress status in most affective disorders, and most studies suggest that the development of affective disorders may be associated with increased oxidative levels (8,9,69).

Mood disorders consist of various psychiatric pathological conditions that involve variable social or emotional behaviors and occur in different combinations. The main known affective disorders are depressive disorder (DD), anxiety disorder (ANX), obsessive-compulsive disease (OCD), panic disorder (PD) and post-traumatic stress disorder (PTSD). A strong correlation has been observed between increased oxidative damage and depression, anxiety (69), panic disorder (70,71).

Clinical trials have been cited as the primary source of evidence that oxidative stress may play a role in the pathogenesis of mood disorders. In this context, it has been stated that mood stabilizers and antidepressant treatments have high antioxidant potential (72). In addition, other studies have shown that some oxidative stress markers return to normal during or after specific treatment for affective episodes, suggesting that antidepressants can actually reduce oxidative stress levels (73).

Multidimensional data obtained in a study aiming to examine the existing evidence on the role of oxidative stress in psychiatric disorders and its academic and clinical consequences support the role of oxidative stress in various psychiatric disorders (74).

Studies show that anxiety disorders can be characterized by low antioxidant defenses and increased oxidative damage to proteins, lipids and nucleic acids. In particular, oxidative modifications in proteins have been implicated as a potential factor in the onset and progression of various psychiatric disorders, including anxiety and depressive disorders (75). Oxidative stress has also been associated with depression, anxiety disorders, and high anxiety levels. Findings that establish a link between oxidative stress and pathological anxiety have inspired a number of other recent studies focusing on the link between oxidative state and normal anxiety, as well as a possible causal relationship between cellular oxidative stress and emotional stress (76). Evidence of increased oxidative stress in the context of psychological stress has also been reported in studies on animals and humans (77).

Conclusion

The COVID-19 pandemic represents a major public health, economic, political and scientific concern in most countries worldwide where COVID-19 cases and deaths have been confirmed (79).

Having a well-informed public about the COVID-19 virus, its causes and mode of transmission can be one of the best strategies to prevent and slow transmission. However, until recently, limited information and scientific knowledge about the virus was available. Scientific understanding of mutation rate, transmission, disease symptoms and severity, herd immunity, and risk groups is still emerging, and this uncertainty poses a challenge to reliably inform the public, leading to confusion about best practices for health maintenance and adverse effects on mental health. (80).

Disease prevention is the most effective way to get rid of the disease. It is necessary to promote positive attitudes and beliefs that allow us to control the transmission of COVID-19, with extensive knowledge and good knowledge, and to practice self-quarantine, socialdistancing and good hygiene measures such as hand washingeyeprotectionandthe use of face masks and disposablegloves.

In preclinical studies, remdesivir(GS5734), an RNA polymerase inhibitör with in-vitro activity, used against various RNA viruses, including Ebola, may be effective in both prophylaxis and treatment against human CoV infections, and Alpha-interferon and lopinavir/ritonavir. It can also be recommended fort he treatment of CoVs(77). With its wide ethnomedical accumulation and rich flora, our country has phytotherapeutic treatments for COVID-19.In this context, among the antioxidant compounds evaluated interms of COVID-19 infections, flavonoids, probiotics, and vitamin C, which are compounds commonly found in fruits, vegetables and some beverages, which ocur through protective effect, reduction of oxidative stress, cerebral lipid peroxidation and regulation of inflammation, İtamin C, D can promote recovery in disease cases (81-83). It can be said that more importance should be given to vaccination and vaccination studies.

In addition to these measures, people’s awareness, attitudes and practices towards COVID-19 may be key in controlling the spread of infection. In addition, anxiety and fear arising from the presence of pandemic situations can affect the behavior of individuals. Given the obvious global effects, the anxiety provoked in individuals about being infected with COVID-19 may lead them to adopt preventive behaviors (37,84).

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