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Oxidative Stress and Toxicity in Reproductive Biology and Medicine

A Comprehensive Update on Male Infertility Volume II

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A Comprehensive Update on Male Infertility Volume Two

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Foreword

Inquiry into human reproductive health and fecundity is an endeavor spanning centuries, even millennia. In fact, the first documented couples' fertility test of sorts, put forward by female physician Trotta of Salerno (circa eleventh century), predated the first observation of human spermatozoa by van Leeuwenhoek and Hamm by almost 600 years. First recorded human artificial insemination was performed in late eighteenth-century England, and the modern andrology came about at the turn of twentieth century, which eventually brought us gamete and embryo cryopreservation, in vitro fertilization (IVF), embryo transfer, intracytoplasmic sperm injection (ICSI), and somatic cell nuclear transfer. We are now at the threshold of new era of precision medicine and andrology, incorporating molecular, biomarker-based approaches into clinical andrology and assisted reproductive therapy (ART).

Notwithstanding environmental, occupational, genetic, and lifestyle factors, age is rapidly becoming the number one contributor to male and female infertility as the couples of reproductive ages increasingly chose to delay parenthood in favor of career and lifestyle. The resultant steady increase in ART cycles realized per year around the world coincides with increased research on reproductive impacts of reactive oxygen species (ROS) and their mitigation by antioxidants. As drug store shelves become increasingly flooded with all imaginable sorts of antioxidant and vitamin supplements, it is crucial to better understand the role of redox potential in both the physiology and pathology of male and female reproductive function. While antioxidants may boost sperm production, viability, and fertility, their indiscriminate use could have the exact opposite effect, upsetting the fine balance between ROS-dependent signaling and ROS scavenging in gametes and reproductive tissues. It is imperative that such treatments do not compromise the sperm cells, particularly in clinical setting where almost all ART cycles nowadays are performed by ICSI and the need to subjectively select single spermatozoon to fertilize the precious human eggs makes it of paramount importance to purify only the fittest spermatozoon from highly heterogeneous human sperm cohorts. In the light of these challenges, the present volume is saddled with an uneasy task of making sense of recent advances in sperm redox research.

Volume II of the book *Oxidative Stress and Toxicity in Reproductive Biology and Medicine: A Comprehensive Update on Male Infertility* edited by Dr. Shubhadeep Roychoudhury and Dr. Kavindra Kumar Kesari considers various influences leading to oxidative stress (OS) in gametes and gonads, including environmental and occupational stressors such as pesticides, heavy

metals, plastics, nanoparticles, and radiation exposure. Importantly, remedies are discussed, such as pharmacological antioxidants and natural herbal remedies. In my opinion, phytomedicine in particular is an important yet underappreciated area of inquiry as it may provide low-cost natural remedies particularly important for developing nations where the high cost and limited access to pharmacological antioxidant supplements may be a barrier to widespread use. This will require meticulous isolation and validation of active compounds present in complex, highly heterogeneous medicinal plant extracts. While the present volume maintains focus on male reproductive function, impacts of OS are also considered in the mind frame of female reproductive system and embryo development. This worthwhile undertaking brings us a thought-provoking collection of in-depth reviews. Without a doubt, a diverse audience of clinicians, scholars, and graduate students will be the beneficiaries.

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Preface

The connection between oxidative stress (OS) and human health to a large extent defines the factors impacting the reproductive potential in the male. Humans are exposed to number of environmental stressors that induce OS. The networking of various such manmade and natural agents, including the environmental toxicants, mutagens, and infectants, may interfere with the normal functioning of the male and female reproductive tracts, thereby affecting reproductive and sexual health. The factors such as radiations, metal toxicity, lifestyle factors, pesticides, nanotoxicity, infections, and chemical toxicity may lead to sperm damage (DNA damage or low count), deformity, and, eventually, male infertility. According to the World Health Organization (WHO), nearly 190 million people struggle with infertility worldwide. This figure could even rise because of the number of couples who are not obtaining proper medical consultation and/or assistance. Therefore, this book contributes towards providing an understanding of the networking of mutagenic factors and their toxic effects on fertility pattern. In this connection, total 19 chapters of this book not only navigate through the impact of OS and toxicity on male reproduction but also suggest protective measures by using several bioactive and natural antioxidants to strengthen the fertility factors. The 19 chapters as Volume II add value to our previous collection of 15 chapters published as Volume I, and discuss an up-to-date view on the impact of OS factors in male reproduction with multidisciplinary approaches with a focus on environmental toxicity.

Chapter 1 highlights the influence of reactive oxygen species (ROS) which is a key player in male infertility along with the diagnosis, available clinical options, and preventive measures against extensive ROS buildup within the spermatozoa. Also, study concludes OS as a major causative agent of male infertility. Chapter 2 discusses the role of environmental toxicants–induced OS and their effects on male fertility factors. The chapter mainly emphasizes on the identification of potential environmental toxicants which may have clinical relevance for early screening and diagnosis of male infertility. It further highlights the role of pesticides, metal toxicity, and possible mechanism of interaction with human reproduction. Chapter 3 mainly discusses the environmental stressors and their effects on the mechanisms causing congenital impairments due to poor sexual health and transmitting altered signal transduction pathways in male gonadal tissues. It also focuses on the pathway(s) of impact of factors such as heavy metals, air pollution, chemical contaminations, drugs, tobacco smoke, and xenobiotics on male reproduction. Chap. 4

discusses the route(s) of their exposure to men and women through various pesticides resulting in different infertility concerns like sperm abnormalities, abnormal sperm count and motility, testicular atrophy, ovarian dysfunction, decreased fertility, and spontaneous abortions. The study also navigates the future possibilities in perceiving the mechanism(s) of reproductive toxicity of different pesticides and their management before any alarm of danger. In connection to environmental toxicants, radiation has been recognized as a major cause of concern for male reproduction. In Chap. 5, the role of radiations in the male fertility has been elucidated. The study focuses the basics of radiation and introduces both ionizing and non-ionizing radiations highlighting their effects on human reproduction. The chapter also intends to describe a detailed literature on the impact of radiation-induced OS on male reproduction and to furthers the understanding of its consequences leading to the phenomenon of male infertility. In this continuation, Chap. 6 elucidates the role of arsenic-induced OS and its association with sex hormone disruption as well as its effect on sperm and semen quality. This is an important chapter that explains the grassroot networking of arsenic through various sources, that is, food chain, ecosystem, agriculture, and ground water contamination, and its effects on human health, especially male reproduction. Chapter 7 discusses the role of various metallic nanomaterials in reproductive health. The study highlights the mechanism(s) of bioaccumulation of metallic nanomaterials which may lead to the generation of ROS in reproductive organs and cause hazardous effects, such as reduced sperm count, sperm motility, hormonal regulation, and morphological and ultrastructural changes. In connection to this, Chap. 8 mainly focuses to identify and elucidate the effect of bisphenol A (BPA) exposure on male fertility. The study also illustrates the mechanisms through which this may occur, while emphasizing the role of OS as a potential pathway. BPA has been recognized as one of the leading chemical contaminants affecting human health through various sources such as food, water, air, ink, medical materials and devices, and occupational exposures. It may enter our body through inhalation, ingestion, and transdermal routes and damage the redox homeostasis by altering the standard equilibrium of oxidative mediators. The chapter mainly discusses the adverse effects of BPA on male fertility with an appropriated citation of both in vivo and in vitro studies. After detailed discussions on the mutagenic factors and male reproduction, Chap. 9 sheds light on the potential impact of herbal drugs to combat OS and thus improve the male fertility parameters. The study highlights the role of OS in the pathophysiology of male infertility, that is, hormonal defects, sexual problems, lifestyle, and genetic factors. The chapter primarily contributes to explore the protective measures against OS through various natural sources such as herbal drugs for the management of male infertility. This is an important chapter navigating possible preventive cares towards early diagnosis of infertility. In this regard, Chap. 10 elaborates various classes of medicinal compounds and their mechanism of killing prostate cancer cells through direct or indirect ROS generation. This chapter identifies and well elaborates the important phytochemicals that inhibit ROS and specifically lead to the death of prostate cancer cells. The study provides clear evidence to generate a novel thought to develop promising drug candidates to

treat prostate cancer patients. Chap. 11 discusses different heat shock factors (HSFs) and their functions including those during spermatogenesis. The chapter highlights different heat shock proteins induced by the HSFs and their activities in these contexts. The novelty of this chapter lies in the identification of several small molecule activators and inhibitors of HSFs from different sources reported so far. Thus, it may contribute to achieve the goal of current and future research and to understand the molecular basis of this distinction, and design therapy to modulate the process as appropriate for the benefit of mankind. Although the Chaps. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 mostly discuss the role of OS in male reproduction, Chapter 12 elucidates the role of ROS in female reproduction. Being viewed as a couple's problem, male infertility literally remains inseparable from the female factors. Hence, the chapter raises several important issues associated with female reproduction such as reduced growth and development of oocytes, ovarian steroidogenesis, ovulation, blastocyst formation, implantation, endothelial dysfunction in the uterus, and fertilization of eggs. It further explores several ROS-induced risk factors to female reproduction such as endometriosis, preeclampsia, maternal diabetes, ovarian epithelial cancer, recurrent pregnancy loss, intrauterine growth restriction, and fetal death. The chapter also demonstrates great impact to understand the possible mechanisms of infertility in the female beyond the male factors. Although male infertility contributes to over 50% of cases worldwide as compared to female infertility, the importance of this chapter in the middle of book navigates towards the therapeutic and protective measures/possibilities. In connection to this, Chap. 13 also assesses the impact of OS on embryogenesis and fetal development. The chapter retracts the evidence available in literature to facilitate an in-depth understanding of the redox regulation during development that may help optimize the pregnancy outcome both under natural and assisted conditions. The focus of the chapter lies on the role of ROS and antioxidants in early development including the processes of gametogenesis, organogenesis, fertilization, and early embryogenesis. In comparison with Chap. 12, this chapter furthers the understanding of the events in ROS formation and the maintenance of the redox homeostasis in embryonic and fetal developments at molecular level.

Meanwhile, Chap. 14 discusses the various methods of analyzing the mammalian embryo culture based on oxidant and antioxidant parameters. The study mainly highlights the role of external factors on embryo culture and the ability of antioxidants to enhance in vitro fertilization (IVF) outcomes. This is an important contribution towards therapeutic measures and treatment perspectives, because the "safe" administration of an antioxidant into culture media during the in vitro process is challenging. Indeed, an optimization of media culture by the addition of enzymatic and non-enzymatic antioxidants in animal models and human embryos in ART has been updated in this study, with an emphasis on comparing the available results and their possible reasons. In connection to this, Chap. 15 elucidates the role of OS in male reproduction and potential use of antioxidants for infertility treatment. The chapter discusses the role of ROS as mediators in male reproductive outcomes, where they are mostly involved to cause changes in Leydig cells, Sertoli cells, and spermatozoa, and it also highlights the defense mechanism

of quercetin as an antioxidant and a novel therapy for OS-induced male infertility. Chap. 16 delineates the role of antioxidants in cellular defense mechanisms against OS-induced male infertility. The study mainly focuses on the effects of ROS in sperm functions and the current concepts regarding the benefits of medical management in men with diminished fertility and ameliorates the effect to improve sperm function. Also, this evidence-based study suggests an increasing rate of infertility that poses a global challenge for human health. It further emphasizes to explore the recommended doses of antioxidants to cure male infertility because an overdose may bring about severe negative effects on male reproduction. As a follow-up, Chap. 17 discusses the evidence-based concepts pertaining to the antioxidants' actions to combat OS-induced male infertility, and the mechanism(s) of induction of reductive stress and its impact on male reproduction. The study also shows that an excessive antioxidant exposure drives the endogenous system towards reductive stress, which is as harmful to sperm health as OS. Therefore, Chaps. 16 and 17 show great impact to understand the dose-response relationship and possible recommendations for consumption of antioxidants by infertile men and women. In connection to this, the study on genetic model could strengthen the understanding of cellular hemostasis apart from the dose response of antioxidants. Furthermore, Chap. 18 presents an *in silico* study wherein the role of *CatSper* family genes and APOB gene regulation in male infertility has been explored. *CatSper* genes play an important role in sperm motility, acrosome reaction, and sperm-oocyte fusion. Therefore, the role of the *CatSper4* gene in sperm tail function and the APOB novel gene has been postulated to be involved in sperm motility. Indeed, understanding the molecular mechanism(s) of regulations of the *CatSper* family genes may navigate to develop future therapeutic approaches for infertile men. Chapter 19 is the final chapter and provides the concluding remarks of the book – both Volumes I and II. In this chapter, the role of OS and toxicity in reproductive biology and medicine is introduced first which follows further discussions on the therapeutic measures. It finally concludes that both the books, Volumes I and II, may leave a major impact on research and academia to navigate both clinicians as well as researchers in the realm of OS and human reproduction. Both book volumes contain an up-to-date review on the impact of OS in male reproduction with a focus on environmental toxicity. Overall, the book will serve as a compendium for clinicians and researchers in updating and discussing the current challenges and future perspectives of OS and toxicity in reproductive biology and medicine with a focus on male infertility.

All the chapters presented in Volumes I and II have follow-up links from each other and conclude that OS-induced free radical formation is the responsible factor for several types of serious health concerns including infertility. This book also focuses on molecular and integrative toxicology in understanding the mechanisms of toxicity associated with free radical generation. The readers of the chapters from both Volumes I and II certainly stand to inculcate a better understanding of environmental challenges and further the understanding of the mutagenic factors in the environment that may lead to infertility not only in the male but also the couple as a whole. Additionally, it provides insights to strategies to reduce the burden of male gonadotoxins, to

enhance men's fecundity, and to help optimize the care of infertile men with a unique feature of an integration of basic science and clinical application.

Finally, we would like to thank all the authors who have contributed to this book. Last but not least, our sincere thanks go to the series editors Dr. W. E. Crusio, Dr. H. Dong, Dr. H. H. Radeke, Dr. N. Rezaei, Dr. O. Steinlein, and Dr. J. Xiao, and the entire Springer editorial team for their sincere assistance and support. Our special thanks go to Dr. Carolyn Spence for her continuous support and suggestions throughout the book editing. We are also thankful to Mr. Vishnu Prakash for his valuable assistance and support during the printing process.

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1 Deciphering the Nexus Between Oxidative Stress and Spermatogenesis: A Compendious Overview

Caleb Joel Raj, C. V. S. Aishwarya, K. V. S. S. N. Mounika, Bishwambhar Mishra, B. Sumithra, Bhushan Vishal, and Sanjeeb Kumar Mandal

Abstract

Oxidative stress (OS) and reactive oxygen species (ROS) are one of the main reasons for the multifactorial concern – male infertility. ROS are active components of cellular metabolism that are intrinsic to cellular functioning and are present at minimal and unreactive levels in normal cells. They are an integral component of the sperm developmental physiology, capacitation, and function. As said "anything in excess is poison," so is the case with ROS. These, when produced in excess to the antioxidants present in the seminal plasma, cause multiple malformations during the process of spermatogenesis such as lipid peroxidation, interfere with capacitation, sperm DNA fragmentation and damage to the membrane of the sperm which in turn reduces the motility of the sperm and its ability to fuse with the oocyte. Exposure of spermatozoa to oxidative stress is a major causative agent of male infertility. Thus, a delicate balance between the beneficial and detrimental effects of ROS for proper functions is of utter importance. In this chapter, the influence of ROS in OS which is a key player in male infertility along with the diagnosis, available treatment, and prevention of extensive ROS buildup within the spermatozoa are highlighted.

Keywords

Infertility · Oxidative stress · ROS · Reproductive health · Spermatogenesis

1.1 Introduction

Infertility, a condition of the reproductive system, is the inability to conceive after a period of 1 year or more of unprotected sexual activity. Millions of men and women that are of reproductive age around the world are affected by infertility (Lindsay and Vitrikas 2015). This has impacted and has been impacting their families and communities both physically and socially. Infertility affects 48 million couples and 186 million people worldwide, according to estimates. Infertility in men is most usually caused by issues with semen ejection, quantity and quality of sperm, or defec-

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tive morphology and motility of sperm. Understanding the causes of reproductive failure is a multifaceted task (Inhorn and Patrizio 2015). Sperm cells' ability to fertilize viable oocytes, initiate and maintain embryonic growth, and carry a pregnancy to term requires the reactive oxygen species (ROS) to function which are also implicated in the development of infertility and thus related with providing better care to patients. This will also open new doors in terms of infertility prevention and control. Oxidative stress (OS) is a predominant cause of male infertility, which is defined as an imbalance in the amounts of ROS and antioxidants (Hassanin et al. 2018). General processes of sperm function like capacitation, hyperactivation, and acrosomal response require a tiny quantity of ROS. When ROS levels go beyond normal, they can cause infertility not just by DNA mutilation and lipid peroxidation, but also by the inactivation of enzymes essential for spermatogenesis and oxidation of proteins in spermatozoa (Chen et al. 2013). Major sources of ROS include immature spermatozoa, stimulation of inflammatory responses, mutations that occur in genes of the spermatozoa, and changing levels of sex hormones (hormonal imbalance). As spermatozoa have poor antioxidant defense mechanisms and a limited ability for detecting and repairing DNA damage, they are particularly sensitive to OS and oxidative DNA damage (ODD). Damage to sperm DNA, RNA transcripts, and telomeres is caused by high OS. Because OS is caused by a shortage of antioxidants in the sperm, adjustments made to one's lifestyle and antioxidant administration can be beneficial in overcoming this issue (Barati et al. 2020). Moving forward, we shall learn more about the biochemistry of ROS, the etiology of OS, the possible diagnosis, and cure to the said condition.

1.2 Oxidative Stress and Male Infertility

OS is bound to occur in the human body whenever the free radicals and antioxidants fall out of equilibrium or undergo an imbalance. OS can also be caused by the body's natural immunological response. Mild inflammation results from this form of OS, which subsides once the immune system has fought off an infection or repaired an injury. In general, the cells in the body produce free radicals during metabolism, and these are neutralized by the production of antioxidants by other cells in the body. This is how the balance between the two is maintained.

Free radicals are the molecules with unpaired electrons. They mainly include ROS and produced as by-products at the end of metabolic processes in which mitochondria, commonly known as the powerhouses of the cell, combine glucose and oxygen to release water, carbon dioxide, and ATP – a major source of energy. Free radicals tend to take part in biochemical processes that help them get rid of that unpaired electron and hence play an active role in the oxidation of membrane lipids, proteins, carbohydrates, and amino acids (Ochsendorf 1999). Figure 1.1 shows the common sources of ROS and free radicals. Antioxidants neutralize these free radicals by donating an electron to the unpaired electrons. Thus, an increase in the ROS exceeding the natural antioxidants produced by the body breaking their defenses leads to damage of the body cells.

The terms ROS and free radicals are often used interchangeably although they do not necessarily mean the same and not all ROS are free radicals (Cheeseman and Slater 1993). In the male, sperm and seminal leukocytes produce ROS, and these include peroxides, oxygen ions, and free radicals. When the ROS production overwhelms the natural defense given by the antioxidants, the resulting OS severely mars the entire process of spermatogenesis and alters the sperm function, ultimately leading to infertility. High levels of OS are one of the major causes of infertility in men. Such an infertility linked with OS is also commonly called the idiopathic male factor infertility.

Earlier, ROS was considered to be toxic to spermatozoa in humans. However, evidence later also suggested that minuscule quantities of ROS are always essential for the spermatozoa to attain their ability to fertilize the ovum (Gagnon et al. 1991). They are also crucial for functions like motility, hyperactivation, acrosome reaction, and

Fig. 1.1 Sources of ROS and free radicals

capacitation (Agarwal et al. 2004). Nonetheless, when in excess, ROS are responsible for causing infertility in two ways. Firstly, they damage the membrane of the sperm, reducing its capability to bind to the egg and also lowering its motility. Secondly, ROS also damage the sperm DNA, jeopardizing the embryo's patrilineal genetic contribution. Despite the well-established link between OS and poor sperm quality, men are infrequently checked for or attended to for this problem. Instead, "mechanical" procedures such as in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), or intrauterine insemination (IUI) are commonly provided (Tremellen 2008).

1.3 Biochemistry of Reactive Oxygen Species (ROS)

The production of ROS is a typical part of cellular metabolism. During the oxidative metabolism that is known to occur in mitochondria, the majority of the energy in the body is produced by the enzymatically regulated reaction of oxygen with hydrogen in oxidative phosphorylation. Free radicals are produced during this reaction (Valko et al. 2007). When one electron is added to dioxygen molecule (O_2), the superoxide anion radical ($O_2^{\bullet}$) is formed, which is the most common type of ROS. The synthesis of ROS also occurs as a product of biochemical reactions that take place in peroxisomes, mitochondria, and other cellular components (Balaban et al. 2005). ROS production is a natural outcome of aerobic metabolism and is essential for tissue oxygen homeostasis. An imbalance in this tissue oxygen homeostasis would lead to a surge in OS in the cellular environment.

Leukocytes, majorly neutrophils and sperm, are the two key sources in the semen where radicals are produced (Aitken et al. 1994; Aitken and Baker 1995). One of the main mechanisms by which neutrophils eliminate infections is through the generation of ROS. This makes it clear that seminal leukocytes can produce OS. However, the relationship between the presence of leukocytes in sperm and male infertility remains a source of debate (Wallach and Wolff, 1995). Several studies have found a correlation between the quantity of seminal leukocytes and the formation of ROS (Whittington et al. 1999; Sharma et al. 2001). Yet, there exist a number of studies that failed to find a noticeable variation in the concentration of seminal leukocytes in fertile and infertile men (Christiansen et al. 1991; Tomlinson et al. 1993).

Three important sources of ROS generation are mitochondria, NADPH oxidase, and 5-lipoxygenase (5-LOX). Figure 1.2 shows the major pathways leading to the formation of ROS. During spermatogenesis, loss of cytoplasm of the sperm occurs by the action of Sertoli cells and this facilitates it to obtain its long, condensed form. Because most antioxidant enzymes are lost when spermatozoa are released into the epididymis without cytoplasm, the intrinsic antioxidant defense that is naturally present in spermatozoa

is reduced, leaving the cells less protected against ROS (Iwasaki and Gagnon 1992). Additionally, they lack the essential cytoplasmic-enzyme repair machinery, limiting their ability to detect and repair DNA damage (Saleh and Agarwal 2002). As a result, they lack a DNA repair mechanism during their transport and deposition in the epididymis or after ejaculation and hence are unable to synthesize DNA, RNA, or translate proteins. Mammalian sperm are prone to damage by OS not only due to the dearth of antioxidant protection, but also due to the presence of a large number of substrates that are open to free radical attack. In such immature sperm (with abnormal morphology) with a small cytoplasm and limited defense mechanism, there is excess accumulation of the cytoplasmic residues that are rich in glucose-6-phosphate in the mid-piece region within the vicinity of mitochondria. NADPH oxidase present within the membrane of the sperm provides a means for the ROS production using NADPH as the fuel (Gomez et al. 1996). Consequently, immature sperm (with abnormal morphology) produce more amounts of ROS than sperm that are biologically normal.

As a result of the loss of cytoplasm in sperm, the plasma membrane surrounding the tail and the acrosome, which is rich in polyunsaturated fatty acids (PUFAs), especially docosahexaenoic acid (DHA), is not provided with enough protection by intracellular antioxidants (Ollero et al. 2000). DHA plays a significant role in spermatogenesis regulation and membrane fluidity (Aitken and Baker 1995).

It has been found that sperm-specific NADPH oxidase (NOX 5) is substantially different from leukocyte NADPH oxidase with the key difference being that in this process, the regulation of NOX 5 activity is not controlled by protein kinase C, unlike the process in leukocytes (Armstrong et al. 2002). However, the correlation between the expression of NOX 5 in infertile men and OS has not been established clearly yet.

Leukocytes were found to be the dominant producers of ROS, which is 1000 times higher than that of sperm at capacitation (Plante et al. 1994). A study was carried out to see if the different sources of ROS have an influence on the functionality of sperm. As a part of this, seminal ROS production by the leukocytes was termed extrinsic ROS production while the ROS production by

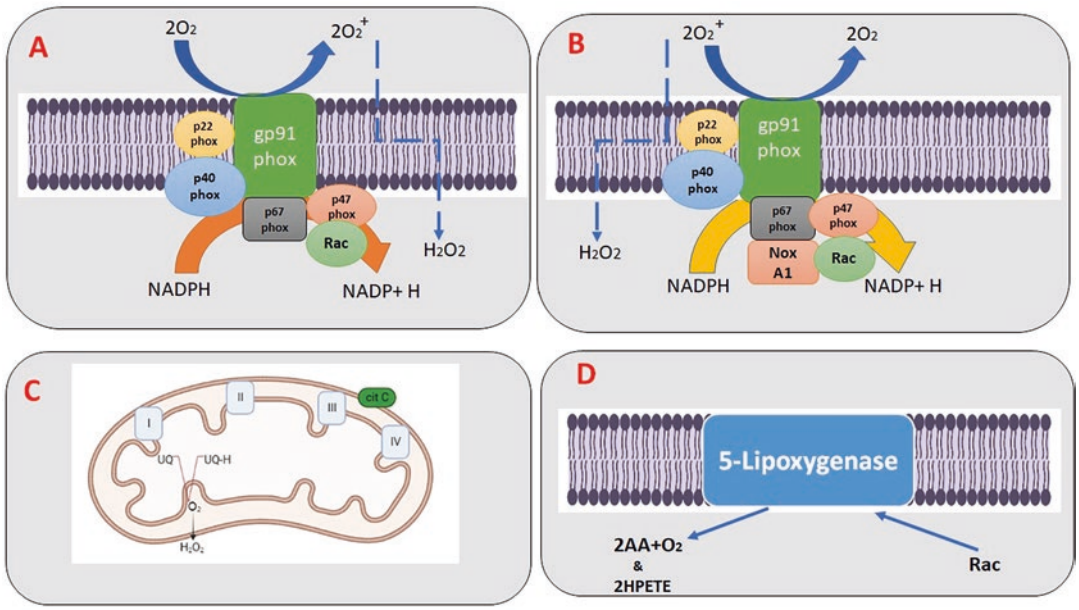

Fig. 1.2 (**a**, **b**) Stimuli for activation of NADPH-oxidase; (**c**) increased mitochondrial generation of ROS induced by stimuli; and (**d**) stimuli for activation of 5-lipoxygenase

sperm themselves was termed intrinsic ROS production. Semen collection was done from a total of 63 random non-leukocytospermic patients. Extrinsic ROS had a much greater impact on sperm count, morphology, and motility in the ejaculate than intrinsic ROS. Furthermore, substantial differences in extrinsic and intrinsic ROS production were found between different patient groups with a high ($\geq 1 \times 10^6$/mL) and low ($<1 \times 10^6$/mL) amounts of leukocytes in the ejaculate. This shows that due to the close connectivity of intrinsic ROS production and sperm DNA, even though leukocytes produce more ROS per cell than sperm, intrinsic ROS production is a more critical variable in terms of reproductive potential (Henkel et al. 2005).

1.4 Influence of ROS on Spermatogenesis

As stated earlier, ROS have deleterious impacts on the spermatogenesis and fertilization processes. Soaring concentrations of ROS in seminal plasma harm spermatozoa by impacting several sperm functions like motility, sperm DNA fragmentation, lipid peroxidation, acrosome reaction, etc. Nonetheless, normal physiological activities including capacitation and hyperactivation, acrosome response, sperm maturation, sperm-zona binding, and oocyte fusion have all proven to be extremely affected by controlled concentrations of ROS (Henkel 2011). Figure 1.3 shows the role of ROS in the various processes of male reproduction.

The following are a few important sperm functions which are affected by levels of ROS.

1.4.1 Lipid Peroxidation

The sperm lipid bilayer is primarily made up of membrane lipids known as PUFAs, which seem to be vulnerable to the substantial concentrations of ROS in seminal plasma. As the levels of ROS surge, it attacks the membrane lipids and results in a decrease in the fluidity of the membrane. As a result, it causes sperm cell dysfunction, affecting the proper functioning of the sperm (Henkel 2011).

1.4.1.1 Lipid Peroxidation

4-Hydroxynonenal (4-HNE), malondialdehyde (MDA), and 2-propenal are key products of lipid peroxidation. MDA is considered to be mutagenic and 4-HNE is considered as genotoxic. As a result, lipid peroxidation's prime products impair spermatozoa by generating DNA adducts, which cause cytotoxicity and DNA damage. Lipid peroxidation thus damages DNA implicitly as well as actively degrading membranes and its activities (Moazamian et al. 2015).

1.4.1.2 Strategies for Prevention of Lipid Peroxidation

There is enough evidence that prove that the inclusion or incorporation of EDTA can considerably reduce the lipid peroxidation of human sperm in the presence of catalyst (e.g., Fe). The antioxidant qualities of seminal plasma, which is highly enriched with a variety of substances meant to protect the spermatozoa against lipid peroxidation, partially compensate for the lack of cytoplasmic defense enzymes. According to a study, iron protects spermatozoa from OS provided they are suspended in seminal plasma. Iron-binding proteins like transferrin and lactoferrin are prevalent in human seminal plasma. Lactoferrin protects the spermatozoa from oxidation by coating their surface (Aitken and Fisher 1994).

The most significant physiological foragers that prevent lipid peroxidation are vitamins C and E, in addition to antioxidant enzymes like glutathione peroxidase, catalase, and superoxide dismutase. Though these may possibly scavenge the peroxidation process, they are not efficient enough to completely eradicate the lipid peroxidation (Riffo and Parraga 1996).

Lipid peroxidation is a cascade of chemical reactions occurring in the cell membrane of the sperm. It has three phases: the first phase is "initiation," the second phase is known as "propagation," and the third phase is "termination" (Takeshima et al. 2021).

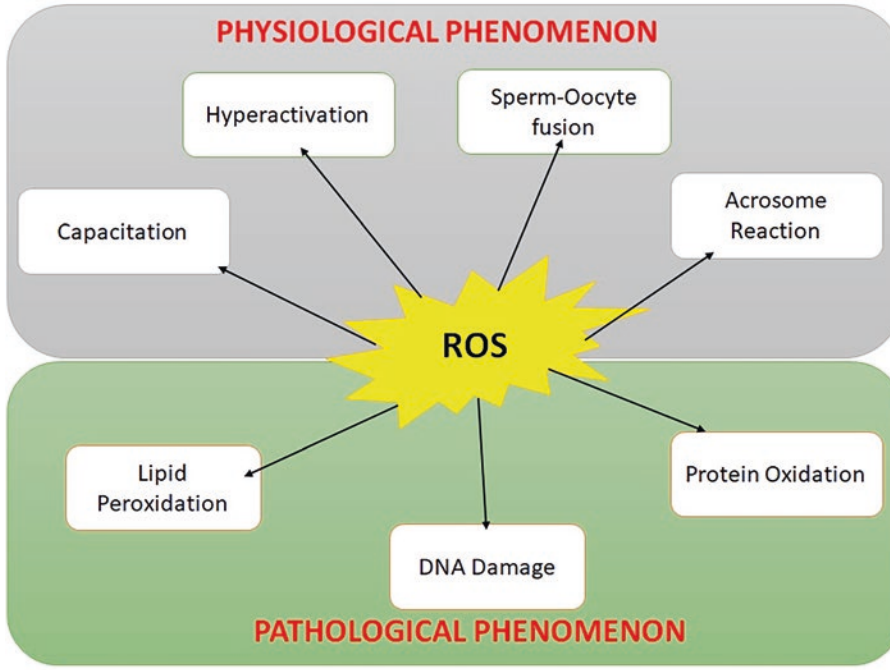

Fig. 1.3 A broad classification of the physiological and pathological role of ROS in male reproduction

- *Initiation*

During the initiation stage, strong oxidizing ROS such as OH* and the hydroperoxyl radical (*HO_2) target membrane lipids at hydrocarbon chains proximal to double bonds, removing hydrogen from adjacent methyl groups, that are particularly reactive for the production of lipid radicals and water. The lipid then absorbs the free electron. This reaction cannot be started by less reactive ROS, such as H_2O_2. The newly generated lipid radical is stabilized by delocalization of the unpaired electrons in resonance structures that are more energetically stable than the original ROS (Sikka 2001). The lipid radical, on the other hand, is a molecule that isn't particularly stable which in turn causes the lipid radical to readily interact with molecular oxygen to produce lipid peroxide (Henkel 2011).

As prior mentioned, less reactive species like H_2O_2 or O_2^- are not capable enough to directly cause lipid peroxidation, but when they react with catalytic concentrations of transition metals like iron or copper, the hydroxyl radical is generated (the Haber-Weiss reaction), which is a direct and potent activator of lipid per oxidation (Aitken et al. 2012). Recent studies have established that H_2O_2, rather than O_2^-, is cytotoxic to human spermatozoa and when these were exposed to this oxidase mixture, they lost motility and the fusion ability, and an upsurge in lipid peroxidation and a depletion of ATP from the cells were connected to these losses.

Ultimately, in initiation, free radicals are propelled by the extraction of hydrogen from the carbon-carbon double bonds of an unsaturated fatty acid (Sikka et al. 1995).

- *Propagation*

Initiation is followed by the propagation phase, at this stage, the reactive lipid peroxide radical molecule formed during the initiation combines with a neighboring fatty acid to produce an additional lipid radical, which subsequently interacts with molecular oxygen to produce lipid peroxide. This method is known as a "radical chain reaction" because it disrupts a significant number of lipid molecules rather than just one or two. As a result, the process of per

oxidation of lipids is capable of oxidizing over 60% of the plasma membrane's unsaturated fatty acid composition (Henkel 2011).

So, altogether in the propagation phase, the production of lipid radicals is followed by a swift interaction with molecular oxygen to produce $*H_2O$ radicals. These radicals again extract hydrogen from an unsaturated fatty acid when metals like copper and iron are present, resulting in lipid radicals and lipid hydrogen peroxide.

- *Termination*

The last stage of the lipid peroxidation cascade is the termination step. The termination of the cascade depends upon the number of lipid radicals available. When the concentration of these lipid radicals is high enough, the possibility of two radicals reacting is significant. The termination step is completed when one lipid radical reacts with another, forming a stable non-radical. The two unpaired electrons from these two radicals form a covalent bond.

1.4.2 Sperm DNA Fragmentation

Sperm DNA intactness and stability is crucial for effective fecundity, embryonic growth, conception, and transfer of inheritable material to progeny. The most prevalent DNA aberration in male gametes is fragmentation, which has been related to reduced semen quality, minimal fertilization rates, poor embryogenesis, and preimplantation development, as well as poor clinical outcomes in assisted reproductive technologies (Veeramachaneni et al. 2006).

Sperm cells are prone to action of ROS because they contain many mitochondria, have a lot of free radical attack sources, and have a limited ability to shield self from OS. The cytotoxic aldehydes produced by per oxidation of lipids which form crosslinks with mitochondrial proteins involved in the ETC (electron transport chain), stimulate the production of reactive oxygen species. ROS and its constituents can cause oxidative damage in the mitochondrial and nuclear DNA, which leads to DNA fragmentation (Fig. 1.4). Damaged DNA is a common feature of faulty human sperm cell, and it has an impact on fertility (Aitken et al. 2007).

According to a significant study, infertile males with elevated interstitial ROS levels had OS-induced sperm DNA damage. Infertile males may have elevated degrees of DNA fragmentation in sperm that could be accounted by the existence of other risks such as varicocele (enlargement of the veins within the scrotum) or other lifestyle difficulties (Kodama et al. 1997). Sperm DNA fragmentation can also be caused by excessive ROS generation and low antioxidant levels in the sperm and by DNA vulnerability produced by a chromatin compaction error during spermiogenesis, which results in a chromatin structural substitution failure from histone to protamine (Mahfouz et al. 2010). The DNA fragmentation index (DFI) of sperm of infertile men affected by varicocele was substantially higher than that of healthy controls. Infertile individuals with varicocele also had a considerably greater OS than infertile patients with normal genital examinations (Agarwal and Said 2005). Diabetic individuals had much higher amounts of sperm DNA fragmentation than normal controls, according to a recent study (Agbaje et al. 2007).

1.4.2.1 Prevention of Sperm DNA Fragmentation

Antioxidants have been shown to inhibit OS-induced DNA fragmentation. It has also been shown to protect sperm from ROS, forage ROS, reduce DNA fragmentation, improve quality of semen, limit sperm cells' cryodamage, inhibit preterm sperm maturation, and stimulate spermatogenesis. After supplementing with vitamins C and E, the number of sperm undergoing DNA fragmentation fell by 13% (Agarwal et al. 2008). Spermatozoa can self-repair modest amounts of DNA damage (Agarwal 2007).

1.4.3 Effect on Sperm Motility

Sperm motility refers to the capability of sperm to swim swiftly. Because sperm must pass through the woman's reproductive system to

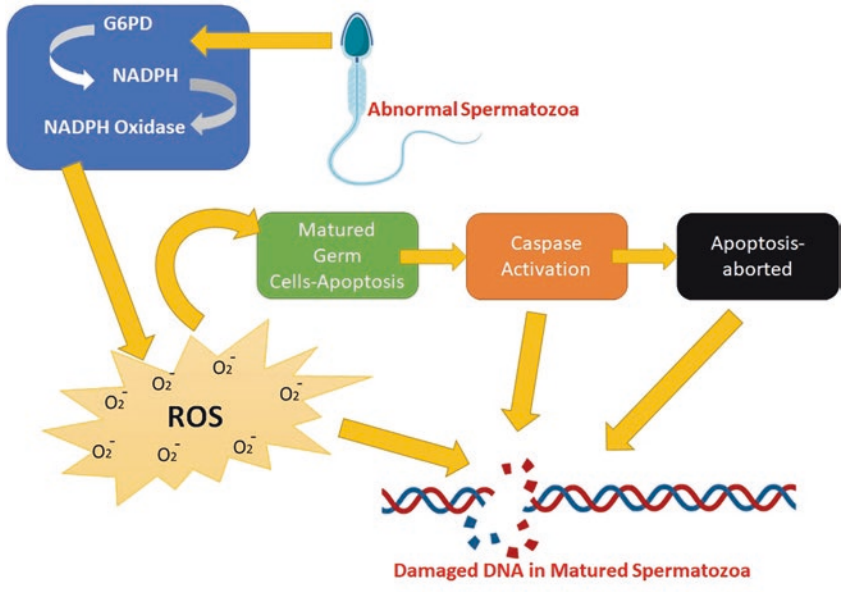

Fig. 1.4 Pathway showing sperm DNA damage and apoptosis due to ROS

reach and fertilize her egg, this is critical in fertility. Male factor infertility can be caused by sperm motility issues.

ROS-induced OS causes axonemal damage and increases morphological abnormalities in the midpiece of sperm, resulting in decreased sperm motility (Kurkowska et al. 2020). When levels of seminal ROS are too high, they may have an adverse effect on the quality and function of spermatozoa (Agarwal et al. 1994). Reduced spermatozoa motility, acrosome response defects, and fertility loss have all been linked to increased seminal ROS generation (Griveau and Lannou 1997). The type, amount, and frequency of ROS exposure significantly influence sperm cell dysfunction. External parameters like oxygen availability and temperature, as well as the concentration of molecular components like ions, proteins, and ROS foragers, greatly impact the extent of damage (Agarwal and Saleh 2002). Two main hypotheses are under discussion for correlating between ROS levels and sperm motility. One theory is that H_2O_2 disperse across the intracellular layer and blocks the action of several important enzymes, including glucose-6-phosphate dehydrogenase (G6PD), via a hexose monophosphate shunt that regulates the cellular availability of nicotinamide adenine dinucleotide phosphate, which is used as an electron donor by sperm to produce ROS via the NADPH oxidase enzyme system (Aitken et al. 1997). Another theory claims that a sequence of incidents leads to a drop in phosphorylation of axonemal protein and spermatozoa immobilization, both of which are associated to a reduction in fluidity of the cell membrane, which is essential for fusion reactions of spermatozoa (de Lamirande and Gagnon 1992). When spermatozoa are kept for incubation overnight, the impairment of mobility is closely attributed to the lipid peroxidation condition of the spermatozoa (Gomez et al. 1998). Additionally, antioxidants (α-tocopherol) have the ability to restore motility of sperm in vivo and in vitro supporting the theory that lipid peroxidation is a primary factor of spermatozoa's loss of motility (Suleiman et al. 1996). Lower spermatozoa motility is linked to reduced G6PD activity and higher levels of interleukin-1, interleukin-10, interleukin-12, and tumor necrosis factors, which could be linked to elevated degrees of OS in fluid part of the semen (Kurkowska et al. 2020).

1.4.4 Apoptosis

Apoptosis is a tissue damage retort defined by biochemical and morphological alterations. It aids in the removal of aberrant spermatozoa (Sakkas et al. 1999). High quantities of ROS damage the membranes, causing the cytochrome C oxidase protein to be released and caspases to be activated, resulting in apoptosis. ROS-independent mechanisms including the Fas cell surface protein potentially trigger apoptosis in sperm.

In samples with low sperm counts, Fas-positive spermatozoa are much more frequent. In individuals with male infertility, increased spermatozoal damage caused by ROS was associated with higher concentrations of cytochrome C and caspases 3 and 9, which act as markers for apoptosis as shown in Fig. 1.4 (Wang et al. 2003). PHGPx, part of the glutathione peroxidases domain, is engaged in antioxidant protection (Maiorino et al. 1989; Thomas and Capecchi 1990) and in managing responses (Schnurr et al. 1996; Hibasami et al. 1998; Sakamoto et al. 2000) and hinders apoptosis (Nomura et al. 2001).

1.4.5 Capacitation and Hyperactivation

Capacitation is the final phase in the growth of sperm cells in mammals, and it is essential to make them capable of fertilizing an egg. After in vivo or in vitro capacitation, sperm will have to go through the final maturation step called activation, which involves the acrosome reaction. Hyperactivation is a movement pattern seen in sperm during fertilization in mammals. It boosts sperm's ability to detach from the oviduct wall, travel about in the labyrinthine lumen of the vaginal canal, penetrate mucosal materials, and finally penetrate the oocyte's zona pellucida, which may be significant for fertilization success (Suarez and Ho 2003).

The signal transduction associated with capacitation of sperm require minimal levels of ROS exposure, whereas overexposure to such metabolites causes OS, which reduces the fertilizing potential of these gametes as well as their ability to sustain the initiation of perfectly natural embryo development. Activity of adenylyl cyclase is stimulated (Zhang et al. 1989; Twigg et al. 2001; Lewis and Aitken 2001; Rivlin et al. 2004), which is followed by protein kinase activation (O'Flaherty et al. 2006); the action of ROS promotes sperm capacitation by inducing oxidation of cholesterol and subsequent outflux from the plasma membrane (Hecht et al. 1992).

1.5 Idiopathic Male Infertility: A Case Study

Infertility is a reproductive health condition observed in about 15% of all married couples around the globe. More than half of them is due to problems related to men. Of which, 60%–75% of instances are idiopathic; this is because the mechanisms that cause these deformities remain unknown. Idiopathic male infertility also known as idiopathic oligoasthenoteratozoospermia specifies that the quality of semen produced is inexplicably low (Malaspina 2001).

Most researchers propose that abnormal epigenetic changes have been the cause of infertility in men. Aberrant DNA methylation has been identified as a possible mechanism compromising male fertility. Abnormal DNA methylation could be the result of a malfunction in the machinery that sets up and maintains normal DNA methylation. Further evidence has been accumulated indicating that gene mutations and single-nucleotide polymorphism (SNP) led to male infertility (Cornwall 2009).

Although the molecular background of idiopathic infertility in males has not been identified distinctly, OS is observed in a lot of these cases. ROS plays a pivotal part in spermatozoa functions during acrosome reaction, fusion of ovum with sperm, and sperm capacitation. Their concentrations should be maintained at a certain level, making sure they are not affecting cellular function and metabolism (Tamura et al. 2012).

In comparison to other groups of infertile men, sperm from oligozoospermic men dem-

onstrated a higher capacity for ROS formation. Seminal ROS are mainly produced by unusual and premature sperm or leukocytes. When the level of oxygen species increases to a pathological level, endogenously produced antioxidants and dietary antioxidants are used to maintain homeostasis (Eid Hammadeh et al. 2009).

A variance between these two contrary factors, in which ROS outnumbers antioxidants, might lead to OS in the cells, which can have a deleterious impact on fertility through a variety of mechanisms. ROS disrupts capacitation and might damage sperm membranes and DNA, reducing the spermatozoa's chances of fertilizing an ovum and produce a normal embryo. ROS inflation disrupts capacitation and may damage sperm membranes and DNA, reducing the sperm's ability to fertilize an ovum and produce a healthy embryo (Bui et al. 2018).

ROS can cause mutagenic by-products and be genotoxic to developing spermatozoa, potentially increasing the chance of disease in the progeny. ROS mutilates DNA and membranes through per oxidation and oxidation events in plasma membranes with high amounts of fatty acids (Wright et al. 2014).

It may be impossible to determine the presence of each component and their interactions in systems, where several tiny molecules of antioxidants are present. As a result, specific indirect tests are utilized to detect seminal ROS. The chemiluminescence test is the most popular method. It measures light intensity produced when luminol probe interacts with ROS in relative light units (RLUs) (Ochsendorf 1999).

Chemiluminescence is a technique for measuring both intracellular and extracellular ROS. Semen samples should have a spermatozoa concentration of 1×10^6/mL or above. They should also be evaluated within 60 minutes of collection to ensure reliable values (Kobayashi et al. 2001). When OS is found, the patient is given vitamin C, vitamin E, coenzyme Q-10, zinc, selenium, carnitine, and lycopene as well as other oral antioxidants (Ahmadi et al. 2016).

Researchers discovered that antioxidant supplements increased the rate of pregnancy while lowering sperm DNA damage. However, substantial further clinical examinations are required to determine the advantage of different antioxidants over another in diverse subpopulations, alongside other key factors like dosage and treatment duration (Robinson et al. 2012).

1.6 Abnormalities That Arise Due to Idiopathic Male Infertility

As previously mentioned, male infertility caused by an unknown cause is known as idiopathic male infertility (Agarwal et al. 2011). It's a major source of concern because its mechanism is unknown. Genetic, environmental, and hormonal aspects all play a major role in this condition (Abid et al. 2008). Regardless of the fact that the molecular basis of idiopathic infertility is unclear, OS appears to be one among the many underlying causes of idiopathic male infertility (Tremellen 2008).

In comparison to the fertile group, spermatozoa from idiopathic infertile males had a much greater rate of DNA fragmentation. Men suffering from idiopathic infertility have considerably higher amounts of MDA, PC, and NT in their seminal plasma (Aktan et al. 2013).

The following are the possible abnormalities that arise due to idiopathic male infertility:

Men experiencing idiopathic infertility have significantly greater levels of seminal ROS and lower antioxidant capabilities than healthy individuals (Sharma and Pasqualotto 2001). As a result, it appears that the prevalence of OS in infertile males is the cause of hitherto inexplicable cases of infertility. According to certain DNA damage tests, it has also been found out that men diagnosed with idiopathic male infertility with quintessential sperm values displayed obscure DNA abnormalities of the sperm. Even in normozoospermic males, idiopathic infertility has notably greater seminal ROS generation along with reduced antioxidant capacity (Sharma and Pasqualotto 2001). Idiopathic infertile males

have a positive seminal oxidation-reduction potential (ORP) which is a measure of the ratio of ROS to antioxidants (Agarwal et al. 2019). To put it concisely, idiopathic infertility carries various abnormalities like DNA damage, lipid peroxidation of the sperm membrane, cell death, and improper fertilization capacity of the sperm.

1.7 Peculiarities Among Global Populations

Infertility is one of the most common health challenges across the global populations. Infertility rates are higher in developed countries than in developing countries, which can be linked to a variety of socio-cultural factors (Boivin et al. 2007). In male, factors that predominantly lead to infertility include spermatogenesis defects, ductal dysfunction, and anti-sperm immune response (O'brien et al. 2010). Some genetic factors also affect spermatogenesis. Genetic factors are also known to affect hormonal homeostasis and the process of formation of sperms in male. Male infertility is a composite condition with a multiplex of clinical symptoms, including azoospermia, spermatogenic quality abnormalities, and hypothalamic-pituitary axis dysfunction (Tournaye et al. 2017).

On a global scale, infertility affects all kinds of population. In Iran, about 20% of the couples suffer from infertility arising due to males (Ferlin et al. 2007). In a recent review by Mojarrad et al. (2021), all the molecular and cellular processes reported among Iranian infertile males have been summarized in order to throw light on male infertility's molecular biology in this population (Mojarrad et al. 2021).

Asthenozoospermia is a condition wherein there is reduction in the sperm motility in the sample of a man's semen. In a case study, it was reported that the consumption of vegetables and fruits greatly diminished the threat of asthenozoospermia among Iranian males, while sweets processed meat escalated the risk of asthenozoospermia (Eslamian et al. 2012). Among the others, a direct association of vitamin D levels (Abbasihormozi et al. 2017), opioid narcotics (Safarinejad et al. 2013), and cigarette smoking was noted (Yu et al. 2014) with sperm motility, in infertile Iranian subjects. Besides the environmental factors, male infertility in the Iranian population was also caused by a combination of environmental influences, gene mutations, and chromosomal abnormalities (Moghbelineja et al. 2018).

It was evaluated that one in six couples in France have faced challenges in pregnancy (Thonneau et al. 1991). Another case-controlled study based on population was carried out in a French military population where a number of infertility risk factors were investigated. It was concluded that those men who were employed as submariners and worked under very hot conditions in nuclear-powered submarines were at a higher risk of posing infertility. The findings of this work imply that infertility in the military population may be linked to the male working in nuclear submarine maintenance or to be precise, the working conditions and exposure to extreme heat (de la Calle et al. 2001).

A study carried out to obtain firsthand information on the toxicological effects of certain hazardous metals on the quality of sperm and their probable role in male infertility among the Pakistani population showed that the measures of the traces of the toxic metals in this population were lower or almost at par with that seen in other kinds of populations around the world. A total of 75 samples of seminal plasma were segregated into classes of 3 – normozoospermia (seminal features meet the normal criteria), oligozoospermia (reduced sperm count), and azoospermia (absence of sperm in the ejaculate) – according to the World Health Organization (WHO). The concentrations of 17 contrasting toxic metals in the samples were investigated. Of these, the levels of nickel (Ni) and cadmium (Cd) were found to be higher in the oligozoospermic and azoospermic subjects when compared to normozoospermic subjects. The study therefore provided a basic interpretation of the effects of acute toxicity of metals on male reproductive health and also suggests that the intake of dietary food and such other products should be avoided as these aid in the alleviation

of the influence of these metals on the fertility in males (Zafar et al. 2015).

In a study carried out to demonstrate a unique and novel method to compute the distribution of male infertility throughout the globe, the results showed that infertility rates inflated in Africa and Central Europe while the male infertility rates in Australia and North America varied from 5–6% and 9%, respectively. Accordingly, at least 30 million men across the globe are infertile and the most soaring rates were noticed in Eastern Europe and Africa (Agarwal et al. 2015).

1.8 Diagnosis and Treatment

The measurement of sperm ROS levels in infertile men can help clinicians figure out who would benefit from antioxidant therapy. To measure seminal ROS levels, a variety of techniques have been developed, which can be divided into direct and indirect assays (Agarwal and Saleh 2002). Because antioxidants decrease the action of ROS, they are used to treat male infertility or mixed with the culture media used in sperm separation procedures (Lanzafame et al. 2009).

Nonetheless, such a treatment's efficacy has been reported to be relatively restricted. This could be due to various factors like patient selection bias, late diagnosis, lack of a double-blind, placebo-controlled clinical study, and the use of endpoints that aren't strong indicators of the presence of OS (Buyse et al. 2010). Before contemplating antioxidant treatment, individuals diagnosed with OS-related infertility should be administered with treatment focused on identifying and treating the underlying cause (Tremellen et al. 2012).

While the effectiveness of changes made to lifestyle in reducing OS has not been exponentially tested, making positive lifestyle changes like vitamin- and mineral-rich diet, weight maintenance, and avoiding consumption of tobacco, alcohol, and narcotics would have some benefits improving sperm health (Kobori et al. 2015).

Pollution, heat, and toxin accumulation have been a major grantor to the development of OS. Exposure to harmful chemicals and vapors should be reduced. The usage of personal protection equipment (PPE) and proper ventilation at work must be standardized (Awasthi et al. 2018).

Chlamydia and *Ureaplasma* infecting sperm and male accessory sex glands have been definitively related to an OS. As both illnesses are curable with the use of antibiotics, all males with a detected condition of OS should be tested for the presence of such pathogens. In a study, men suffering from *Ureaplasma* infection or *Chlamydia* were randomly chosen and half of them were assigned 3 months of antibiotics (Gallegos et al. 2008).

At 3 months, the group of men that were treated with antibiotics showed significant decrease in leukocytes number in their semen and low ROS production. They also exhibited greater sperm motility and a considerable increase in natural conception in comparison to the control group. Non-steroidal anti-inflammatory (NSAID) medicines, in combination to antibiotic treatment, may diminish the formation of free radicals by seminal leukocytes (Vicari et al. 2016).

As a result, the best treatment for infection-related OS appears to be a combination of both anti-inflammatory drugs and antibiotics in separate terms/durations (Garrido-Mesa et al. 2013). Vitamins B, folate, B12, and B6 have been shown to improve the enzymatic effectiveness of the methylenetetrahydrofolate reductase (MTHFR) and cystathionine-synthase enzymes, which remove homocysteine from the bloodstream (Weir and Scott 1999). Although the accurate outcome of combinational therapy in improving the integrity of sperm, the usage of multiple antioxidants having different effects on cellular pathways, in combination with a leukocyte ROS reducing agent is most likely to be useful (Tremellen 2012).

1.9 Conclusions

OS has been found to be a major contributory factor to male infertility. Given the known potential of multiple, contradictory actions of ROS on sperm activity and consequently male fertility, we must recognize that ROS might have both negative and positive effects on spermatozoa when it comes to activating critical cellular processes. Therefore, a balance between ROS formation and its rummaging antioxidative capability is crucial for the physiology and function of these cells, as well as their pathology. As discussed earlier, spermatozoa are competent ROS producers because in order to ensure successful conception, a sufficient quantity of ROS is necessary to handle critical oxidative processes such as capacitation, hyper activation, acrosome reaction, and signaling pathways. However, several internal and extrinsic stimuli may enhance the generation of ROS, which might overrun the antioxidant system. This leads to male infertility causing peroxidative damage to the plasma membrane and DNA damage of the sperm, both of which considerably impair the natural functions of the sperm. Various cases of sperm DNA repair are present in which the oocyte machinery using different mechanisms mend the faulty DNA based upon the kind and severity of chromatin damage, as well as the oocyte's ability to repair it. Although antioxidant therapy is not always the go-to treatment after the evaluation of OS, the focus should lie upon treating the underlying cause rather than providing treatment right away. According to the different case studies discussed earlier, "combinational therapy" has proven to be effective in treating OS-related male idiopathic infertility. Along with this therapy, slight changes made to lifestyle, a controlled and balanced diet, and antioxidant supplementation as and when essential can go a long way in producing positive and most effective outcomes.

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2 The Role of Environmental Toxicant-Induced Oxidative Stress in Male Infertility

Mohammad Mustafa, Sajad Ahmad Dar, Sarfuddin Azmi, and Shafiul Haque

Abstract

Infertility is a serious public health issue affecting around 15% of couples globally. Of the 60–80 million people of reproductive age affected by infertility, 40–50% are due to male factor while 30–40% of cases are still idiopathic. The recent global deterioration in sperm quality raises apprehensions regarding the toxic effects of environmental pollutants on reproductive health of males. Environmental toxicants have shown strong evidences for inducing oxidative stress affecting spermatogenesis severely, thereby leading to reduced sperm motility, count, and DNA damage. Reactive oxygen species (ROS) influences the spermatozoa development and transit process both internally and externally. Low level of ROS is indispensable for critical physiological sperm processes like sperm capacitation, motility, acrosome reaction, hyper-activation, sperm-oocyte interaction, etc., while excessive ROS disrupt antioxidant molecules which is detrimental to normal functioning of the sperm. Hence, identification of potential environmental toxicant may have clinical relevance for early screening and diagnosis of male infertility.

Keywords

Environmental pollutants · Pesticides · Heavy metals · Reactive oxygen species · Oxidative stress · Fertilization · Infertility

2.1 Introduction to Male Infertility

Infertility is defined as failure to conceive spontaneous pregnancy over more than a year after regular and unprotected sexual intercourse. The World Health Organization (WHO) has classified male infertility as a disease affecting 7–15% or 60–80 million couples worldwide (Datta et al. 2016). It is reported that 1 out of every 20 males in the reproductive age group experiences infertility (Rowe et al. 2000). Of the total infertility cases, 40–50% are contributed by male factors including genetic factors, varicocele, cryptorchidism, and hypogonadism, while 30–40% of cases are still idiopathic with normal hormonal, genetic, and biochemical parameters (Jarow 2007). In most couples, identifying a common reason of infertility is challenging since the

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pathology is often multifactorial and illusive. Reports suggest that environmental toxicant, genetics, epigenetics, age, lifestyle, sex, heat, stress, and obesity can affect male infertility (Agarwal et al. 2014). However, the exact pathophysiology is still poorly known. Global industrialization has amplified the risk of possible environmental toxin exposure to human beings. The exponential growth in automobiles and industrial sectors are held responsible for increased male infertility. Numerous environmental toxicants identified recently can affect male fertility severely.

2.2 Environmental Risk Factors and Male Infertility

The identification of high-risk environmental factors could contribute to exploration of disease etiopathology as well as the early detection and screening of high-risk patients and treatment strategies for male infertility. Environmental pollutants like organophosphate pesticides (OPs), persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), heavy metals, dioxin, bisphenol A (BPA), plastics, oils, solvents, explosives, disinfectants, radiation, etc. are severely affecting male fertility (Hauser et al. 2003; Geng et al. 2015; Babu et al. 2013; Ghafouri-Khosrowshahi et al. 2019). Humans in particular are exposed to environmental toxins by consuming contaminated food and water, use of consumable goods such as plastic ware, cosmetics, exposure to polluted air, etc. (Wong and Cheng 2011). Environmental toxicants can severely affect spermatogenesis process resulting in deprived semen quality, low sperm count, DNA damage, and irregular sperm morphology, primarily through the mechanism of oxidative stress (OS). OS and reactive oxygen species (ROS) have been identified as potential mechanisms for idiopathic infertility (Aitken et al. 2004; Sharpe 2010).

Sperm cells are surrounded by ROS both internally and externally during its formation and transit through the reproductive tracts of both male and female (Ribas-Maynou and Yeste 2020). The ROS are generated by both endogenous sources such as mitochondria, peroxisomes, endoplasmic reticulum, phagocytic cells, and exogenous sources such as environmental factors. Low levels of ROS are needed for regulation of essential normal sperm functions such as sperm capacitation, motility, acrosome reaction, hyper-activation, sperm-oocyte interaction etc., while excessive ROS disrupt the balance between antioxidant-oxidant molecules in seminal fluid which is detrimental to normal function of sperm (Ribas-Maynou and Yeste 2020). Environmental pollutants have been linked to an increase in ROS generation and a decrease in sperm quality. They can also affect male infertility by their endocrine-disrupting properties (Fig. 2.1). Environmental pollutants can affect male infertility by causing changes in hormone action, synthesis, transportation, and metabolism (Gore et al. 2015). In the last two decades, with the advancement in the area of environmental toxicology and developmental biology, research in the field of endocrine-disrupting chemicals (EDCs) has increased substantially, proving their hazardous impact on human life. Many countries in the world have banned the use of EDCs such as PCBs, BPA, and OCPs after experiencing their hazardous effects (Meeker 2012).

2.3 Pesticides Exposure and Risk of Male Infertility

Pesticides are chemicals or mixtures of chemicals commonly used to prevent, kill, repel, or mitigate pests. Pesticides have greatly helped in increasing agricultural yield to meet the food needs of fast-growing global population, while also lowering the risk of vector-borne diseases (Aktar et al. 2009). Pesticide exposure of humans and assessment of its hazards to human life needs attention regardless of the type of pesticide used. In the United States alone, approximately 750,000 new people are exposed to pesticides yearly (Perry et al. 2011). The fate of pesticide is determined by xenobiotic biotransformation enzymes, its metabolism, and degradation into

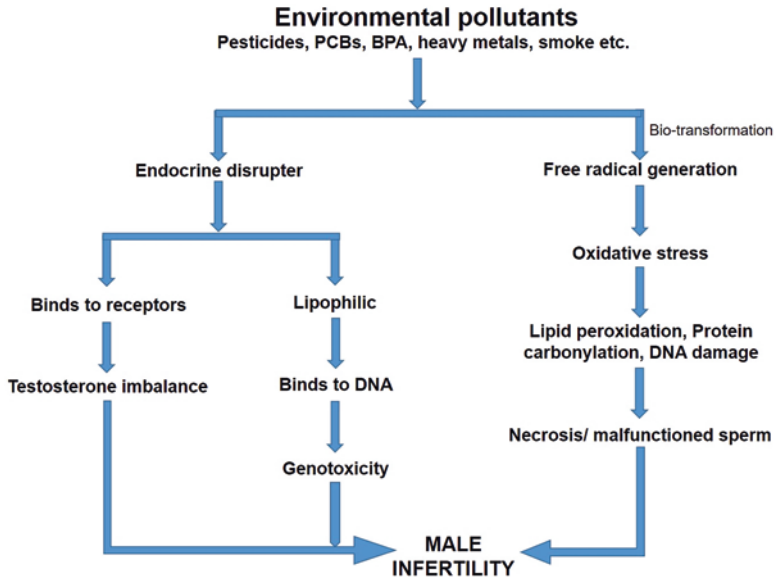

Fig. 2.1 Environmental toxicant induced toxicity and its role in male infertility

smaller weight metabolites that can stay longer in environment. Pesticides can affect the male reproductive system in many ways, including causing reproductive toxicity through direct cell structure damage or biotransformation into metabolites (Roy et al. 2017). The dose, frequency, duration, route of exposure, and genotoxic effect are the major determinants of pesticide hazard (Silva Pinto et al. 2020). The pesticides are categorized into organochlorine, organophosphate, carbamate, and pyrethroid insecticides.

2.3.1 Organophosphate Pesticides (OPs)

Pesticides such as OPs are widely used to control pests in agricultural crops, ornamentals, buildings, and homes (Jayaraj et al. 2016; Koureas et al. 2014). Humans are exposed to OPs either through their occupation or through their surroundings. The extent of OP exposure may be affected by the chemical type, the exposure route and period, age, gender, and genetic susceptibility. Similarly, occupational exposure can be affected by a variety of factors like nature of jobs, duration of exposure, precaution practice, personal protective equipment, and legislations (Mehrpour et al. 2014). OPs are potent neurotoxic chemicals that are characterized by their inhibitory action on acetylcholinesterase (AChE) enzyme activity in the cholinergic nerve terminal (Nili-Ahmadabadi et al. 2018). Acute exposures to OPs lead to the hyper-activation of the nicotinic and muscarinic receptors and often result in death if not managed properly (Peter et al. 2014). OPs are found to be associated with decreased sperm concentration and motility, resulting in infertility in men (Ghafouri-Khosrowshahi et al. 2019). The toxic effect of OPs on the spermatogenesis have also been evidenced in animal studies (Babazadeh and Najafi 2017). Chlorpyrifos, methyl parathion, and parathion are among the pesticides that have been shown to reduce sperm counts by disrupting the seminiferous epithelium by germ cell proliferation (Babazadeh and Najafi 2017; Perry et al. 2011). Furthermore, OPs have the ability to cross the epididymal epithelium and enter into stored spermatozoa due to their lipophilic nature, resulting in disruption of sperm structure and function (Adamkovicova et al. 2016). The positive correlation between OP exposure and total testosterone level was reported in Thai farmers (Panuwet et al. 2018). Increased luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels in pesticide- exposed workers engaged in spraying insecticides has

also been reported (Jamal et al. 2016). An epidemiological study from Southern Iran has shown the incidence rate of primary infertility is substantially higher among farm workers as compared to normal population (Neghab et al. 2014).

OPs are bio-transformed by xenobiotic metabolizing enzymes, such as cytochrome P450 (CYPs) and the paraoxonase (PON). Therefore, OPs are not bioaccumulative in nature; the existence of secondary metabolites in biological matrices, especially dialkyl phosphates, confirms its exposure (Androutsopoulos et al. 2013; Sokoloff et al. 2016). The activity of plasma cholinesterase that affects testosterone levels is considered as biomarker for the chronic exposure to OPs (De Silva et al. 2006). OS induced by OPs may have negative impact on sperm production and gonadal function. Ghafouri-Khosrowshahi et al. (2019) reported higher OS in rural farmers accompanied by increase in serum lipid peroxidation (LPO) levels and decreased total antioxidant capacity (TAC).

2.3.2 Organochlorine Pesticides (OCPs)

Organochlorines, which include chlorinated pesticides and PCBs, are one of the forms of environmental pollutants. OCPs are absorbed through the skin, inhaled through the air, and ingested through food and water (Jayaraj et al. 2016). Of these, taking non-vegetarian food may cause greater exposure to organochlorines since they are lipid soluble and bioaccumulative in nature. Hence, their effects are more seen in the higher trophic organism such as human beings. In addition to this, OCPs are of great concern because they are chemically stable, endocrine disrupting in nature, strongly lipophilic, as well as have a very long half-life (Abhilash and Singh 2009) (Table 2.1).

Organochlorine hydrocarbons are classified into dichlorodiphenylethanes, cyclodiene compounds, hexachlorocyclohexane, endosulfans, and other related compounds. They may exert their toxicity through several mechanisms. Previous studies have reported environmental exposure to persistent OCPs results in their presence and accumulation in human follicular fluid (Younglai et al. 2002), seminal fluid (Toft et al. 2006), as well as in embryos and fetuses (Waliszewski et al. 2000). It results in abnormal sperm count, impaired sperm motility, and reduced fertilization capacity (Sengupta and Banerjee 2014). Many researchers have concluded that OCPs may increase OS and contribute to adverse reproductive outcomes (Pathak et al. 2010; Kumar et al. 2014). An in vitro study has also reported that lindane, the gamma-isomer of hexachlorocyclohexane (HCH) may cause OS and immunotoxicity (Mrema et al. 2013). Furthermore, Banerjee et al. (2001) have shown OCPs induce alteration in lipid peroxidation, protein oxidation, glutathione redox cycle, and excessive DNA damage.

Spermatozoa are extremely vulnerable to the deleterious effects of ROS because they are rich in polyunsaturated fatty acids (PUFA) in their plasma membrane and cytoplasm. ROS causes LPO leading to disintegration of membrane, increased permeability, decreased sperm motility, DNA damage, and apoptosis (Schuppe et al. 2008; Alahmar 2019). Moreover, OCPs are known to act as environmental xeno-estrogens and structurally resemble the sex hormones. Because estrogens contribute significantly in regulation of several reproductive processes, it is possible that exposure of humans to xeno-oestrogens in the environment could influence

Table 2.1 Environmental half-life of organochlorine pesticides (OCPs)

OCPs	Half-life in soil (years)	95% disappearance (years)
Aldrin	0.3–3.0	3.0
Lindane	1.2–6.5	6.5
Dieldrin	2.5–8.0	8.0
Dichlorodiphenyltrichloroethane (DDT)	2.8–10	10.0
β-Hexachlorocyclohexane (β-HCH)	7.2–7.6	7.6

male fertility. Several evidences have accumulated during the past decade to support the notion that environmental estrogens that may cause reproductive disorders (Bulayeva and Watson 2004).

Polymorphism in xenobiotic metabolizing gene may interfere with the proper metabolism of OCPs, and thus it induces OS causing excessive free radicals generation and alteration in antioxidant interference along with disruption in steroid hormonal synthesis (Mustafa et al. 2010, 2013). Dalvie et al. (2004) evaluated sperm parameters in regularly dichlorodiphenyltrichloroethane (DDT) exposed workers and observed that sperm morphology scores were lower than that of WHO or Tygerberg criteria in 84% of the workers, with the highest recording at 6%. In a large epidemiological study, a strong association between the percentage of DNA fragmentation index (% DFI) and p,p′-DDE levels has been reported. They have suggested that DDT/DDE can damage sperm DNA/chromatin if the extent of exposure is high (de Jager et al. 2009). In a study consisting a total number of 212 infertility cases in the United States, Hauser et al. (2003) registered that a mean serum level of DDE was 254 ng/g of lipid. Pant et al. (2004) reported higher levels of p,p′DDE and p,p′DDD in the sperm of infertile men as compared to normal population.

Lindane causes OS in adult rat testes, epididymis, and alters sperm dynamics (Joshi and Goyal 2004; Chitra et al. 2001). Several studies have shown that exogenous lindane therapy lowers serum testosterone levels, suggesting that lindane inhibits testicular steroidogenesis (Ronco et al. 2001; Saradha et al. 2008). It causes alterations in Leydig and Sertoli cells by impairing their functions (Suwalsky et al. 2000). In rats, lindane doses of 10 mg/kg/day for 15 and 45 days caused considerable loss in sperm production and mortality. Control rats had 100% positive fertility, while rats exposed to lindane for 15 and 45 days had 20% and 50% negative fertility (Dalsenter et al. 1997). Treating male mice with endosulfan dosage of 3 mg/kg body weight for 35 days caused defective sperm tail, morphed acrosome, coiled tail, decreased testosterone, and enhanced LH, confirming testicular dysfunction leading to infertility (Ali et al. 2012).

2.3.3 Bisphenol A (BPA)

BPA is a typical EDC affecting biosynthesis of steroid hormones. It has major public health concern due to its widespread use, toxicity, and persistent nature. It's a common chemical used in a variety of products used in our everyday lives, such as medical supplies, dental sealants, epoxy resins, can linings, and polycarbonate plastics. Its exposure has shown association with general male infertility (Manfo et al. 2014; Vitku et al. 2016), but the results are ambiguous (Yuan et al. 2015; Mínguez-Alarcón et al. 2016). Ingestion of contaminated food and wastewater are the major sources of BPA exposure whereas drinking water, air, and dust particles are other sources of exposure to humans and animals (Vom Saal and Welshons 2014). BPA is linked to affects on male reproductive function due to the breakdown of estrogen- or androgen-mediated pathways (Jambor et al. 2018). The hormone level analysis reflected that BPA may cause high FSH and lower inhibin B and estradiol:testosterone ratio (Meeker 2012). Its can decrease antioxidant capacity because of high free radical level causing its detrimental effect on sperm physiology and subsequent cause of infertility. Even at the lowest environmentally permissible dose, BPA can stimulate testicular seminoma cell proliferation through both putative membrane estrogen receptors (ER) and likely G-protein-coupled receptors (GPCR) (Bouskine et al. 2009).

BPA has shown its negative effects on spermatogenesis in both in vitro and in vivo studies. It has shown dose-dependent effect on sperm fertilizing ability, spermatozoa motility, and mitochondrial damage as a result of increased intracellular superoxide levels (Singh et al. 2015; Lukacova et al. 2015; Rahman et al. 2015). At high level, BPA can bind to the androgen receptor (AR) and act as antagonist. It may interfere with LH receptor-ligand binding and affect the expression of 17-hydroxylase/17,20 lyase and aromatase in Leydig cells, impairing steroidogenesis. However, BPA is considered as less potent than estradiol (Patel et al. 2015; Chen et al. 2013; Vitku et al. 2016). The Centers for Disease Control and Prevention has reported the biologically active levels of BPA in the urine

samples of >90% of American people (Calafat et al. 2008; Welshons et al. 2006). In China, strong association of polycyclic aromatic hydrocarbons (PAH) with male infertility was detected in 100% of test candidates reported (Xia et al. 2009). In another study, the average BPA levels in the urine have shown to be associated with poor sperm quality. It is worth noting that the average concentration of BPA in urine of infertile men is about 70 times lower, while in control group is 2000 times lower than the USEPA's recommended daily permissible amount. These numbers reveal that BPA can cause toxicity at far lower doses than the USEPA's daily recommended intake (Li et al. 2009).

2.4 Phthalates: Steroidogenesis and Spermatogenesis

Phthalates are one of the classes of EDCs which are ubiquitously distributed in the environment. They are used as plasticizers for polyvinyl chloride plastics. Global explosion of plastic use in daily life has increased its exposure to humans. Phthalates have the ability to damage male fertility by causing OS in the testes as well as disrupting endocrine synthesis pathway. The induced OS caused depletion of antioxidant capacity, especially in GPX (glutathione peroxidase) and GST (glutathione S-transferase) and increase LPO, CAT (catalase) and SOD (Cu/Zn superoxide dismutase) activity (Asghari et al. 2015). Reports suggested that di(2-ethylhexyl)phthalate (DEHP) affects the gene expression of antioxidant-related genes such as SOD1 and GPX expression at a dose of 100 μg/mL; however, decreased expression of anti-apoptotic factor (Bcl-2) and increased expression of pro-apoptotic factor (Bax) were noted at doses of 1, 10, and 100 μg/mL (Wang et al. 2012).

The ability of phthalates to bind and to activate peroxisome proliferator-activated receptors (PPARs) has long been known (Lapinskas et al. 2005). The phthalates binding to PPARs can cause increased intracellular OS by the mechanism of activating certain ROS generating enzymes (Mathur and Cruz 2011). Phthalates can also reduce Leydig cell activity by inducing ROS, which lowers the levels of steroidogenic enzymes. According to its toxicological profile, monoethylhexyl phthalate (MEHP) is ten times more toxic to Leydig and Sertoli cells than DEHP, indicating that DEHP is the pre-toxin action by metabolizing into MEHP (Koo et al. 2002).

2.4.1 Dioxins

Dioxins are lipophilic, persistent organic compounds that are highly resistant to degradation, allowing them to remain in the atmosphere for long duration. They are part of the "dirty dozen," a collection of hazardous chemicals known as POPs. These are the outcome of industrial processes including chlorine bleaching of pulp and paper, pesticide manufacturing, and medical waste and plastics incineration (Hewitt et al. 2006). The complex mixture of dioxins contains 75 dioxin congeners and 135 furan congeners; of the total, 7 and 10 congeners can bind to aryl hydrocarbon receptor (AhR) for its activation (Van den Berg et al. 2006). Dioxins and dioxin-like compounds such as polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans, and certain polychlorinated biphenyls have shown the similar structural and biological properties. Dioxin exposure has been considered as a risk factor for adverse male reproductive outcomes. The adverse effect of a single in utero 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure leads to reduction in sperm count in adult rats (Foster et al. 2010). TCDD has been reported to cause depletion in antioxidant enzymes may increase OS (Yoshida and Ogawa 2000).

2.4.2 Polychlorinated Biphenyls (PCBs)

PCBs are lipophilic, persistent organochlorines that were previously used in cutting oils and lubricants, as well as electrical insulators. Because of their toxicological effects on humans and laboratory animals, PCBs use and manufacture were banned in several countries in the late 1970s.

However, they are still present in food today, primarily in fish, poultry, and dairy products (Freels et al. 2007). PCBs are lipophilic, non-biodegradable, and bioaccumulative chemicals; hence, they tend to accumulate in higher tropic levels of the food chain (Gupta et al. 2018). The epidemiological studies reveal that prenatal exposure to PCBs may disrupt spermatogenesis and steroidogenesis processes resulting in reduced sperm count and male infertility (Guo et al. 2000). PCBs may induce OS by increasing ROS levels, which can damage membrane lipids by LPO and subsequently causes disruption of membrane (Halliwell and Gutteridge 1990). Both, in vivo and in vitro studies have reported deleterious effect of PCBs on LPO in Leydig cells (Aly et al. 2009; Venkataraman et al. 2008).

Petersen and colleagues included 266 fertile men for estimating exposure of PCBs by evaluating hormone concentration and semen quality. Their study showed that the ratio of testosterone to estradiol, as well as the levels of FSH and sex hormone binding globulin (SHBG), was related to serum PCB levels. In contrary to this, no association was reported between semen volume, sperm concentration, count and morphology, and abstinence duration by others (Petersen et al. 2015). Despite the fact that PCBs are not currently in use, their residues are continuously being reported in human beings. Hence, questions about no association of male infertility with PCBs or inverse correlation of PCBs with indicators of male reproductive function persist.

2.4.3 Heavy Metals and Male Infertility

Heavy metals such as lead (Pb), cadmium (Cd), arsenic (As), barium (Ba), mercury (Hg), and uranium (U) are dense elements with potential toxicity, especially in environmental context (Bánfalvi 2013) and are indicators of male infertility. Heavy metals are difficult to metabolize, hence toxic heavy metals can bioaccumulate in human body (Kamath and Bhattacharya 2012). Lower mean levels of zinc and higher level of heavy metals like Cu, Pb, and Cd in the serum and blood of infertile men indicates changes in their metabolism, which could be linked to the development of infertility among these men (Venkatesh et al. 2009; Fatma et al. 2012). Spermatogenesis and sperm function are depleted as a result of heavy metal-induced OS. Infertile men have higher levels of heavy metals and oxidant levels, as well as lower levels of enzymatic and non-enzymatic antioxidants and necessary antioxidant micronutrients (Venkatesh et al. 2009; Fatma et al. 2012). Cd, Pb, and selenium (Se) have been found in seminal plasma, which could affect semen parameters and DNA damage in human spermatozoa (Xu et al. 2003). The routine monitoring of heavy metals in humans could be helpful in improving the general health conditions including screening of high-risk male infertility.

2.5 Metabolism of Environmental Pollutants

Exposure of an organism to environmental toxins activates biological mechanisms in order to metabolize and detoxify xenobiotics by biotransformation. Biotransformation, also known as metabolic transformation, is a chemical process that converts organic compounds into relatively greater polarity compounds. It is essential for survival because it transforms ingested nutrients into substances necessary for normal body functions. Phase I and phase II reactions are the two forms of biotransformation reactions. In phase I reactions, adding a functional group to the parent compound activates xenobiotics, while in phase II reactions, a covalent linkage is built between the functional group and an endogenous water, resulting in the formation of a soluble conjugates like glucuronic acid to facilitate excretion. The cytochrome P450 (or CYPs) and glutathione S-transferases (GSTs) enzymes play an integral role in the biotransformation processes.

Cytochrome P450 enzyme family of proteins is involved in the synthesis and metabolism of both internal and external substances. The ferric

(Fe^{3+}) form of heme iron in CYP P450 is reduced to the ferrous (Fe2+) form during ligand binding. These enzymes catalyze mono-oxygenation reaction resulting in incorporation of one oxygen atom into the substrate (RH).

$$O_2 + 2e^- + R \quad H + 2H^+ \rightarrow H_2O + ROH$$

The polymorphism or mutations in CYP P450 genes may cause improper metabolism of xenobiotics leading to excess free radical generation. Phase II enzymes are involved in the hydrophilization of phase I molecules by conjugating them with glutathione, completing the detoxification step, and contributing to the excretion of conjugated compounds. GSTs catalyze this reaction as follows:

$$GSH + R \quad X \rightarrow GSR + H \quad X$$

GST enzyme brings electrophilic substrate close to GSH and allows the sulfhydryl group on GSH to be activated, resulting in completion of nucleophilic reaction and hydrophilic conjugate formation (R-X) (Eaton and Bammler 1999).

OCPs and PCBs can be activated by phase I and phase II metabolizing enzymes such as CYP1A1, CYP1B1, UGT1A6, and NQQ1 through the AhR pathway (Yan and Cheng 2006). Methoxychlor is primarily demethylated and hydroxylated by cytochrome P450 enzymes such as CYP3A4, CYP3A5, and CYP2B6. Phthalates are metabolized in a similar way to BPA, with glucuronidation reactions demonstrating a key mechanism in the detoxification process, while OPs are metabolized to the typical metabolite dialkyl phosphate (DAP) through the enzyme PON1. The functions of the parent compounds are retained in the metabolites of OPs.

2.6 Environmental Toxicant-Induced Oxidative Stress

The imbalance of oxidant molecules in cells causes OS. The sperm metabolism, fast cell division, and increased mitochondrial activity contribute significantly to the excessive free radical generation in sperm. The sperm is highly vulnerable to oxidative damage than any other cells because of the large amounts of unsaturated fatty acids in their cell membranes, scarcity of cytoplasm in a mature sperm, and limited quantity of antioxidants in the sperm cells (Saleh and Agarwal 2002). Moreover, sperm morphology also resists antioxidant enzymes to work against ROS and protect the membrane covering acrosome and tail of the sperm.

Free radicals can reduce sperm fertility potential by affecting various sperm parameters such as sperm count, motility, and genetic material. The OS plays a significant role in the production of abnormal sperm, as well as the reduction of sperm count, sperm transformation, and sperm DNA damage (Asadi et al. 2017). Thus, substantial amount of antioxidants in seminal plasma can protect fertility potential of sperm. Almonds, avocados, cabbage, and sweet potatoes are rich in vitamin E and should be included in our diet. They have strong antioxidant properties, neutralizing free radicals and inhibiting ROS, thus combating OS level in sperm (Alahmar 2019). In normal conditions, ROS produced in the sperm is constantly deactivated by seminal antioxidants (Showell et al. 2011) (Fig. 2.2).

Furthermore, ROS mediates sperm hyperactivation, which is necessary for fertilizing the oocyte (Barati et al. 2020; Griveau and Lannou 1997). Because of this partial consumption of ROS, sperm cells have low levels of ROS after spermatogenesis and epididymal maturation.

To investigate the relationship between exposure to OCPs and OS, Koner et al. (1998) used sub-acute doses of DDT and lindane in rats and reported high thiobarbituric acid reactive substance (TBARS) levels in serum after 8 weeks of treatment. Antioxidant status was also compromised in their study. According to Samanta and Chainy (2002), endosulfan causes LPO and OS in the testes of mice and rats. OCP-induced OS is the culmination of a multi-step pathway resulting in an imbalance between free radical and antioxidant levels. This imbalance causes increased peroxidation of membrane phospholipids, increased membrane permeability, loss of membrane integrity, enzyme inactivation, DNA damage, protein oxidation, and dysfunction of glutathione redox

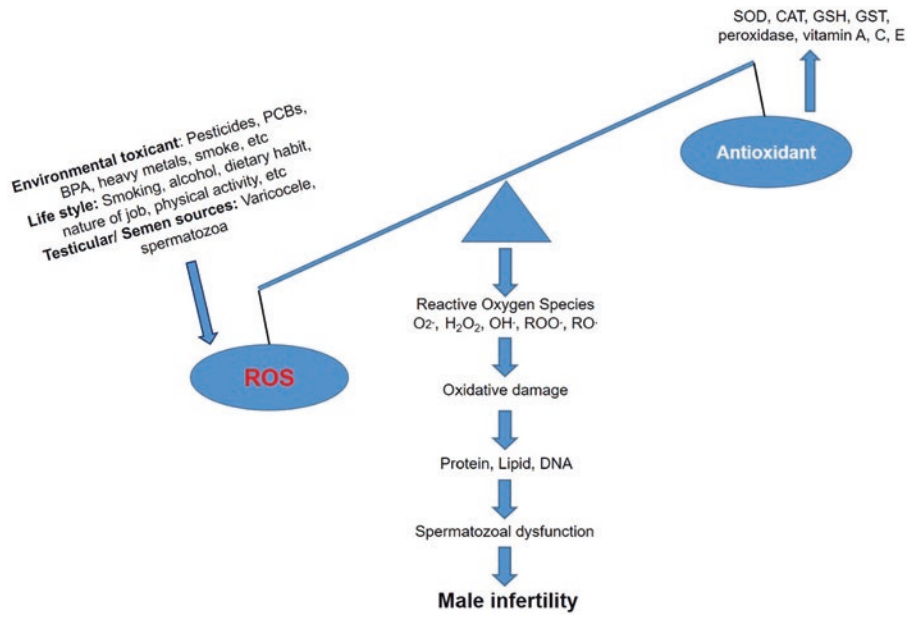

Fig. 2.2 Environmental toxicant-induced oxidative stress in male infertility

system (Banerjee et al. 2001; Koner et al. 1998; Pathak et al. 2011). HCH, DDT, and endosulfan have been shown to enhance OS in vivo and in vitro by producing a large number of free radicals (Srivastava and Shivanandappa 2005; Agarwal et al. 2005). Significant association of β-HCH, γ-HCH, α-endosulfan with content of malondialdehyde (MDA), and protein carbonyls suggests that OCPs play a role in the formation of ROS (Pathak et al. 2010). The study observed a negative association of GSH with OCPs which may be due to utilization of GSH to develop conjugates with electrophilic metabolites of OCPs or because of GSH being oxidized more efficiently by glutathione peroxidase.

2.7 Formation of Free Radicals

ROS or free radicals are oxygen derivate molecules consisting of free electrons or radicals that make them extremely reactive. Despite the fact that not all ROS are free radicals, the terms are often used interchangeably (Cheeseman and Slater 1993). Several forms of free radicals exist in biological system such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), proxyl radical (ROO), or hydroxyl (OH^-). The superoxide anion radical (O_2^-) is formed when one electron is added to dioxygen (O_2). It is the most common type of ROS. Further, this anion would then be converted either directly or indirectly to secondary ROS such as the hydroxyl radical (OH), peroxyl radical (ROO), or hydrogen peroxide (H_2O_2). Other than ROS, the reactive nitrogen species (RNS), consisting of nitric oxide (NO), dinitrogen trioxide (N_2O_3), and peroxynitrite ($ONOO^-$), are also present in sperm cells. Identification of the possible sources of ROS and RNS could be helpful to explore the underlying mechanism for the development and consequences of male infertility. It would also aid in the advancement of alternative therapeutic options as well as male reproductive health. ROS may be produced either endogenously or exogenously in the male germ line.

2.8 Sources of ROS

Reactive oxygen consists of at least one unpaired electron in the outer shell, which makes them extremely reactive molecules. It is worth to highlight that the spermatozoon produces ROS as a result of its metabolic activity.

In spermatozoa, ROS can be generated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system at the plasma membrane level and/or the NAD-dependent redox reaction at the mitochondrial level, which is the most common mechanism (Dutta et al. 2019). The retention of excess cytoplasm caused by spermatogenesis arrest may trigger the NADPH system through the hexose-monophosphate shunt, which provides electrons for ROS generation and OS. Researchers have reported that environmental pollutants like BPA can affect the enzymatic H_2O_2/peroxidase and NADPH/CYP450 pathway and generate ROS (Babu et al. 2013).

Dysfunctional or immature spermatozoa in semen supply additional free radicals affecting mitochondrial function and motility of sperm (Agarwal et al. 2014). The most common ROS in human spermatozoa is superoxide ($O2^-$), which reacts with itself to create hydrogen peroxide through dismutation reactions. Further, transition metals such as iron and copper catalyze the reaction between H_2O_2 and O_2 resulting in formation of highly reactive hydroxyl radical (OH^-). At normal physiological conditions, low levels of ROS are essential for sperm maturation, hyper activation, capacitation, acrosome reaction, as well as fertilization. However, ROS imbalance causes lipid peroxidation, sperm DNA fragmentation, and apoptosis, all of which lead to infertility.

Capacitation, for example, necessitates NO and H_2O_2, which primes the spermatozoa to start the acrosome reaction that also needs ROS (Lamirande et al. 1998; Herrero et al. 1999). Furthermore, ROS mediate sperm hyperactivation or contact with the oocyte, making it necessary for fertilization to occur (Barati et al. 2020). Environmental toxins contribute significantly to the OS in testes of up to 80% of clinically reported infertile men (Tremellen 2008; Agarwal et al. 2005). On the other hand, the level of OS, may vary greatly based on chemical composition (Kabuto et al. 2004; Dhanabalan and Mathur 2009) (Fig. 2.3).

In addition to this, environment pollutant-induced OS may activate phosphatidylinositol 3-kinase (PI3K)/c-Src signaling pathway, which can increase the risk of male infertility. Sertoli cells are essential for normal spermatogenesis, owing to their role in nutrient supply, cell junction maintenance, and germ cell mitosis, and meiosis process. The possible mechanism behind this is the breaking of the cell junctions and adhesion between Sertoli-Sertoli cells and/or Sertoli-germ cells through the PI3K/c-Src/focal adhesion kinase (FAK) pathway. Also, tight junctions (TJ) and adherens junctions (AJ) are more susceptible to OS resulting in increased permeability (Lucas et al. 2009; Sandoval and Witt 2008; Rao et al. 2002). The environmental factor-induced OS may activate PI3K/c-Src signaling pathway which subsequently cause testicular damage. The incubation of spermatozoa under OS increases production of H_2O_2 which results in decreasing the rate and motility of sperm (Aitken and Clarkson 1987).

In living systems, the free radical components strike major cellular targets that include basic protein degradation resulting in decrease in sulfhydryls or thiols in the body and DNA destroyed by free radical-induced oxidative damage to chromatin structure. The structural damage to chromatin makes the individual susceptible to infertility. Because of this, Ca^{2+} membrane permeability increases causing damage to mitochondria, DNA, and proteins, which leads to cell swelling and apoptosis.

Exogenously ROS is produced by varicocele, infections and leukocytospermia, alcohol and tobacco consumption, physical exercise and heat stress, radiations and pollution, etc. which are responsible for exogenous ROS generation in humans.

2.9 Oxidative Damage to Sperm DNA

DNA integrity and accuracy are important factors for transfer of healthy genetic material from parents to offspring; otherwise, defective embryo could be formed. It could be a better diagnostic and prognostic marker to test the functional competence of the sperm. Hence, other than the con-

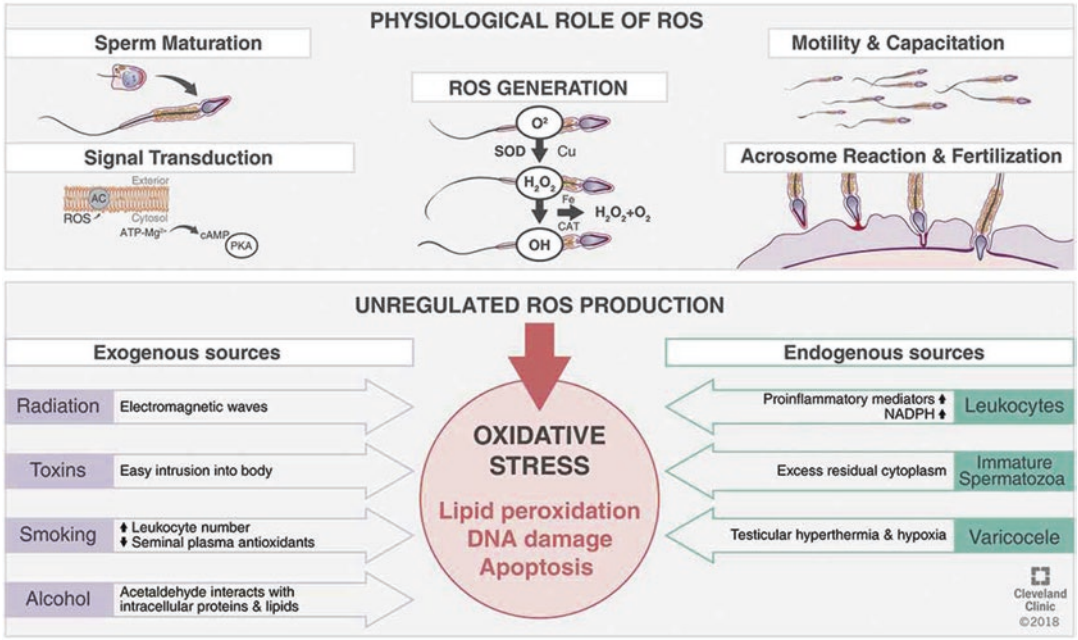

Fig. 2.3 Role of ROS in the sperm physiology and its toxic effects on spermatozoa (Dutta et al. 2019)

ventional semen analysis, usefulness of the molecular biomarkers should be explored (O'Brien and Zini 2005). Industrial explosion, environmental toxicant exposure, and life style changes of human being altogether may cause enhanced levels of ROS in abnormal spermatozoa or leukocytes conferring male infertility.

Both the exogenous and endogenous sources of ROS have deleterious effects on DNA integrity. The inheritance of abnormal genetic material to offsprings may cause autosomal dominant disorders, neuropsychiatric disorders, childhood cancers, etc. It has been reported that seminal leukocytes have the capacity to produce 1000-fold more ROS as compared to other cells with aerobic respiration (Plante et al. 1994). Therefore, excessive generation of ROS or decreased antioxidant capacity is recommended as potential threat and major cause for male infertility because it can increase DNA fragmentation, reduced sperm motility, and increased sperm death. High ROS concentration is reported in the sperm of the men whose partners have experienced abortion. The destruction of sperm membrane and DNA damage have been observed in these men, which may be the possible reasons for abortion.

2.10 Endocrine-Disrupting Chemicals (EDCs) and Male Infertility

EDCs are the substances that can disrupt male and female endocrine function through interacting with hormone receptors. Male infertility is more susceptible to EDCs because it can lead to reduction in fertility biomarkers, especially low sperm counts and testosterone level (Rehman et al. 2018; Slutsky et al. 1999; Jouannet et al. 2001). Humans are exposed to EDCs mainly through ingestion, dermal contact, inhalation, etc. (Joensen et al. 2009; Vilela et al. 2014; Zhu et al. 2016; Nordkap et al. 2012). Hence, the presence of more than the permissible limits of EDCs in our daily consumables such as foods, water, plastics, shampoos, clothing, toothpastes, soaps, fertilizers, paper, carpets, utensils, bedding, toys, cosmetics, etc. needs greater attention.

Environmental pollutants like OCPs, PCBs, BPAs, dioxin, etc. are known endocrine disrupters, which can disrupt hormone synthesis. OCPs including endosulfan and DDT as well as its

metabolites (DDE) are potential EDCs that can disrupt the hypothalamic pituitary testes axis and bind to sex steroid receptors affecting endocrine system in that specific tissue (Mehrpour et al. 2014). Pesticides may also affect neuroendocrine regulation at the testicular level, resulting in decreased testosterone secretion and increased free radical production (Petrelli and Figà-Talamanca 2001; Abdollahi et al. 2004). The exposure to OCPs raises the risk of morphological abnormalities in farm workers including fall in sperm count and a decreased level of viable sperm. Pesticides such as parathion and methyl parathion may cause genotoxicity in sperm, possibly through the OS mechanism. These OPs can also lower the concentration of the sperm by damaging the seminiferous epithelium (Perry et al. 2011). Lifeng et al. (2006) identified that sperm motility could be affected by fenvalerate pyrethroid pesticides. Male farmers in three different Malaysian communities exposed to malathion and/or paraquat showed significantly lower sperm concentration, pH, and mean volume motility of sperm cells compared to the non-exposed population (Hossain et al. 2010). Both malathion and parathion have been shown to reduce the body weights, weight of reproductive organ, and the sperm counts in rats (Narayana et al. 2006; Geng et al. 2015). The potential mechanism of action of OPs is that they alter the fold of Bax and Bcl-2 protein expression as well as reduce LH, FSH, and testosterone levels, resulting in sperm count depletion (Geng et al. 2015).

2.11 Conclusion

Available evidences suggest that the male reproductive system is highly vulnerable target of environmental toxicity. The metabolism of environmental toxicants in human body increases ROS generation, which at lower levels is required for critical physiological sperm processes like capacitation, motility, acrosome reaction, oocyte fusion, and fertilization. Higher ROS levels are detrimental to reproductive health of males and can cause a reduction in sperm count and motility, protein alterations, LPO, and DNA damage. The availability of antioxidants in seminal plasma improves the motility and fertilizing ability of spermatozoa by deactivating excessive ROS. Hence, for the normal functioning of spermatozoa, a balance between the benefits and risks of ROS and the antioxidants is essential. The identification of potential environmental toxicants in the body may have clinical relevance for early screening and diagnosis of male infertility.

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3 Effect of Environmental Stressors, Xenobiotics, and Oxidative Stress on Male Reproductive and Sexual Health

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Abstract

This article examines the environmental factor-induced oxidative stress (OS) and their effects on male reproductive and sexual health. There are several factors that induce OS, i.e. radition, metal contamination, xenobiotic compounds, and cigarette smoke and lead to cause toxicity in the cells through metabolic or bioenergetic processes. These environmen-

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tal factors may produce free radicals and enhance the reactive oxygen species (ROS). Free radicals are molecules that include oxygen and disbalance the amount of electrons that can create major chemical chains in the body and cause oxidation. Oxidative damage to cells may impair male fertility and lead to abnormal embryonic development. Moreover, it does not only cause a vast number of health issues such as ageing, cancer, atherosclerosis, insulin resistance, diabetes mellitus, cardiovascular diseases, ischemia-reperfusion injury, and neurodegenerative disorders but also decreases the motility of spermatozoa while increasing sperm DNA damage, impairing sperm mitochondrial membrane lipids and protein kinases. This chapter mainly focuses on the environmental stressors with further discussion on the mechanisms causing congenital impairments due to poor sexual health and transmitting altered signal transduction pathways in male gonadal tissues.

Keywords

Environmental stressors · Oxidative stress · ROS · Male reproductive organs · Xenobiotics

3.1 Introduction

Infertility affects 40–50% of men worldwide due to male factor abnormalities (Kumar and Singh 2015). The current review reveals the association of oxidative stress (OS) on male fertility factors such as decreased sperm motility, damage to sperm DNA, and a higher risk of recurring abortions and hereditary illnesses (Alahmar 2019). Reactive oxygen species (ROS) triggers the acrosome reaction at a low level. However, this physiological activity is also hindered by the redox imbalance (Bisht and Dada 2017).

The term OS in toxicology refers to the generation of ROS or reactive nitrogen species (RNS) that may damage (or even benefit) cells. Typical by-products of cellular respiration include superoxide (O_2^-) and hydroxyl (OH^-) radicals and other oxidants, including hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$). Protein redox regulation is also critical for optimal cellular function. Therefore, OS is defined as an increase in the quantity of oxidized biomolecules such as macromolecules including fatty acids, proteins, and nucleic acids and small molecular weight peptides or antioxidants such as tocopherol, ascorbate, and glutathione (James and Phillip 2018). Metal particles, pesticides, particulate pollutants, smoke, and other incalculable chemicals contribute to various illnesses, where intracellular or external sources may cause such stress (Manisalidis et al. 2020). They can influence the germ cell population of seminiferous tubular regions of the male gonad and result in human diseases, including reproductive problems (O'Donnell et al. 2017), cancers (Aminjan et al. 2019), diabetes (Maresch et al. 2018), chronic lung disease, and neurological disorders (Sone et al. 2010).

On the other hand, antioxidant depletion may cause free radicals to build up, contributing to the disease processes. Long-term exposure to elevated amounts of pro-oxidant substances may lead to mitochondrial DNA structural abnormalities and functional modification of numerous enzymes and cell structures that contribute to errors in gene expression. The primary enzymes are responsible for the ROS, while secondary enzymes have an indirect influence such as assisting other endogenous antioxidants. For example, in the case of glucose-6-phosphate dehydrogenase, regenerated NADPH, which is required for the activity of the original enzyme, is produced (Banafsheh and Sirous 2016; Sharifi-Rad et al. 2020). The male reproductive system is recognized to be impacted by different types of pesticides (Lwin et al. 2018). Diet and consumption of fruits and vegetables with a high residual pesticidal concentration may usually expose individuals to pesticides and may cause depletion of sperm in the male. Many insecticides may have a detrimental impact on the male reproductive system (Roychoudhury et al. 2021; Madhu et al. 2011, 2014). Several pesticides act as environ-

mental stressors (Hiroshi et al. 2020). Understanding the molecular mechanism of the effects of such environmental stressors could provide a wealth of knowledge for identifying biomarkers for diseases induced by pesticides and other environmental toxins (Franco and Panayiotidis 2009; Gibb 2008). Many of them influence the active redox-sensitive signalling pathways, such as ROS metabolism and antioxidant defence systems, nitric oxide (NO) signalling pathway, p53 signalling pathway, growth factor (TGF) transformation, hypoxia signalling, signalling of morphogenic bone protein (BMP), and tumour necroses (TNF) (Sone et al. 2010). Environmental stressors of chemical origin may induce OS that significantly impact male reproductive health, which is highlighted in this study, whereas research addressing the hazards related to early exposures in foetal life and infancy remains scarce. However, developing a large mother-child cohort with biobanked blood specimens provides a ray of hope for filling the information gap (Hymie and Zul 1999). The purpose of this review is to inform the male reproductive system of the inherent and extrinsic variables involved with OS and their effect on human reproductive functions.

Stresses are encountered in daily lives. Cataclysmic catastrophes, stressful life events, everyday difficulties, and ambient stressors are the four types of physical environmental stressors (Hymie and Zul 1999). Examples of catastrophic events include sudden catastrophes, including floods, severe earthquakes, tempests, nuclear power plant crashes, volcanic eruptions, and chemical factory mishaps. A few examples are moving to a new residential neighbourhood, starting a new career, significant construction activity in the current residential area, and other stressful life events. In some cases, stress is also brought about by the whispering of the air conditioner, constant dust in an industrial neighbourhood, and slight hiss from the central heating system (Hymie and Zul 1999).

Colds or extreme temperatures are other environmental stresses that may cause pain. Noise, congestion, air quality, colours, light, insects, and other everyday environmental stressors are only a few examples. Noise is the initial stressor (Münzel et al. 2018), and it detects any unpleasant sound. When it comes to crowding stress, both animals and humans have been found to become more stressed. Colours, light, insects, and other physical surrounding environmental stressors are the most apparent physical stressors that may cause direct or indirect stress in everyday lives (Münzel et al. 2018).

Various high-stress occupations, such as business, fighting, or combat training, have been linked to reducing plasma testosterone levels (Al-Damegh 2014; Steeno and Pangkahila 1984). Furthermore, emotional stress related to the diagnosis or treatment of infertility in couples has been linked to oligospermia (Rooney and Domar 2018), perhaps contributing to the differences in semen quality seen during the assessment.

In this paper, we provide a complete summary of the most recent findings on the mechanism of ROS generation, the physiological functions of ROS, and ROS pathophysiology, as well as the influence of OS on male infertility in humans. Many molecular elements of heavy metal-induced toxicities have yet to be clarified or recognized. The distinct biological mechanisms of action of each metal are recognized to have unique characteristics and physico-chemical properties. Cadmium, lead, chromium, arsenic, mercury, etc. are examined for their toxicity, genotoxicity, and carcinogenicity, as well as their structural processes of occurrence, manufacture, and utilization in the environment.

3.2 Methods

Published literature have been searched in electronic databases Google Scholar, PubMed, and Medline using the keywords such as “male reproductive health”, “sexual health”, “environmental stressors”, “xenobiotics”, and “oxidative stress”. Environmental stressors, xenobiotics, and oxidative stress were all mentioned in many publicly accessible sources from regulatory agencies, including the International Agency for Research

on Cancer (IARC) and the World Health Organization (WHO). Male reproductive impacts on other vertebrate models and articles published in language other than English were considered as exclusion criteria. In all cases, the articles chosen were thought to lend credence to the argument being made in the present review. The papers that matched the inclusion criteria were thoroughly evaluated.

3.3 Environmentally Linked DNA Methylation

OS and methylation mistakes are inextricably linked with DNA patterns (Gruber et al. 2018). One of its (poly A) mRNAs with intense expression levels in the primary embryo coding (Menezo et al. 2020) is the enzyme DNA methyltransferase (DNMT1), which is responsible for methylation maintenance. For the understanding of DNA methylation patterns, which provide the chemical foundation for imprinting in gametes and early embryos which is critical because changes may lead to transgenerational epigenetic diseases like autism (Menezo et al. 2015). Lack of OS protection in the culture raises the chances of methylation mistakes. Furthermore, owing to delays in the maternal-zygotic transition phase, a reduction in methylation activities is occasionally seen shortly after conception.

Protamine-bound DNA that may be modified by environmental stress factors are shown in Fig. 3.1. Histone-bound DNA comprises less than 15% of the sperm genome, which is shown in a simplified secondary structure. Sperm CG-rich, histone-bound fractions have higher levels of DNA methylation remodelling, which is also observed in repetitive DNA sequences. Environmental variables may also affect histone's location in relation to protamines. After dietary stress, some histone modifications at certain loci alter as well. There is a link between stress in life and changes in the expression of certain short ribonucleic acids (sRNA) types, such as tRF, miRNA, and the piWI-interacting RNA (Donkin and Barrès 2018).

3.3.1 Influence of Environmental Epigenetics in Metals Exposure

Toxic metals found in the environment, such as chromium, arsenic, lead, cadmium, selenium, and mercury, which have been linked to human diseases such as cardiovascular, cancer, neurological problems, autoimmune diseases, and their effect on the epigenome have just lately been studied (Ray et al. 2014). Prolonged environmental exposure to metal compounds such as nickel, arsenic, cadmium, and chromium, for example, causes male reproductive and sexual health problems, malignancies, and other diseases in those individuals who are exposed (Gibb et al. 2000; Yuan et al. 2007). This capacity has been shown in cell culture and animal study models for a long time (Kumar 2018).

It is one of the most common reproductive disorders, and environmental epigenetics, particularly in major metal exposures, has been shown to have an important role in its development (Menezo et al. 2015). The epigenetic signature of spermatozoa results from a dynamic modification of epigenetic marks that occurs, first, in the testis during germ cell progression, then along the epididymis, where spermatozoa continue to acquire molecules carried by epididymosomes (Cescon et al. 2020). Pathways of heavy metal exposure are shown in Fig. 3.2.

3.3.1.1 Arsenic

Arsenic is a common environmental pollutant found in water, soil, and airborne particles. Epidemiological studies have linked arsenic exposure to the development of tumour of the male reproductive organs and bladder, lung, kidney, and liver cancers (Marshall et al. 2007;Hong et al. 2014). Several reports suggest that low semen volume and sperm motility are linked to arsenic exposures. Arsenic's effects were found to be concentrated in the hypothalamus and brain, resulting in hormone dysregulation and lower sperm counts (Biswas et al. 1999; Sarkar et al. 2003; Jana et al. 2006; Ahmad et al. 2020; De-Luca et al. 2021). On the other hand, a significant quantity of arsenic is found in the testes, epi-

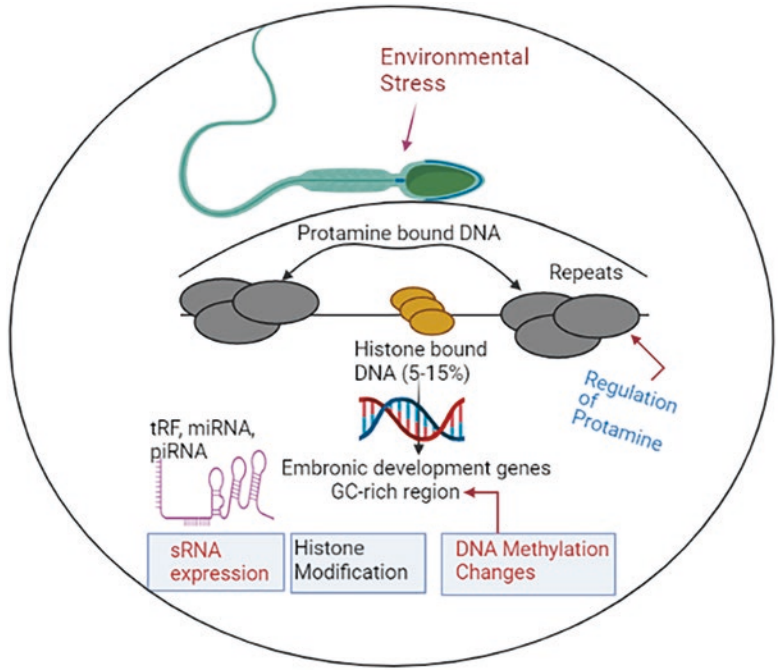

Fig. 3.1 Environmentally related DNA methylation in sperm

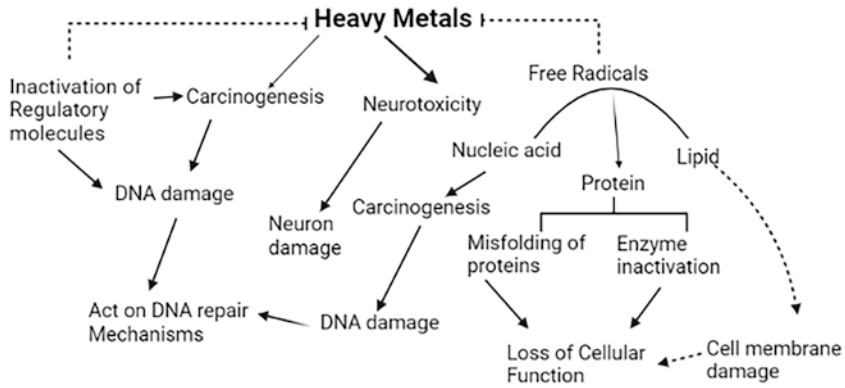

Fig. 3.2 Pathways of heavy metal exposure

didymis, seminal vesicle, and ventral prostate indicating a potential direct impact on testicular tissues (Morris and Cronin 2005). Arsenic is electropositive and may bind to high electron sulphhydryl in proteins, affecting the function of vital testosterone-producing enzymes. Glutathione and other antioxidant enzymes were found to be attached to arsenic, decrease cell capability, and cause oxidative stress (Morris and Cronin 2005).

Arsenic, both organic and inorganic, is also a carcinogenic metal. Arsenite, not arsenate, is thought to cause the increased cancer risk, perhaps owing to the cell's capacity to absorb arsenite quicker than arsenate (Bertolero et al. 1987; Hughes et al. 2011; Shankar et al. 2014). Inorganic arsenic particles – trivalent arsenite [As(III)] and pentavalent arsenate [As(V)] as environmental carcinogens – affect the status of DNA methylation in cells (Cheng et al. 2012). Exposure has been linked to dose-dependent global DNA hyper-methylation, as measured by metal concentrations in drinking water and biological fluids or tissues (Niedzwiecki et al. 2013).

Genome-wide analyses of DNA methylation from Bangladesh showed an effect on skin lesions with changing methylation 6CpG sites, one of which correlates to the RHBDF1 gene (Seow et al. 2014). However, there was a difference in DNA methylation between arsenic-induced and non-arsenic-induced urothelial carcinomas in Taiwanese individuals (Yang et al. 2014). Hypermethylation of the transposons repeat was discovered in LINE-1, the p16 promoter, and other particular sequences with arsenic concentrations (Kile et al. 2012). According to a prospective American birth cohort study, prenatal exposure to low amounts of arsenic seems to alter the neonatal blood DNA methylation pattern (Koestler et al. 2013). As a consequence, arsenic was thought to induce cellular transformation in male gonads through chromatin-based mechanisms. Figure 3.3 schematically shows the male reproductive toxicity of arsenic III.

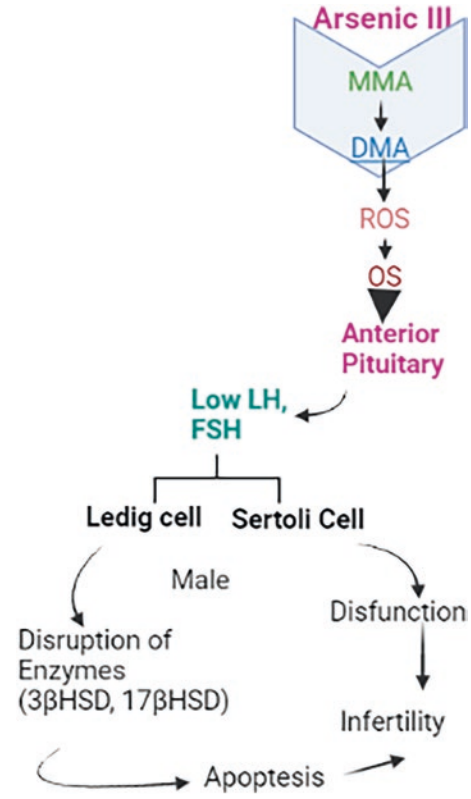

Fig. 3.3 Male reproductive toxicity of arsenic III

3.3.1.2 Nickel

As a non-biodegradable heavy metal, nickel is a concern to the environment as well as human health. Human activities such as municipal waste, smelting, fertilizers, pesticides, and industrial effluents all contribute to the pollution of the environment with trace amounts of nickel at the range of 0.005–100 ppm (Sharma et al. 2021; Fabiano et al. 2015).

Nickel is a carcinogenic and hazardous metal that may occur in both water-soluble (e.g. $NiCl_2$, $NiSO_4$) and water-insoluble (e.g. Ni_3S_2, NiO) forms and is a source of environmental concern in both forms (e.g. $NiCl_2$, $NiSO_4$). It is widely used in batteries, welding, carbon nano-particle synthesis, plating, jewellery, coins, and medical devices (Arita and Costa 2009). These nickel compounds are weakly mutagenic in most animals. The propensity of nickel to induce epigenetic alterations is believed to cause its carcinogenic risk (Arita and Costa 2009; Rizvi et al. 2020). Structural alteration of chromatin, such as heterochromatinization, linked with gene suppression, may play a role in nickel-induced carcinogenesis. Promoter hypermethylation of the tumour suppressor gene p16 was linked to Ni-induced carcinogenesis (Govindarajan et al. 2002) and inflammatory responses in broiler chickens (Deng et al. 2016). Ji et al. (2008) examined the molecular connection between nickel-induced epigenetic alterations and cellular transformation in human bronchial epithelial (16HBE) cells.

Like other heavy metals, nickel increases ROS generation, lowers glutathione and other antioxidant levels, increases cell membrane lipid peroxidation, causes apoptosis, and contributes to oxidative DNA damage (Aitken and Koppers 2011). Damage to the sperm membrane decreases sperm motility and the capacity to fuse with the egg. It is interesting to note that the mitochondrial inner membrane contains the cytochrome C oxidase enzyme, which aids in the oxidation of cytochrome c^{2+} to cytochrome c^{3+} and reduces oxygen to water. As a result, it is crucial for oxidative phosphorylation and energy metabolism (Fig. 3.4). In the testes, a particular isomer allows for maximum aerobic metabolism and the consequent generation of ATP required for spermato-

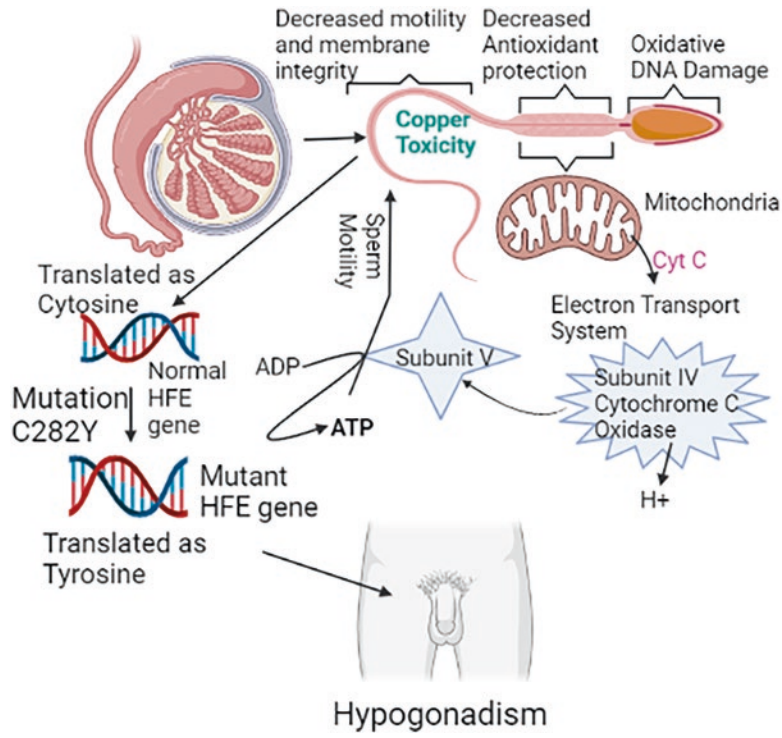

Fig. 3.4 The mitochondrial inner membrane contains the cytochrome C oxidase enzyme, which aids in the oxidation of cytochrome c^{2+} to cytochrome c^{3+} and reduces oxygen to water. As a result, it is crucial for oxidative phosphorylation and energy metabolism. In the testes, a particular isomer allows for maximum aerobic metabolism and the consequent generation of ATP required for spermatozoa motility (Adapted from Tvrda et al. (2015))

zoa motility (Tvrda et al. 2015). However, damage to the sperm DNA impairs paternal genetic contribution to the embryo and raises the likelihood of infertility, miscarriage, or severe illness in the child (Tremellen 2008; Aitken and Koppers 2011).

3.3.1.3 Lead

Lead is a naturally potent toxicant and impacts human health (Rădulescu and Lundgren 2019; Tiwari et al. 2013). Tobacco also contains lead (Ashraf 2012). Studies on the effects of heavy metal exposure on male reproductive tissues/organs are lacking from the perspective of environmental high exposure levels. However, consequences at low exposure levels have been seen, with cadmium, lead, and mercury showing the strongest effects (Wirth and Mijal 2010; Ren et al. 2017). Exposure of experimental animals to lead has been associated with DNA strand breaks and chromosomal alterations at various locations. Compared to controls, adult primates exposed to lead had lower levels of DNA methyltransferases DNMT3A and DNMT1 and lower levels of H4K8ac, H3K9ac H3K4me2, and H4K12ac (Hyun-Wook et al. 2015). Telisman et al. (2007) discovered statistically significant relationships between BPb, ALAD, and/or EP and reproductive parameters, suggesting a plum-related increase in the amount of immature sperm and its abnormalities. These reproductive consequences were found at modest exposure to lead (BPb median 49 μg/L, 11–149 μg/L).

Doumouchtsis et al. (2009) stated that lead may affect mostly endocrine glands. The stunned thyroid-stimulating hormone, growth hormone, and follicle-stimulating hormone (FSH)/luteinizing hormone (LH) ratio on the hypothalamic-hypophysial axis seems to be causing thyroid growth hormone-releasing hormone and gonadotropin-releasing hormone stimulation.

A host of genetic, environmental, occupational, and lifestyle considerations combine to exert unfavourable impacts on men's health. In most investigations, even moderate to low exposure to lead usually had key reproductive changes (Pizent et al. 2012). Several previous investigations indicate that occupational lead exposure

impairs semen quality in the blood of the exposed individuals at a level of >40 µg/dL (Kumar 2018). Effect of lead on males has been tabulated in Table 3.1.

3.3.1.4 Chromium

Chromium may be found involved with various OS conditions, including 0, +3, and +6, Cr(VI). Cr(III) is the ultimate oxidative form (Cheng et al. 2012). It has been related to respiratory malignancies in epidemiological studies (Salnikow and Zhitkovich 2008). A major source of chromium exposure to animals is drinking water via industrial chromium, mainly Cr(VI). If mixed with drinking water, it may cause many types of cancer, including leukaemia, bone, prostate, lymphoma, renal, brain, lung cancer, gastrointestinal, and nasal cancers due to its high mutagenic characteristics (Nickens et al. 2010). Chromium is believed to cause OS, DNA strand breakage, DNA-protein cross-links, and the creation of persistent chromium-DNA adducts as the major mechanism of chromium-induced cytotoxicity (Zhitkovich 2005).

Chromium(VI) has an unstable electronic structure that has mutagenic and carcinogenic effects on cells. By increasing OS, chromium(VI) affects the male reproductive system. Total sperm disorders are caused by the loss of seminiferous tubules, chromatin fragmentation, mitochondrial disorders, and blood test barrier problems (Aruldhas et al. 2005). Aruldhas et al. (2005) found that chromium(VI) is highly toxic to testicular organs. Bonnet monkeys when subjected 6 months of chromium(VI) treatment at 100, 200, and 400 ppm in their potable water was found to trigger total disruption of spermatogenesis with premature release of germ cells in the lumen of seminiferous tubules (in various phases of development). The spermatocytes showed chromatin fragmentation, mitochondrial swelling, and vacuolation. In addition, the existence of phagocytic sperm cells has indicated the breakdown of the blood-testis barrier. Thus, the authors hypothesized that chromium(VI) might interfere with spermatogenesis (Aruldhas et al. 2005; Pereira et al. 2021).

Table 3.1 Effect of lead on males

Exposer to lead	Observed effects	References
Low levels of environmental lead in the blood	Reduced growth and delayed pubertal development in adolescent boys were associated with low ambient blood lead levels	Hauser et al. (2008)
The relationship between blood lead levels and pubertal onset in Russian boys was studied. There were 481 boys with blood lead levels averaging 3 g/dl and 28 per cent with values less than 5 g/dl	For peripubertal boys, greater blood lead levels were related to pubertal onset 6–8 months later than those with lower blood lead levels of less than 5 g/dl. Pubertal development was slowed in high lead environment	Williams et al. (2010)
Boys with elevated lead levels (more than 10 g/dl)	Females with elevated blood lead levels had a delayed breast stage of sexual development. FSH and LH levels were dramatically lowered in both sexes, but testosterone levels were reduced in men with elevated lead	Tomoum et al. (2010)

3.3.1.5 Copper

Major sources of copper are fish, meat, vegetables, seeds, whole grains, nuts, chocolate, and potatoes (Morris et al. 2006). Current industrial sources include agricultural chemicals, municipal garbage, construction, automobiles, and electricity (Bertram et al. 2002). Copper has two oxidation states: cupric (Cu^{2+}) and cupric cuprous (Cu^{+}). Cu^{2+} is highly soluble; therefore Cu^{+} is listed in a submicromolar range (Arredondo and Nunez 2005). In biological systems, copper is primarily present in the Cu^{2+} form because Cu^{+} is rapidly oxidized into Cu^{2+} in the presence of oxygen or other electron acceptors. Copper oxidation is reversible because Cu^{2+} may absorb a strong reductant electron like ascorbate (Løvstad 1987) and reduced glutathione (Kachur et al. 1998;

Tvrda et al. 2015). Dietary copper is primarily linked to serum albumin and transported to the liver. The remainder is bound to ceruloplasmin and is discharged into the bloodstream (Hellman and Gitlin 2002). A tiny amount of copper is eliminated in bile. Ceruloplasmin is the main copper-binding protein, where each molecule contains six copper atoms. Around 80% of seminal ceruloplasmin is found in Sertoli cells inside the tests (Orlando et al. 1985; Aldred et al. 1987; Tvrda et al. 2015). Copper is linked to metallothioneins (MTs), copper, and zinc proteins (Krezel and Maret 2007). MTs are known to detoxify a range of heavy metals in the reproductive system of male mice, rats, and humans (Mukhopadhyay et al. 2009; Ren et al. 2003; Dutta et al. 2021). Two main MT isoforms and their mRNAs have been mainly shown to protect germ epithelium in Sertoli and spermatogenic cells (Betka and Callard 1999). In addition, Sugihara et al. (1999) discovered that testing, which was specifically created in spermatocytes on day 8 of postnatal development, corresponded with the entrance of germ cells into meiosis, which was a new type of testing. As a result, it is currently used as an early diagnostic tool to differentiate between the male germ lines.

Copper exposure at work may potentially alter the epigenome. Copper reduces global acetylation of histone H3 and H4 in human hepatocytes (Hep3B cells) via direct suppression of histone acetyltransferase (HAT) activity without altering histone deacetylase (HDAC) activity (Kang et al. 2004). An in vitro, study showed that copper could bind to key histidine residues in histones H4, H3, and H2A (Karavelas et al. 2005). Copper binding to H2B's C-terminal peptide (H2B94-125) may interfere with Lys120's ubiquitination, which has been linked to gene silencing (Zavitsanos et al. 2011). In addition, copper interacts with DNA and histones, causing chromatin structural changes and altered gene expression. According to researchers, males with reproductive difficulties exhibited higher copper levels (Stanwell-Smith et al. 1983; Huang et al. 2000; Aydemir et al. 2006; Govindaraju et al. 2013; Hardneck et al. 2021).

3.3.1.6 Mercury

Widespread exposure to the environmental pollutant mercury is thought to be harmful to men's reproductive health (Hg). The hazardous consequences of mercuric chloride ($HgCl_2$) are not well understood; however; Hg may disrupt male reproductive function (Martinez et al. 2014). Although it is not a natural metal, it is a significant environmental hazard. Food (mainly fish) and different industries such as gold mines, electric motors, metal smelting, and coal-burning consume and magnify mercury effects regularly. Mercury exposure mainly affects the brain and placenta. As a result, it is most harmful while in foetus development. Methylmercury (MeHg) influences the expression of many neuronal growth factors in cell culture models, influencing neurite outgrowth and neural stem cell differentiation (Parran et al. 2003). A study linked rising mercury levels in male hair samples to the selenoprotein-P plasma-1 (SEPP1) promoter (Goodrich et al. 2013; Martinez et al. 2020). Experimental animals have been utilized in a bulk of studies on histone alteration induced by environmental mercury. Long-term exposure to inorganic and organic mercury caused Leydig cell disintegration, which hindered 3-b-hydroxysteroid dehydrogenase (3-b-HSD), a key enzyme in testosterone synthesis, and reduced testosterone levels (Chowdhury et al. 1985; Vachhrajani and Chowdhury 1990; Zhang et al. 2020; Massányi et al. 2020).

Mercury was linked with reduced sperm production and reproductive failures (Bjørklund et al. 2019; Kushawaha et al. 2021). Mercury-induced necrosis and sperm abnormality seem to involve two separate mechanisms. Mercury leads to higher ROS and MDA levels and reduces total antioxidant (TAC) and superoxide (SOD) activity, resulting in reduced membrane intactness and cell death. In addition, OS also leads to a reduced transmembrane potential (MTP) for mitochondrial function that can increase cAMP levels and release Ca^{++}, which increases spontaneous acrosome reactions (AR) and reduces the ability to reduce the power of sperm to fertilize finally. In all, mercury leads to necrosis-driven cell death

instead of apoptosis. Necrosis, therefore, seems to be the main signalling route in sperm abnormalities caused by mercury and may be the result of substantial damage to sperm cell ultrastructure (Kushawaha et al. 2021). Proposed mechanisms of mercury-induced spermatozoa toxicity are presented in Fig. 3.5.

3.3.1.7 Cadmium

Cadmium is a toxic transition metal found in the environment that has been linked to malignancies of the testes, liver, kidney, pancreas, and bone and is a recognized risk factor for cardiovascular diseases (Tellez-Plaza et al. 2012). Consumption of tobacco products, battery production, and certain electroplating operations are the primary sources of cadmium in the environment. Even though cadmium may cause OS, it is mutagenic and has a low DNA-binding affinity. This has led to the idea that cadmium may induce carcinogenesis through epigenetic processes (Arita and Costa 2009). Acute cadmium exposure has been found to reduce DNA methylation by inhibiting DNA methyltransferase (DNMT) activity in a non-competitive manner. Chronic cadmium exposure, on the other hand, causes worldwide DNA hypermethylation and increased DNMT activity. Furthermore, higher methyla-

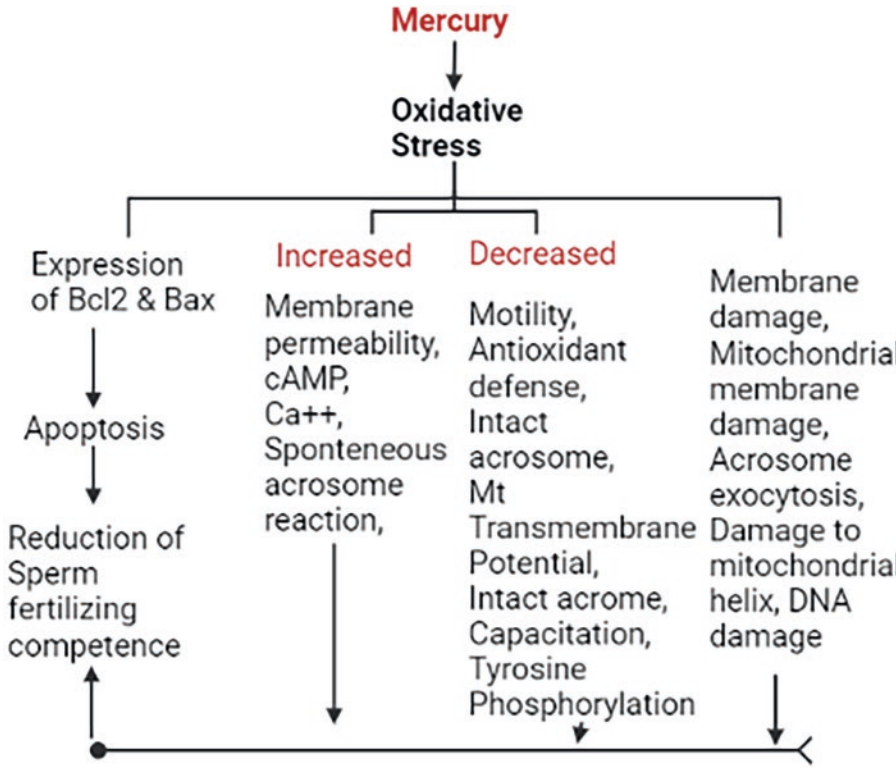

Fig. 3.5 Proposed mechanisms of mercury-induced spermatozoa toxicity. Mercury-induced spermatozoa necrosis and death appear to be caused by two distinct pathways: mercury causes an increase in reactive oxygen species (ROS) and malondialdehyde (MDA) levels, as well as a reduction in total antioxidant capacity (TAC) and superoxide dismutase (SOD) activity, all of which lead to decreased membrane integrity and cell death. Oxidative stress also causes a reduction in mitochondrial transmembrane potential (MTP), which can lead to an increase in cAMP levels and increased intracellular Ca^{++} release from spermatozoa, which can lead to an increase in spontaneous acrosome reactions (AR) and a reduction in capacitation, which can reduce sperm fertilization ability. Overall, mercury causes ROS-induced cell death via necrosis rather than apoptosis. In mercury-induced sperm mortality, necrosis seems to be the main signalling route, possibly owing to significant damage to sperm cell ultrastructures (Adapted from Kushawaha et al. (2020, 2021))

tion was also related to increased cell proliferation, followed by transformation (Takiguchi et al. 2003).

The mechanism by which Cd influences male fertility is increasingly associated with the generation of ROS in the testes (Morielli and O'Flaherty 2015). ROS consists of hydroperoxyl radicals, peroxyl and hydroxyl, nitric oxide, nitrogen dioxide, and superoxide. ROS equilibrium is maintained via ROS generation and the antioxidant system. This disruption of homeostasis leads to OS that impedes sperm and somatic cell growth and function or causes apoptosis (Morielli and O'Flaherty 2015). Cadmium promotes testicular ROS generation. For 5 days, cadmium (6.5 mg/kg) exposure to adult rats increased OS, including increased peroxidation and nitric oxide. It reduced GSH, catalase, superoxide dismutase (SOD), glutathione peroxidase, and glutathione reductase. As a result, the BCL-2-associated-X-protein (Bax) and tumour necrosis factor (TNF), a pro-apoptotic protein that regulates expression, are increased (Elmallah et al. 2017). Rat treated for 13, 25, and 39 days with cadmium (1 and 5 mg/kg) increased ROS generation and reduced seminiferous tube diameters, decreased numbers of spermatogonia, SCs, LCs, and reduced sperm motility and sperm numbers (Mahmoudi et al. 2018). Cadmium exposure to adult mice (1 mg/kg intraperitoneally) increased lipid peroxidation after 5 and 8 weeks and lowered testicular SOD, catalase, and peroxidase levels resulting in increased sperm abnormalities and decreased sperm count (Acharya et al. 2008).

Cadmium has a biological half-life of 7–26 years in the kidney and 3–4 months in the blood. Thus, cadmium accumulates in the ovaries, testes, semen, liver, kidneys, and placenta over time due to its poor rate of excretion (Varga et al. 1993; Akinloye et al. 2006) with a preference for the male reproductive organs (Varga et al. 1993; Ronco et al. 2005; Danielsson et al. 1984; Zhu et al. 2020). It influences the function of mature sperm and decreases sperm motility and progressive motility as shown after in vitro treatments with human and mouse sperm (Zhao et al. 2017). Table 3.2 presents the effect of cadmium on testicular cells.

3.4 Air Pollution and Oxidative Stress (OS)

Carbon monoxide, nitrate, sulphur dioxide, and ozone are all components of air pollution. The most robust connections in that combination are aerosols and particulate matter, including solid and liquid particles (Farhat et al. 2011; Brook et al. 2004). The composition and effect of air pollution vary significantly depending on the pollutant source and environmental factors such as weather, seasons, industrial activity, and traffic congestion. It is now well accepted that some air pollutants may damage sperm and reduce their viability.

3.4.1 Particulate Matter (PM)

Fine particulate matter (PM) has been linked to low sperm quality, although the mechanism is unknown (Zhou et al. 2021). After exposure to $PM_{2.5}$, the luteinizing hormone levels, the quantity of sperm, testosterone, and the ultrastructure of BTB in the testis of rats decreased (Liu et al. 2018; Zhou et al. 2021; Chen et al. 2020). Furthermore, $PM_{2.5}$ exposure impaired male reproduction in mice by causing OS through the PI3K/Akt signalling pathway (Xi-Ning et al. 2015). In addition, PMs irritate the upper bronchi and induce inflammation of the lungs (Nemmar et al. 2002). The atmosphere contains three types of PM: coarse, fine, and ultrafine particles. PM_{10} refers to big "coarse" particles of less than 10 μm in diameter. These coarse particles come from several sources, including silica-based crystal particles (such as soil, sand, and volcanic ash), natural material combustion (such as wood smoke), and equipment wear (e.g. vehicle braking and tyre erosion). Fine particles have a diameter of fewer than 2.5 μm and are referred to as $PM_{2.5}$, whereas ultrafine particles have fewer than 0.1 μm diameters and are referred to as $PM_{0.1}$ (Pope et al. 2004). Industrial combustion of fossil fuels and traffic-related sources are the primary sources of fine and ultrafine PMs. These fractions are particularly significant

Table 3.2 Effect of cadmium on testicular cells

Species	Cell	Action	References
Rat	Sertoli cell	Blood-testis barrier disruption (ST), cytoplasmic vacuolation (ST), Dhh and Fshr expression (IN), cytoskeleton disarrangement (ST), ultrastructure alteration (ST)	Xiao et al. (2014), Li et al. (2018), and Zhu et al. (2018)
Pig	Sertoli cell	Apoptosis (ST), DNA damage (ST)	Zhang et al. (2018)
Mouse	Sertoli cell	Mitochondrial alteration (ST)	Bizarro et al. (2003)
Human	Sertoli cell	Blood-testis barrier disruption (ST)	Xiao et al. (2014)
Rat	Adult Leydig cell	Leydig cell number (IN), Leydig cell development (IN), Leydig cell volume (IN), Testosterone synthesis (IN), Leydig cell tumour (ST), Leydig cell regeneration (IN), cytoplasm vacuolization (ST)	Blanco et al. (2007, 2010), Cupertino et al. (2017), Wu et al. (2017), Mahmoudi et al. (2018), and Tian et al. (2018)
Rat	Foetal Leydig cell	Insl3 expression (IN), testosterone synthesis (IN), steroidogenic gene expression (IN)	Hu et al. (2014) and Li et al. (2018)
Mouse	Adult Leydig cell	Testosterone secretion (IN), steroidogenic gene expression (IN), Leydig cell tumours (IN), Leydig cell cytoplasm alteration (ST), Leydig cell number (IN)	Hu et al. (2014) and Mahmoudi et al. (2018)
Human	Sperm	Motility (IN)	Zhao et al. (2017) and Mahmoudi et al. (2018)
Rat	Spermatogenesis	Massive germ cell death (ST), spermatogonia number (IN)	Cupertino et al. (2017) and Mahmoudi et al. (2018)
Rat	Sperm	Sperm count (IN), sperm motility (IN), early embryonic development (IN), in vitro fertilization rate (IN)	Zhao et al. (2017) and Mahmoudi et al. (2018)

Adapted from Zhu et al. (2020)
BTB blood-testis barrier, *IN* inhibition, *ST* stimulation

because they are breathed deep into the airways (Donaldson et al. 2001).

OS is caused by urban PM and particle capacity. A variety of PM types may produce oxygen free radicals. In addition, PM exposure for a lengthy period increases the formation of atheromatous plaques (Shaw et al. 2011). There are many potentially hazardous compounds in urban PM which possess the ability to cause OS. Both leukocytes and noninflammatory cells have ROS and inflammation pathways that mediate the cellular response to PMs. Several PM sources enhance various pro-inflammatory molecules in macrophages and endothelial cells (Shaw et al. 2011). PMs can also disrupt endothelial function and increase leukocyte adhesion to arteries indicating their possibility in early phases of atherogenesis. In a human lung epithelial cell line exposed to PM_{10}, Chirino et al. (2010) reported ROS production and decreased glutathione and other antioxidant enzyme activity, such as SOD and glutathione reductase.

3.4.2 Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are organic pollutants that are mainly colourless, white, or light yellow solid compounds made up of two or more fused aromatic rings containing carbon and hydrogen atoms (Suman et al. 2016). Human activities, cause the release of PAHs into the environment as a consequence of rapid industrialization and urbanization (Mojiri et al. 2019). Several biological processes, including changes in semen quality, may play a role in revealing the link between PAH exposure and male infertility. Researchers investigating PAH exposure and sperm quality noted that 1-OHP might impact sperm quality even at non-occupational levels (Kim et al. 1999). ROS in sperm can harm plasma membranes, DNA integrity, motility, and overall semen quality (Chen et al. 2012). PAHs are well-known carcinogenic and teratogenic organic contaminants. They are also long-lasting pollutants. Natural activities such as volcanic eruptions and forest fires and human actions (e.g. incomplete combustion of fuel oils) and oil spills may cause PAH pollution (Dabestani and Ivanov 1999). PAHs that are often found in the environment include anthracene, benzo(a)anthracene, benzo(a) pyrene, fluorene, fluoranthene, naphthalene, and phenanthrene (PHEN). This mainly impacts the immunological and respiratory systems of animals. Combustion particles may have a role in developing cardiovascular diseases (CVDs) in humans in a variety of ways. The release of pro-inflammatory mediators into the blood may be triggered by OS in pulmonary macrophages and epithelial cells. These mediators can induce systemic effects and damage endothelial cells (Holme et al. 2019).

Furthermore, PAHs present in cigarette smoke may induce significant OS-induced damage to biological macromolecules (e.g. DNA, proteins, and lipids) (Halliwell and Gutteridge 2007). According to studies conducted on both plants and animals, when PAHs are metabolized, reactive electrophilic metabolites and ROS are generated (Bonnet and Boucherat 2018; Sun et al. 2006). In a model referred to as the "redox hypothesis of hypoxic pulmonary vasoconstriction (HPV)", the mitochondrial electron transport chain (ETC) detects hypoxia and reduces ROS production (ROS being produced as an inevitable by-product of oxidative phosphorylation), causing intracellular calcium inflow secondary to redox-sensitive potassium channel inhibition. Endothelium-depleted distal pulmonary arteries (Michelakis et al. 2002) and isolated perfused rat lungs both show that hypoxia and proximal ETC inhibitors limit the synthesis of activated O_2 species and promote HPV, respectively. Hypoxia, according to the "ROS hypothesis", increases the generation of ROS in the mitochondria, causing intracellular calcium concentrations to rise, resulting in an increase in exogenous H_2O_2. It has been shown that HPV can infect both isolated lungs and pulmonary artery smooth muscle cells (PASMCs) in vitro, demonstrating that this is appropriate (Adesina et al. 2015). Antioxidants such as catalase and glutathione peroxidase also reduced the HPV response.

3.4.3 Benzene

A colourless organic solvent produced from petroleum refining, benzene is one of the aromatic hydrocarbons. Human leukemogenic benzene is a common contaminant. In men exposed to high amounts of benzene, the sperm are more likely to have an aberrant number of chromosomes, which may affect fertility and foetal development. Furthermore, benzene affects sperm motility which is often associated with dynein-ATPase activity reduction (Priyanka et al. 2013).

Benzoquinones and other benzene metabolites may cause an increase in the ROS, lipid peroxidation in vivo, and the formation of hydroxylated deoxyguanosine residues. ROS may induce double-stranded DNA breaks by oxidizing nucleotides, which are subsequently converted into double-stranded fractures during DNA replication. Homologous recombination, which is not error-free, may be used to mend these fractures. Double-stranded DNA breaks induced by ROS and other factors may result in increased mitotic

recombination, chromosomal translocations, and aneuploidy (Zhu et al. 2013). Proteins, lipids, and DNA are among the molecular targets of ROS-induced damage, leading to cancer. Benzene metabolites have been shown to cause oxidative damage in HL60 cells (Shen et al. 1996) and lead to lipid oxidation in an animal model (Gaido and Wierda 1987), suggesting that ROS may play a role in benzene-induced toxicity.

3.4.4 Volatile Organic Compounds (VOCs)

VOCs are major indoor air pollutants from many sources, such as building materials, coatings, purifiers, furniture, adhesives, combustion materials, floor, and wall coverings, and evaporate at room temperature. They are linked to respiratory discomfort, OS, and a reduction in lung function (Nurmatov et al. 2015). Lipid peroxidation, protein oxidation, and DNA damage are all side consequences of ROS interactions with biological components (Bogdan et al. 2015).

Long working hours, along with unprotected exposure to organic solvents, have been linked to changes in employees' neurochemical processes, resulting in physical and emotional stress. When paints, varnish, lacquer, and glue are exposed to the brain, the anterior pituitary gland, which regulates gonadotropin release, is activated (Yilmaz et al. 2001; Priyanka et al. 2013). Dihydrotestosterone through 5-alpha-reductase or aromatase may be transformed from testosterone in the peripheral to a more active form, such as estradiol. Also of importance are inhibin B and the Mullerian inhibiting substance (MIS hormone), both of which are produced by the Sertoli cells in the testes. It is the anterior pituitary gland's FSH and LH as well as GnRH, the hypothalamus' gonadotropin-releasing hormone (GnRH), that control these processes. Hypothalamus-pituitary-gonadal axis hormones work together to keep male sexual function healthy and promote sexual desire in males (Gurung et al. 2021).

Male exposure to organic solvents may result in different injuries and reproductive functions. This may be caused via inhalation of hazardous vapours and hamper testosterone production and testicular functions, including disease induction because of the vulnerability of chemical absorption of irritants through the skin.

3.5 Persistent Organic Pollutants (POPs) and Endocrine Disruptors (EDs)

In the ecosystem and in animals, POPs build and move up the food chain, eventually affecting wildlife, the environment, and even humans. Bonefeld-Jørgensen et al. (2014) reported that the combined effect of serum POPs on hormone and/or dioxin receptor function is a risk factor for human health in epidemiological and in vitro/ex vivo studies.

Substantially stable lipophilic contaminants, including ovotoxicity, ovulatory failure, and early ovulation, are stable lipophilic pollutants causing significant reproductive problems such as polybrominated diphenyl ethers (PBDEs) and polychlorinated dibenzo-p-dioxins (PCDDs). The majority of POPs affect transcription factors, including the AhR and the steroid hormone receptors (Ewa and Anna 2013). Many of these substances are considered to influence the health of men. Besides, the widespread use of polychlorinated biphenyls and other organochlorine pesticides such as DDT or hexachlorobenzene (HCB) have also been extensively used as plasticizers, isolators, paints, and flame retardants, as well as insulated condensers, polychlorinated isolators, and transformers (Vested et al. 2014).

3.6 Drug-Induced Oxidative Stress (OS)

Epimutations in sensitive genomic areas may be caused by pharmaceutical medications in developing germ lines. Health consequences of recreational drug use may be mediated in part by changes in the epigenome. For example, MeDIP-seq was used to compare sperm methylation changes in adult males treated with chemother-

apy for osteosarcoma at least 10 years prior to research including untreated men (controls). More than 2000 DMRs (P = 0.0001) were discovered to be linked with chemotherapy (P = 0.0001). These DMRs were identified mainly in CpG desert areas spread across all chromosomes, including the sex-specific ones. Further research used restriction landmark genomic scanning (RLGS) to look at the methylation patterns of male rat germ cells that had been subjected to various dosages of combined testicular cancer therapy such as bleomycin, etoposide, and trans-platinum (BEP) (Marcho et al. 2020). Overall, 143 locations were discovered that were substantially different in spermatids and mature spermatozoa than controls, based on the direction of methylation patterns (Chan et al. 2012; Marcho et al. 2020).

Male sexual and reproductive organs, particularly the testes and epididymis, may be subjected to drug-induced OS. As a result, they interfere with spermatogenesis by causing OS and death of germ cells or target Sertoli cells. They also impair the activity of the Leydig cells by producing ROS and lowering the levels of steroidogenic enzymes (Sedha et al. 2015).

Several tissues and organ systems, including the kidney, liver, cardiovascular system, ear, and neurological systems, have been identified as vulnerable to toxicity by drug-induced OS (Damian et al. 2012). Furthermore, pleiotropic negative effects of OS have been documented in a range of disease states and have also been linked to a number of drug-induced toxicities (Damian et al. 2012). It has a complex aetiology, and antibiotic exposure has been linked to an increased risk of low birth weight (Francesca and Marcello 2015). In the absence of cytoplasmic-enzyme repair mechanisms, spermatozoa are unable to repair damage caused by OS. Spermatozoa are unusual in their vulnerability to oxidative damage because of this property (Saleh and Agarwal 2002). The production of ROS is required for reproductive function, while OS has a negative impact on fertility. Reduced spermatogenesis, aberrant sperm morphology, reduced seminal fluid volume, poor testosterone levels, and increased OS have all been linked to heavy alcohol use (Alahmar 2019).

3.7 Tobacco Smoke and Male Infertility

Over 4000 ingredients have been detected in cigarette smoke, including nicotine, acrolein, ammonia, acetaldehyde, phenols, polyphenols, PAHs, carbon monoxide, hydrogen cyanide and trace metals (Pryor 1997; Koul et al. 2001; Kamisaki et al. 1997).

When someone smokes a cigarette, several types of smokes are produced. To be considered a "mainstream smoker", one must inhale cigarette smoke after lighting a cigarette and before exhaling it. Exhaled mainstream smoke is that that enters the environment after passing through the cigarette and the smoker's lungs. The burning tip of the cigarette emits sidestream smoke, which is dispersed into the air. An inhalation of mainstream tobacco smoke and an exhalation of sidestream tobacco smoke together constitute "active smoking". Smoking may have a negative impact on sperm quality, particularly in terms of sperm concentration, motility, and morphology (Merino et al. 1998; Harlev et al. 2015; Rehman et al. 2019).

In the semen of smokers, these substances create an imbalance between ROS and antioxidants (Lavranos et al. 2012; Omolaoye et al. 2021). An imbalance between ROS and the body's natural antioxidant defences cause OS. An increase in ROS may lead to an imbalanced (usually reduced) TAC. Regarding seminal OS indicators, cigarette smoking has been linked to higher ROS and lower ROS-TAC values. Many polyunsaturated fatty acids in the plasma membrane are ROS substrates and few scavenging enzymes in the spermatozoa make them especially vulnerable to ROS damage (Alvarez and Storey 1995; Rehman et al. 2019; Zhang et al. 2020).

Imbalance between ROS and antioxidants has an impact on the overall quality of sperm. Smoking has been reported to cause a 48% rise in seminal leukocyte concentrations and a 107%

increase in seminal ROS levels (Saleh et al. 2002).

In various categories of adults and young individuals, advanced techniques were employed to compare smokers and non-smokers. Although some consistent changes were found involving DNA methylation variations in the F2RL3 at specific locations (Zhang et al. 2014), AHRR (Monick et al. 2012), and GPR15 (Dogan et al. 2014) genes, which have emerged as strong candidates to predict smoking-related adverse health outcomes. In addition, several processes, including direct damage by radical species and the inflammatory response induced by cigarette smoking, contribute to increased OS in smokers (Bruno and Traber 2006). Male fertility is hindered due to the egative impact of OS on sperm characteristics, including viability, morphology, and function (Harlev et al. 2015; Dutta et al. 2021).

Aldehydes may, for example, deplete GSH, alter protein-sheet and protein-sheet groups, and smoke-tart complexes, which permeate across cell membranes, causing superoxide radicals formation and contributing to the formation of hydroglycolic acids (Basant et al. 2008).

3.8 Xenobiotics and Male Infertility

Environmental xenobiotics may have a significant effect on male sexual and reproductive health. Industrial chemicals have the potential to harm human testicular functions. Sex hormones, without a doubt, play a substantial role in the normal development and functioning of male reproductive capacity. Disorders (libido and sex) or male sex behaviour in relation to cells during spermatogenesis and the quality of sperm may be classified as male reproductive toxicity (Mattison et al. 1990; Figa-Talamanca and Hatch 1994), as well as alterations of the endocrine system, central nervous system (CNS), or reproductive organs (McMaster 1993).

Production of ROS, the occurrence of redox couples such as chromium (Cr(VI)/Cr(III)), and the development of reactive intermediates of the original molecule may all cause oxidative damage as shown in animals and humans. Free radicals and oxidant species are potentially harmful and poisonous substances that may cause cellular and organ malfunction. In addition, overproduction of these species has the potential to harm DNA, lipids, and proteins (Pagano 2002; Stohs and Bagchi 1995).

Enzyme and transporter functions may be altered, ion channels blocked, certain receptors activated, and DNA transcription can be violated if the xenobiotic's structure corresponds to the macromolecule-binding sites. When nucleophiles produce endogenous adducts, reversible covalent contact does not require structural fitness (nucleophilic amino acids or nucleic acids). Gene mutations, carcinogenesis (if the harmed gene is involved in cell reproduction and differentiation), and protein malfunctions may all occur due to these interactions, leading to cell death or tissue toxicity (Trang and Huyen 2018).

Enzymes for removing endocrine disruptors such as phthalates, dioxins, polychlorinated biphenyls (PCB), and pesticides are critical in the reproductive system since they have been proven to harm reproductive tissues. For example, 1,2-dibromo-3-chloropropane (DBCP), an occupational xenobiotic nematocide that damages the seminiferous epithelium partly reversibly and reduces sperm counts and sterility, is one of the well-studied male testicular toxins. Also, dichlorodiphenyldichloroethylene, a workplace and environmental contaminant, lowers sperm count and contributes to male infertility (Bonde and Giwercman 2014).

Environmental, exogenous, and endogenous stressors induced deterioration in sperm quality, and elevated sperm mortality through various pathways are presented in Fig. 3.6.

3.9 Discussion

A number of environmental and occupational hazards have an impact on male reproductive function (Table 3.3). Many molecules have not yet been recognized. Heavy metal exposure, whether occupational or environmental, now

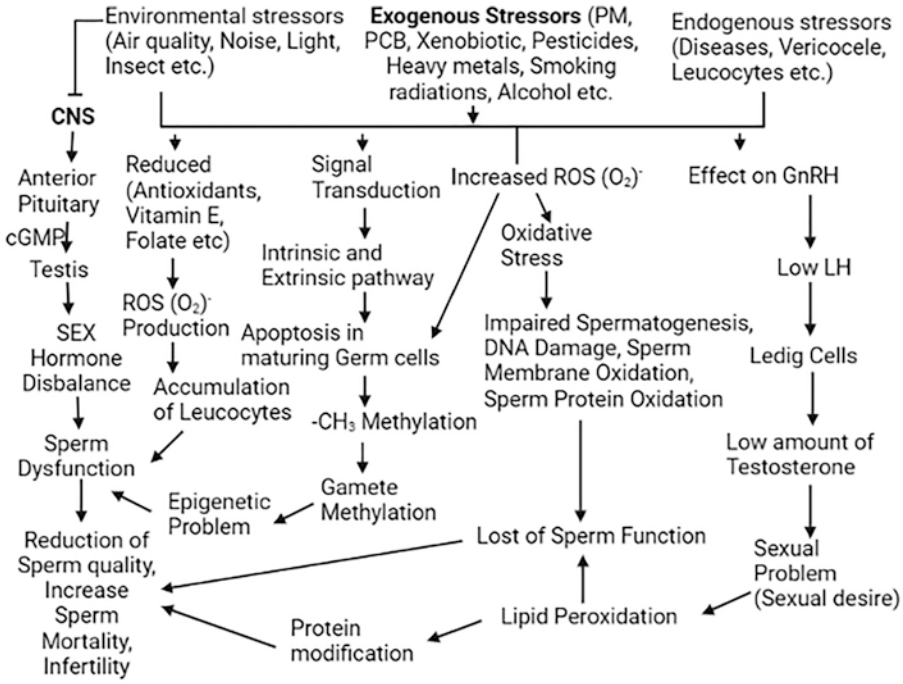

Fig. 3.6 Environmental, exogenous, and endogenous stressors induced deterioration in sperm quality and elevated sperm mortality through various pathways

Table 3.3 Role of endocrine-disrupting chemicals in causing sperm epigenetic abnormalities

Exposure	Species	Method	Sperm epigenetic aberrations	References
DEHP	Human	HRM-PCR	The methylation of LINE-1 DNA was shown to be inversely related to occupational exposure to DEHP	Tian et al. (2019) and Marcho et al. (2020)
Phthalates	Human	450K array	There was a strong correlation between high levels of anti-androgenic urinary phthalate metabolites in the environment (MEHP, MBP, and MCOCH) and the majority of sperm DMRs, which were enriched in genes involved with blastocyst quality, growth and development, and cellular function and maintenance. There were also 17 DMRs linked to poor blastocyst quality	Wu et al. (2017) and Marcho et al. (2020)
BPA	Human	qPCR	Those exposed to BPA had higher ACHE gene locus sperm 5hmC than workers not exposed to BPA. ACHE 5hmC was not associated with urine BPA in any way among the participants	Marcho et al. (2020)
Bisphenol A(BPA)	Human	5-hMeDIP-seq	Comparing occupationally exposed males with control groups, researchers found 9610 more sperm DMRs. There was no analysis of the relationship between 5hmC and urine BPA levels	Zheng et al. (2017) and Marcho et al. (2020)
Dioxins	Human	WGBS	When young people with high levels of peripubertal blood dioxin (TCDD) (8–9 years of age) were compared to those with low levels of peripubertal blood dioxin, 52 sperm DMRs were found. DMRs were enriched in regions of functional significance for cell development and function	Pilsner et al. (2018) and Marcho et al. (2020)
Dioxins	Human	450K array	When compared to controls, Operation Ranch Hand veterans with high blood TCDD levels had methylation changes in 36 sperm gene regions, including H19. A comparison of these results with those from a prior study, 143 revealed 5 loci with methylation directionality that were significantly overlapped	Kelsey et al. (2019) and Marcho et al. (2020)

poses a risk to reproductive health (López-Botella et al. 2021). Inhibiting the Leydig and Sertoli cell activity, interruption of spermatogenesis, and elevated testicular OS have unfavourable consequences (direct and indirect ROS production). The anterior pituitary gland's FSH and LH and GnRH, the hypothalamic GnRH, govern these processes. Hypothalamic-pituitary-gonadal hormones maintain male sexual functions healthily and stimulate the male desire (Gurung et al. 2021).

Men under stress have abnormal sperm motility, morphology, DNA fragmentation, and viability, as well as hormonal changes (Chen et al. 2020; Dutta et al. 2021). Notably, symptoms of male reproductive system problems include failure to ejaculate, bone density loss, muscular atrophy, and, most significantly, a lack of sexual development if the condition is genetic or acquired before puberty (Gurung et al. 2021).

Involvement of redox-dependent effects in the toxicity of environmental stressors and xenobiotics on OS may lead to increased public awareness and transformative policy changes that will reverse the recent increases in common human diseases and disorders, including male reproductive abnormalities. This study revealed that environmental epigenetics might become a powerful concept for thoroughly assessing the exposome effect on male reproductive and sexual health from an overall evaluation of the existing database. In pulmonary macrophages and epithelial cells, OS may cause pro-inflammatory mediator release into the circulation. These inflammatory mediators may harm endothelial cells (Holme et al. 2019).

Free radicals and non-radical derivatives generated as a by-product of cellular metabolism are known as ROS, stimulating cellular development at lower levels but may cause tissue damage and decrease cell function at higher levels. Therefore, under physiological circumstances, intracellular OS is carefully balanced by ROS generation, detoxifying enzymes (e.g. SOD), and antioxidants (e.g. carotenoids) to preserve cellular homeostasis (Taverne et al. 2013).

The pro-oxidant-to-antioxidant ratio rises under pathological circumstances, mitochondrial energy metabolism changes, and the heart reverts to foetal metabolism, switching from beta to glucose oxidation. Thus, the balance of oxidants and antioxidant enzymes is disturbed. The levels of SOD, CAT, and GPX were found to be lower in smokers as compared to non-smokers. In addition, MDA levels in plasma were significantly greater among smokers than non-smokers, and there was seldom convincing proof of the direct causative link between these factors and a couple's infertility (Dogan et al. 2014). It is believed that OS reduces the transmembrane potential (MTP) for mitochondrial medicines that boost cAMP levels and sperm intra-cell Ca^{++} release, resulting in an increase in spontaneous AR and a reduction in sperm fertility. Overall, mercury causes necrosis rather than apoptosis. Thus, necrosis seems to be the predominant signalling channel in mercury-induced sperm mortality and may occur from structural damage to sperm cells (Kushawaha et al. 2021).

During germ cell development, epigenetic signatures begin to establish in the testis and proceed to the most significant level of complexity in sperm. Surprisingly, such a signal does not remain constant throughout sperm maturation along the epididymis but somewhat varies with time. Epididymal epithelial cells provide a significant contribution here, which is explained by epididymosomes. Several environmental variables, including pollution, heavy metal, stress, and nutrition, impact human health, mainly via epigenetic mechanisms. It is widely understood that the epigenome serves as a link between the genome and the environment, and those epigenetic markers may be passed down across generations (Hyun-Wook et al. 2015).

ROS usually cause OS when they accumulate to levels higher than the organism's antioxidant capability. Multiple causes, such as increased leukocyte activity owing to inflammation in the male reproductive tract, varicocele, or the presence of immature spermatozoa, may explain elevated ROS levels, as can the external sources

such as exposure to harmful chemicals, radiation, or xenobiotics. Some physiological activities, like capacitation, need a modest quantity of ROS, while too much ROS may harm sperm. The cytoplasmic volume of spermatozoa is also limited. Due to the low supply of ROS-metabolizing enzymes, these cells are more susceptible to ROS (Pereira et al. 2021).

The hypothesis of OS suggests that cellular damages in chronic pancreatitis came from the acinar cell and were mediated mainly by free radicals with a short half-life obtained from oxygen (Braganza and Dormandy 2010). According to their hypothesis, free radicals originating from oxygen are produced because of discrepancies between the processes which make them disable or quench them. Glutathione-(2S)-2-amino-4-[(1R)-1-[(carboxymethyl) carbamoyl] is an endogenous peptide. Carbamoyl butanoic acid[-2-sulfanylethyl] (GSH) is a key mediator produced in a highly controlled way in the cytoplasm of all mammalian cells. Availability of cysteine and the rate-limiting enzyme glutamate-cysteine ligase activity are important factors of GSH production (GCL) (Braganza and Dormandy 2010).

Furthermore, cysteine's sulphhydryl (thiol) group (SH) functions as a proton donor and is responsible for glutathione's biological action (Lu 2009). Environmental stressors and xenobiotics have long been thought to have a role in autoimmunity via complicated interactions between genetic, environmental, and epigenetic variables. Environmentally induced epigenetic alterations in gametes are a fascinating biological subject in and of themselves, but they also constitute a major worldwide public health issue due to their direct effect on disease aetiology.

Altering the testicular gene expression in test functions and over disturbing conditions is unlikely to result in OS alterations. However, in normal physiological activity, modest and controlled ROS levels, including capacitation, hyperactivation, acrosome responses, and signalling systems that ensure correct fertilization, have been demonstrated to play a critical role. Moreover, there is growing evidence of sperm reduction by increasing ROS (Hiroshi et al. 2020).

3.10 Conclusions and Future Perspectives

ROS harm sperm in a variety of ways, including by interacting with the sperm plasma membrane, which is rich in polyunsaturated fatty acids and therefore promote sperm tail motility loss. ROS can cause peroxidation of the sperm acrosome membrane and a reduction in acrosin activity, which would reduce the chances of fertilization. The mitochondria of stressed out spermatozoids generate large amounts of ROS, which may lead to changes in the mitochondrial DNA of several cells involved in spermatogenesis. Male infertility has been linked to various genetic abnormalities, including Y-chromosome microdeletions, which affect genes involved in spermatogenesis. Considering the above, inclusion of heavy metal evaluation in the biological samples of patients might facilitate for personalized diagnosis and prognosis in avoiding male infertility. This would also help assisted reproductive procedures. Intrinsic, extrinsic, or environmental stressors impact male reproductive health in different ways. These mechanisms need additional investigation. Finally, such exposure continues to harm the male reproductive health. Hence, it is critical to safeguard men's reproductive health against these dangerous toxins.

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Pesticide Toxicity Associated with Infertility

4

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Abstract

Pesticides have benefited mankind in many ways like agriculture, industrial and health sectors. On the other hand, conversely their deleterious effects in both, humans and animals are also alarming. Pesticides including organophosphates, organochlorines, carbamates, pyrethrins and pyrethroids are found sufficiently in the environment resulting in everyday human exposure. This is of a huge concern because most of the pesticides are known to target all the physiological functions of both humans and animals. Indeed, reproduction, being one of the most important physiological processes, that is affected by the daily exposure to pesticides and leading to infertility issues. The present study summarizes the exposure of men and women to certain pesticides resulting in different infertility concerns like sperm abnormalities, decreased fertility, abnormal sperm count and motility, testicular atrophy, ovarian dysfunction, spontaneous abortions, disruption of hypothalamic–pituitary–gonadal axis, etc. So, this article will be helpful in perceiving the mechanism of reproductive toxicity of different pesticides and their management before any alarm of danger.

Keywords

Pesticides · Reproductive toxicity · Infertility

4.1 Introduction

The global population is predicted to hit 9.1 billion by 2050, which has been increased from 2.5 billion since 1950 (Carvalho 2006). This rapid expansion is preceded by the critical issue of food security and availability. As a result of these factors, pesticide production and use have notably increased in recent few decades. Pesticides have benefited humans by improving crop yield and lowering the incidence of enormous diseases (food-borne and vector-borne) (Kim et al., 2017). However, large number of pesticides is found to cause serious health effects to non-target species, including humans depending on the type of pesticide and the mode of exposure (Duzguner and Erdogan, 2010). The non-target organisms can

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get pesticide exposure, either directly through working with pesticides or indirectly through food and water (Mostafalou & Abdollahi, 2017). Pesticide toxicity in humans have been found to cause serious health effects, viz. neurological, psychological and metabolic disorders, hormonal imbalance and even cancer (Kim et al., 2017; Ganie et al., 2022). The widespread use of pesticides has a greater impact on reproduction, which is a highly selective process that ensures species continuity. In well-designed epidemiological studies, mixed pesticide exposure in occupational environments has been linked to detrimental reproductive or developmental effects, mainly when personal defensive equipment is not worn. Organophosphates, carbamates, pyrethrins, pyrethroids and particularly organochlorines all have the capability of causing reproductive or developmental toxicity in laboratory animals and humans (Sharma et al. 2020), as shown in Table 4.1. Male and female fertility are negatively affected by lifestyle and environmental factors (Alamo et al. 2019). Infertility, being a major issue in the society, affects 15% of couples in their reproductive years (Fritz and Speroff 2011). Infertility is the inability of an organism to reproduce naturally and has been widely studied in relation to pesticide exposures. Pesticides have been found to cause endocrine disruption and have been reported to elevate OS in reproductive organs, thereby inducing cytotoxic and genotoxic effects (Hajam et al., 2022). Insecticides and herbicides, particularly organophosphates, organochlorines, phenoxyacetic acids and triazine compounds, are responsible for majority of the disorders (Mustafalou et al. 2017). The current study summarizes the deleterious impacts of different pesticides on male and female reproductive health that eventually paves the way for reproductive infertility.

4.2 Male-Mediated Reproductive Effects

Male reproductive system dysfunction is a major concern in the livestock industry. Decline in spermatogenesis, alterations in the pathways of reproductive enzymes, anti-androgenic effects and decrease in sperm motility are the major effects in male infertility caused by pesticides particularly insecticides. Insecticide expo-

Table 4.1 Some of the most commonly used pesticides and their reproductive toxicities on male and female reproductive systems of different animals

Pesticide	Reproductive effects	Reference
Diazinon (DZN)	Lessening in seminiferous tubule dimensions and germ cell amount. Testicular atrophy, tubule deluminization, degeneration and damage to the seminiferous epithelium in rats	Toman et al. (2016)
Methoxychlor	Substantial reduction in the weights of the epididymis, seminal vesicles, ventral prostate and testes in rats	Latchoumycandane and Mathur (2002)
Endosulfan	Increased production of abnormal and dysfunctional spermatozoa in rats	Rao et al. (2005)
Methomyl	Reduction in fertility index, testes' weight, sperm motility and seminiferous tubule degeneration in rats	Shalaby et al. (2010)
Parathion	Significant harm to epithelium of seminiferous tubules by the proliferation of germ cells in recently married males from a rural region of China	Perry et al. (2011)
Mancozeb	Significant decline in epididymal and testicular sperm count in rats	Joshi et al. (2005)
Dichlorvos	Rise in endometrial hyperplasia, reduced pregnancies and live births in rats	Yu et al. (2013)
Cypermethrin	Blocks the secretion and activity of progesterone in female rats	Sangha et al. (2013)
Atrazine	Disruption of steroidogenesis in swine granulosa cells, retrogressive alterations in the Sertoli cell cytoplasm, reduction in the sperm output in workers exposed to it	Basini et al. (2012), Swan (2006)
Malathion	Increased apoptosis in granulosa cells of caprine animals	Bhardwaj and Saraf (2016)

sure induces male reproductive toxicity by damaging testicular cells like Sertoli cells, Leydig cells and germ cells or causing alterations in homeostasis of hormones (Kara and Oztaş 2020).

4.2.1 Effects on the Testes

Number of researchers reported significant effects of diazinon on the structure and functions of the testes in rats (Adamkovicova et al. 2014). Joursaraei et al., 2010 discovered a significant decline in the size of seminiferous tubules and the number of germ cells after the intraperitoneal administration of diazinon. Exposure of diazinon has also been reported to be linked with degenerative changes like necrosis of the epithelium of seminiferous tubules, deluminization of seminiferous tubules and testicular atrophy, thus leading to infertility (Toman et al. 2016). Oral exposure of diazinon has been found to cause significant histological alterations like a significant damage of basal germinal epithelium and vacuoles in the rat testes (Damodar et al. 2012). Dutta and Meijer (2003) have reported that 2 weeks' of exposure to diazinon causes significant decline in germ cells, spermatozoa and the diameter of seminiferous tubules. In diazinon-exposed animals, a significant decrease in number of Sertoli, spermatogenic and Leydig cells were found (Hatjian et al. 2000). According to a histological study, the basal germinal epithelium and vacuoles in the rat testis were disrupted and sloughed off after oral administration of diazinon (Damodar et al. 2012). Latchoumycandane and Mathur (2002) reported that methoxychlor-treated rats showed a significant decrease in weight of testes, epididymis, seminal vesicles and ventral prostate. Besides, the activities and levels of major antioxidant enzymes, viz. superoxide dismutase and catalase, and GSH cycle enzymes, viz. glutathione peroxidase and glutathione reductase in testes, were also diminished. They have also demonstrated that methoxychlor-exposed rats showed increased generation of hydrogen peroxide and peroxidation of lipids. Methoxychlor has been found to cause a significant decrease in the StAR protein expression and the 3beta-HSD and 17beta-HSD activities with a concurrent elevation in level of hydrogen peroxide in rat testes (Vaithinathan et al. 2008). Endosulfan causes irregular spermatozoa to develop and reduces the number of sperm counts in rats (Rao et al. 2005). Methomyl exposure have been found to be associated with significant decline in fertility index, testes weight and weight of accessory male sexual glands, level of serum testosterone, motility and count of sperm in rats, thus increasing abnormalities in sperm cell. It also resulted in mild to severe seminiferous tubule degeneration, as well as an incomplete seizure of spermatogenesis (Shalaby et al. 2010). In male rats, subchronic carbaryl exposure caused deformation of the seminiferous tubules, disruption in spermatogenesis, interstitial space oedema and depletion of the sperm cells in testes (Rao et al. 2005). Oral administration of Mancozeb (a fungicide) to the rats caused a significant reduction in the testicular weight, epididymis, seminal vesicle, ventral prostate and testicular sperm counts (Joshi et al. 2005).

4.2.2 Effects on Sperm Count and Morphology

Several studies have linked infertility in males to the number of sperm (Hanke and Jurewicz. 2004). Some epidemiological studies found that working on a farm raises the likelihood of particular morphological anomalies in farmers' sperm, such as a reduced sperm count per ejaculate and substantial drop in the percentage of viable sperm. The diminution of the epididymal muscles resulted in the production of immature sperm (Aamer et al., 2015). Yucra et al. 2006 reported the alterations in the structure of sperm in rats exposed to certain organophosphate (OP) pesticides. Endosulfan has been reported to cause an increase in the number of impaired sperm in the rat epididymis and has been found to cause the necrosis of the seminiferous tubules and Leydig cells (Jaiswal et al. 2005). Endosulfan also causes an increase in dysfunctional sperm

and in addition to that it causes a substantial decline in the sperm motility and count (Rao et al. 2005).

4.2.3 Effects on Sperm Concentration and Motility

The number of sperm per millilitre is referred to as sperm concentration or density. Organophosphates (parathion and methyl parathion) have been suggested to affect the concentration of sperm by causing severe harm to the epithelium of seminiferous tubules by the proliferation of germ cells (Perry et al. 2011). Pesticide exposure has a major impact on the prostate glands and seminal vesicles, which contribute thirty percent and sixty percent of the seminal volume, respectively(Yucra et al. 2006). Exposure to these OP pesticides might also bring about a significant drop in the seminal volume. The coordination of the midpiece and tail is needed for sperm motility to generate enough energy to travel. Adenosine triphosphate (ATP) is the major source of energy for spermatozoa. Protein kinases and phosphatases possess a crucial role to play in the synthesis of ATP. Organophosphates have been found to covalently phosphorylate these enzymes alone or more serine, threonine, tyrosine or histidine residues. Therefore, any agent that interferes with the assemblage of different tail-protein components and alters the process of ATP synthesis can cause sperm motility decline (Perry et al. 2011). Lifeng et al. (2006) found that motility of the sperm can be used to predict fertilization capacity indirectly. They discovered that low-dose exposures to majority of pesticides that are toxic to the reproductive system, for instance, Fenvalerate (a synthetic pyrethroid), can impede sperm motility. Fischer rats were used to test the impacts of atrazine on the fertility of males (Kniewald et al. 2000). The number of sperm was found to be increased in those who were exposed to atrazine due to the declined motility of the spermatozoa. Atrazine intervention also resulted in significant reduction in motility and the number of sperm in the epididymis of rat testes. A histological examination also revealed disorganization of spermatocytes and Leydig cells were found to possess unusual morphological features. Atrazine caused declinatory changes in the cytoplasm of Sertoli cells. Atrazine has also been reported to decline sperm production in workers that they are exposed to it (Swan 2006). In male animals, certain insecticides which belong to the pyrethroid class of pesticides have been reported to alter the functions of the reproductive and endocrine systems. , These can lead to substantial reduction in the concentration of sperm, motility, sperm head distortion, upsurge in abnormal count of spermatozoa, sperm DNA alteration and increase in aneuploidy rates. It may also affect the sex hormone levels. Dimethoate, an organophosphate insecticide, has also been reported to decline the viability, movement and density of the sperm in male mice (Farag et al. 2007).

4.2.4 Effects on Male Sex Hormones

Malathion-exposed rats possessed lower levels of plasma FSH, LH and testosterone than controls, according to a report (Mehrpour et al. 2014). Quinalphos, a widely used organophosphate insecticide, has been found to decrease the enzymatic activity of prostatic acid phosphatase. Also, in the accessory sex glands, the amount of fructose was found to be reduced. Furthermore a significant reduction in the testosterone, FSH and LH plasma levels were reported. Quinalphos, by inhibiting the release of pituitary gonadotropins, is thought to have suppressive effects on the activities of prostate gland and seminal vesicles (Ray et al. 1991). Figure 4.1 depicts some of the most common pathways of male reproductive system damage that can be intensified by pesticide exposures.

4.3 Female-Mediated Reproductive Effects

Pesticides have been reported to disrupt reproduction in females by affecting a multiple number of reproductive tissues and functions.

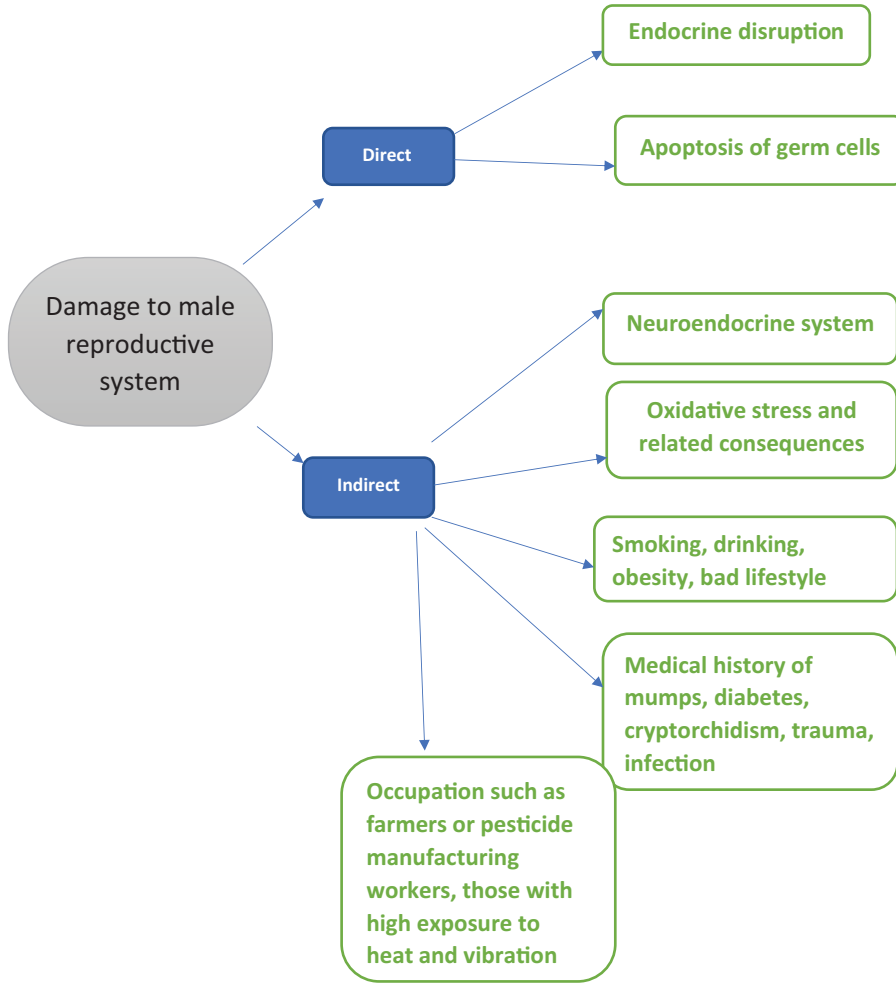

Fig. 4.1 Some familiar mechanisms of damage to male reproductive system (Mehrpour et al. 2014)

Disruption of hormone synthesis, maturation of the follicles, ovarian cycle, extension in gestation period, stillbirth and infertility are all related to pesticide exposure, which leads to damage in DNA, inflammation and induction of apoptosis (Sharma et al. 2020). Pesticide exposure affects the hypothalamus, pituitary, ovary, uterus, fertility and reproductive senescence, as mentioned below.

4.3.1 Effects on Hypothalamus and Pituitary

There is currently little research on the impact of pesticide toxicity on the hypothalamus/pituitary in adults. In GT1-7 cells, the organophosphate pesticide, chlorpyrifos and the organochlorine pesticide, methoxychlor, significantly increased GnRH mRNA levels (Gore 2001). Stoker et al. (2005) reported that a carbamate pesticide, molinate, represses the LH pulse frequency, thereby causing delay in the ovulation in rats. It has been found that atrazine activates the release of hormones secreted by the pituitary gland (Fraites et al. 2009) and inhibits the release of luteinizing hormone secreted by the pituitary gland in rats (Goldman et al. 2013). As depicted in Fig. 4.2, certain pesticides result in the impairment of hypothalamus–pituitary–gonadal axis by decreasing the GnRH level which in turn induces a drop in the release of LH and FSH and further

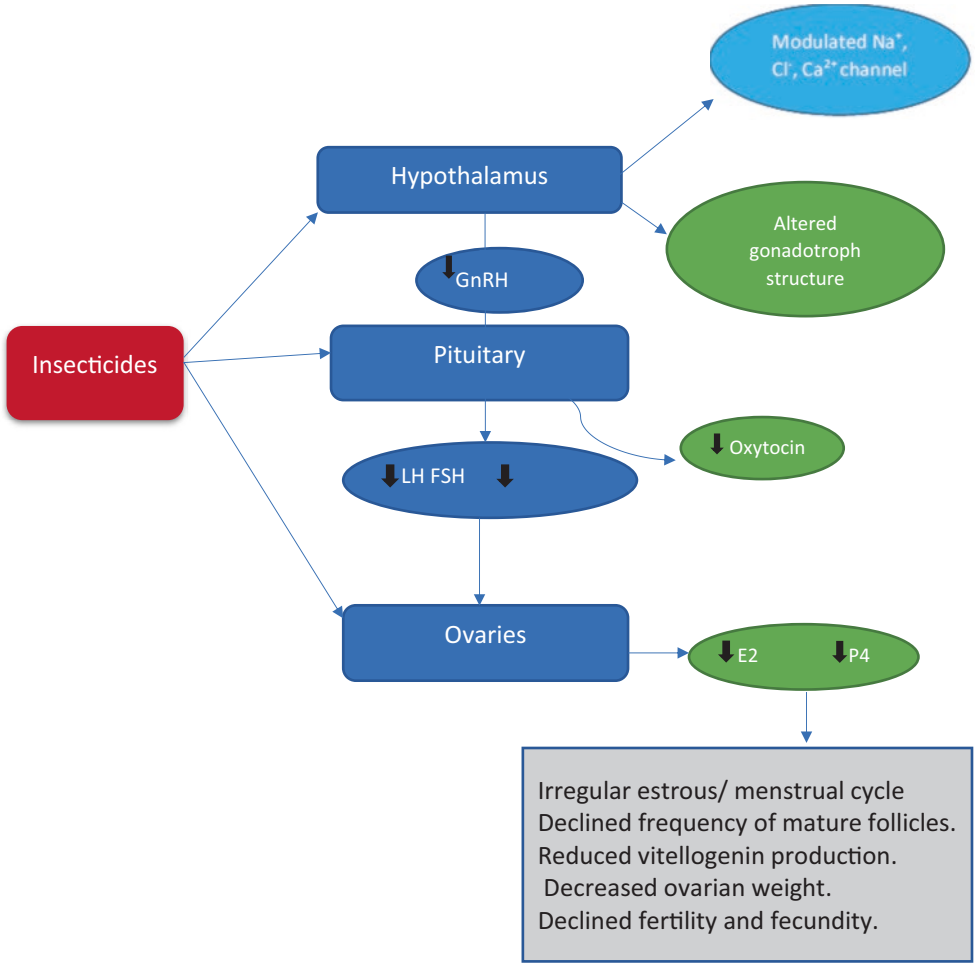

Fig. 4.2 Disruption of hypothalamus–pituitary–gonadal axis induced by insecticides

declining the secretion of oestrogen and progesterone (Sharma et al. 2020).

4.3.2 Effects on the Ovary

Exposure to pesticides, particularly insecticides in females, has been found to cause reproductive dysfunction essentially by interfering with the physiology of the ovaries. Changes in hormone production, follicular maturation, the ovulatory phase and the ovarian cycle are all examples of disrupted ovarian activities, which can result in decreased fertility, a longer period to conceive a child, stillbirths, impulsive abortions and defects in the embryo or foetus in the prenatal period. Ovarian toxicity triggered by insecticides is also influenced by oxidative stress and endocrine dysfunction. By suppressing the antioxidant defence mechanism and reactive oxygen and nitrogen species, oxidative stress in cells increases damage to DNA and the expression of inflammatory and apoptotic markers. The hypothalamic–pituitary–gonadal axis is disrupted by insecticide exposure, which contributes to ovarian dysfunction. Many insecticides, in general, interfere with ovarian physiology and thereby reproductive effectiveness. Organochlorine pesticides have been shown to have a negative impact on the ovary, decreasing formation of ovarian follicles, viability of oocytes and ovarian weight, as well as increasing atresia in animal studies (Tiemann

2008). For example, methoxychlor decreased the weight of ovaries, enhanced the prevalence of cystic ovaries, restricted the growth of ovarian follicles and induced atresia in rodents (Aoyama and Chapin 2014). It has been found that under in vitro conditions, endosulfan causes a reduction in the competence and viability in oocytes of buffalo (Nandi et al. 2011). In rats, methoxychlor has been found to reduce the healthy ovarian follicles while increasing the number of atretic follicles (Koc et al. 2009). Certain pyrethroids have also been reported to accelerate the follicular atresia in rats (Sanghaet al. 2013), and a carbamate pesticide has been found to decline the quantity of minor follicles in mice (Shanthalatha et al. 2012).

Malathion, an organophosphate pesticide, has been found to cause an increase in apoptosis of goat granulosa cells when exposed to it (Bhardwaj and Saraf 2016).

It has been reported that, in both women and animal models, exposure to pesticides impairs the ovary's ability to produce sex steroid hormones. For instance, exposure to an organochlorine pesticide, heptachlor, has been found to be linked to a slower decrease in the ratio of oestradiol and metabolites of progesterone in women following ovulation (Luderer et al. 2013). Basavarajappa et al. (2012) analysed that in isolated mouse antral follicles, an organochlorine pesticide, methoxychlor, restricted the secretion of testosterone, oestradiol androstenedione and progesterone. A pyrethrin pesticide, cypermethrin, has been reported to impede the activity of 3β-hydroxysteroid dehydrogenase (an enzyme responsible for progesterone biosynthesis) in rats (Sangha et al. 2013), and it also stalled the secretion of progesterone from the corpus luteum of bovines (Gill et al. 2011). Besides, atrazine has been found to cause a rise in sex steroid hormone levels and the enzymes which are involved in steroidogenesis and steroid biosynthesis in vivo. In addition, atrazine also amplified the estrogenic to androgenic ratio in rats (Quignot et al. 2012). It also elevated the synthesis of progesterone and oestradiol as well as the aromatase activity (an enzyme that helps in the conversion of testosterone into oestradiol) in the primary granulosa cells of rats (Tinfo et al. 2011), and it also impaired the process of steroidogenesis in the granulosa cells of swine (Basini et al. 2012).

4.3.3 Effects on the Uterus

In many studies, pesticides have been shown to disrupt the structure and function of the uterus in different animal models (Gore et al. 2015). For instance, an organochlorine pesticide, methoxychlor, promoted the weights of uteri in rats (Yu et al. 2013); however, carbendazim lowered the weights of uteri in rats (Rama et al. 2014). Furthermore, Aoyama et al. (2012) reported that a mixture of organophosphate pesticides such as dimethoate, dichlorvos and malathion increased hyperplasia of endometrium in rats. The organochlorine o,p-DDT has been found to elicit the estrogenic responses in the uteri of immature mice and rats with surgically removed ovaries (Kwekel et al. 2013).

4.3.4 Effects on Fertility

In a series of surveys, pesticides have been shown to disrupt the uterine structure and function in various animal models. For example, 2,2′,4,4′,5,5′-hexachlorobiphenyl (CB-153) and DDE exposures have been related to a greater chance of foetal loss in women (Toft et al. 2010). Besides, exposures to organochlorine pesticides have also been correlated to an amplified time to pregnancy in women (Chevrier et al. 2013). Furthermore, Yu et al. (2013) discovered that a mixture of organophosphate pesticides, viz. dimethoate, dichlorvos and malathion caused a significant reduction in pregnancy and normal live birth rates in rats.

4.3.5 Reproductive Senescence

Grindler et al. (2015) compared the women with high levels of beta-hexachlorocyclohexane and mirex to women with low levels of beta-hexachlorocyclohexane and mirex and reported

that women with high levels of beta-hexachlorocyclohexane and mirex had a younger mean age at menopause. In addition, it has been observed that rats exposed to the organochlorine pesticide, methoxychlor, during pregnancy had an earlier occurrence of reproductive senescence (Gore et al. 2011).

4.3.6 Breast Milk Contamination

Pesticides invade breast milk from a woman's body stores as well as from acute maternal exposures. The organochlorine family of pesticides has the largest body burden, which includes dieldrin, lindane, endosulfan, mirex, aldrin, chlordane and DDT. Breastfed babies have higher levels of organochlorine in their blood as compared to bottle-fed babies (Ribas-Fito et al. 2005). It has been analysed that if a mother has a heavy organochlorine body burden, her baby will get as much exposure to organochlorines during the first 6 months of breastfeeding as an adult would in 25 years (Bergkvist et al., 2012).

4.4 Insecticides-Induced Toxicity Mitigation Strategies

To prevent the harmful effects of insecticides, a number of methods may be used. There are three types of potential treatments that tend to be effective in avoiding insecticide-related havoc (Fig. 4.3). The first and the foremost is the integrated pest management (IPM). The IPM seeks to minimize reliance on toxic chemical insecticides while also ensuring long-term management of insect pests. The detoxification of insecticidal toxins from the atmosphere or the sources of plant food is the second approach to combat their toxicity. The detoxification group includes the biological/microbial detoxification, phytohormone therapy and other chemical methods such as advanced oxidation system. The final choice is amelioration that can be achieved by the use of plant extracts, dietary supplements, nanoparticles and other methods.

4.5 Conclusion

Pesticides are being enormously used in the modern agriculture, and the concerns about the protection of these toxic chemicals are dramatically increasing. Pesticide toxicities are found to be greater in developed countries with low occupational conditions and a lack of safety and health for employees who are not a top priority for industrial authorities. It is not possible to remove the pesticides immediately from cultivation in the coming future. However, being aware of the threats that these pesticides can pose to human health and the environment, strict measures need to be taken to restrict their rampant use. So, the government must collaborate with the pesticide manufacturers, agriculturalists, public health authorities, environmental protection organizations, etc. in order to minimize the damages that are caused by the use of these toxic pesticides at both acute and chronic levels. Health practitioners and manufacturers must take the appropriate steps to ensure effective pesticide control by stringent laws and toxicity regulations. This includes raising consciousness about the proper use of pesticides when they are absolutely necessary, wearing adequate safety security masks by farmers and the use of proper applicators to reduce the toxicity associated with these unsafe compounds. Furthermore, all countries that are yet to develop must implement the strategies of IPM that involves biological, social and cultural interventions. This can only be accomplished by careful planning and determination to limit the usage of these noxious compounds. So, suitable and stringent legislation is necessary for the control, coordination, selling, storage, use and dumping of these harmful substances, particularly those used in the agricultural fields. The chemical composition and toxicological characteristics of a pesticide should be evaluated on a regular basis, and the consent for marketing should be granted for a particular period of time that should not exceed more than a few years. It is also necessary to establish the appropriate commissions and agencies with the concerned authority to withdraw the consent if it is found being misused or

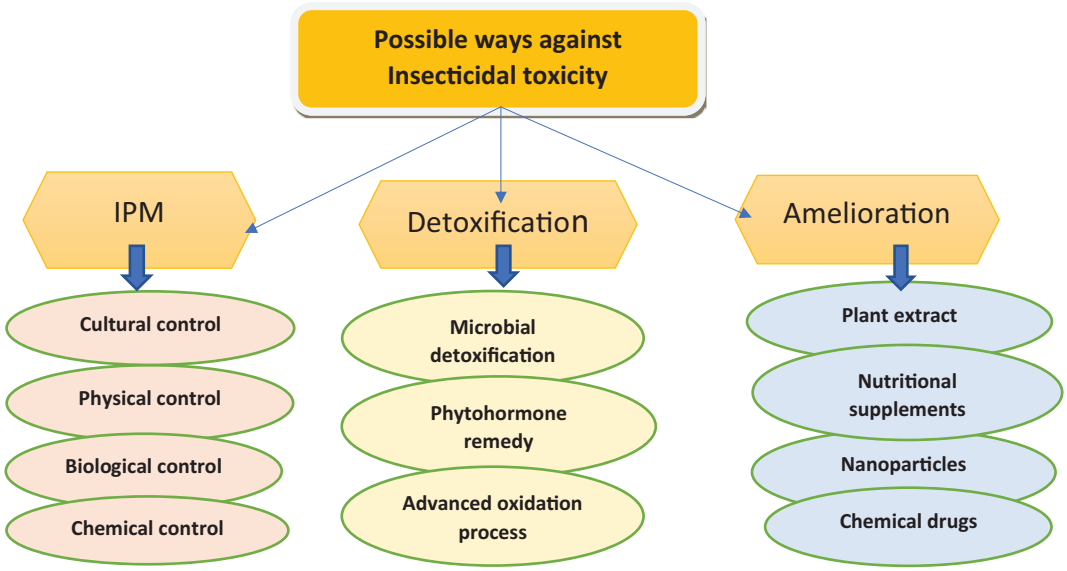

Fig. 4.3 Potential strategies for limiting the toxic effects induced by insecticides (Sharma et al. 2020)

even overused. The majority of acute pesticide exposure, as well as unwanted health and environmental harm, can be avoided or reduced if preventive measures are introduced and enforced. Nations have already suffered a lot of damage, and there will be more if the developed countries do not react now.

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5 Impact of Radiation on Male Fertility

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Abstract

In today's time, environmental aspects, lifestyle changes, and person's health coalesce to form stupendous impact on the fertility. All of us are knowingly or unknowingly exposed to several types of radiation. These can lead to collection of early and delayed adverse effects of which infertility is one. A spurt in the number of cases of male infertility may be attributed to intense exposure to heat, pesticides, radiations, radioactivity, and other hazardous substances. Radiation both ionizing and non-ionizing can lead to adverse effects on spermatogenesis. Though thermal and non-thermal interactions of radiation with biological tissue can't be ruled out, most studies emphasize on the generation of reactive oxygen species (ROS). In addition, radiation pathophysiology also involves the role of kinases in cellular metabolism, endocrine system, genotoxicity, and genomic instability. In this study, we intend to describe a detailed literature on the impact of ionizing and non-ionizing radiation on male reproductive system and understand its consequences leading to the phenomenon of male infertility.

Keywords

Male fertility · Radiations · ROS

5.1 Introduction

Worldwide, as many as 48.5 million or 15% of couples are affected by infertility. Males are found to be solely responsible for 20–30% of infertility cases and contribute to 50% of cases overall (Agarwal et al. 2015). The various factors which impose male infertility in modern times include environmental factors, lifestyle, biochemical factors, etc. But several studies in recent decades have proved a very huge impact of radiation exposures on reproductive health causing infertility. Strong evidences suggest that long-term exposure to very commonly used household

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devices like mobile phones, Wi-Fi, luminous watches, wireless routers, bluetooth devices, smoke detectors, and laptops can increase the probability of infertility. Besides these, radioactive substances released into the environment from various sources like nuclear power plants also cause increase in such occurrences. Radiation exposures during medical diagnostic and therapeutic procedure can also account for the same. The principal mechanisms include production of reactive oxygen species (ROS) and DNA damage.

Human testes and sperm are overly sensitive to radiation; owing to reasons like the following: (A) testes are located outside the abdominal cavity (Abuelhija et al. 2013) in thin external sac of the skin and is protected by scantier tissue than any other organ (Houston et al. 2006), (B) testicular cells have high proliferation and growth rate (Vogin and Foray 2013), and (C) sperm lack general cellular DNA repair mechanisms and antioxidant pool due to their highly specialized and compact structure. Radiation can be either ionizing or non-ionizing.

5.1.1 Ionizing Radiation

Ionizing radiation (IR) exists as atomic or subatomic particles or a very high-energy electromagnetic waves, all of which can ionize the nucleus of a substance (Ahmad and Agarwal 2017). IR includes X-rays, y-rays, and α-particles.

IRs are more dangerous to living cells as compared to the non-ionizing radiation because the electromagnetic waves of IR contain enough kinetic energy per quantum to break the bonds between molecules causing the ionization of these molecules. This phenomenon leads to initiation and propagation of chemical reactions causing damage to living cells. Since charged particles like electrons, protons, or neutrons are released during this process of radioactive decay, hence any molecule can cause irradiation.

Sources of IR can be natural or artificial. Examples of some natural IR sources include gamma (γ) rays generated during the radioactive decay of uranium, products of radon gas degeneration in the atmosphere, radionuclides of natural origin, and cosmic rays. Sources of artificial IR exposure include therapeutic (diagnostic or medical procedures) like X-rays used in medical diagnostic procedures and radiation therapy (RT), radionuclides present in eating and drinking materials, γ- rays which are generated as derivate in the nuclear industry, and remnant radiations during atmospheric nuclear testing (du Plessis et al. 2014). Occupational hazards during industrial manufacturing or military fallouts can also result in irradiation. Figure 5.1 shows a vicious cycle formed by radiation exposure, cancer development, and its treatment with radiotherapy or chemotherapy and their detrimental effects on male fertility.

5.1.2 Non-ionizing Radiation

These can be broadly classified into two types: (A) ELF-EMF – extremely low-frequency (ELF) electromagnetic fields (EMF) or power line (60 Hz) – and (B) RF-EMF, radiofrequency electromagnetic fields which are produced by wireless radio wave/microwave products. Non-ionizing radiation emitted as ELF-EMF are considered as non-thermal and do not cause serious irradiation in the living systems and hence are not considered as a potential health hazard in general. The higher energy radiations of the electromagnetic spectrum like radio frequencies (RF), microwaves, lasers, infrared, visible spectrum, and ultraviolet rays (from lowest to highest frequency), however, contain energy which can cause molecular excitation (changes in rotational, vibrational, or electronic structure of atoms and molecules) causing excitation of electrons from lower to higher energy states through the matter it passes. In the biological systems, these radiations can produce several thermal/non-thermal effects (depending on frequency and power level) which can range from burns, photochemical reactions, and accelerated radical reactions such as photochemical aging to non-thermal biological damages, similar to ionizing radiations. Although long-term exposure leads to the effects similar to IR (Lancranjan et al. 1975).

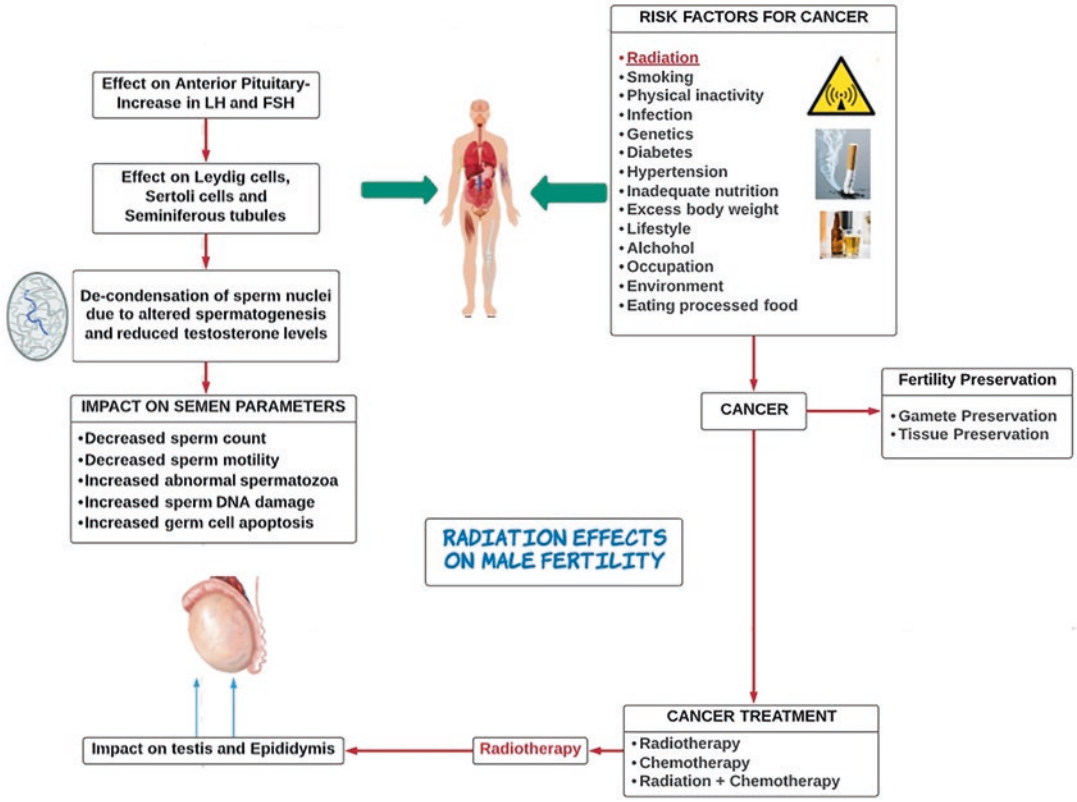

Fig. 5.1 Different sources and impact of radiation exposure on male fertility (Reproduced from Ahmad and Agarwal 2017)

5.2 Ionizing Radiation and Spermatogenesis

As mentioned earlier, human testis is very sensitive to radiation and even a low-dose exposure can impair spermatogenesis. Rowely et al. showed that an exposure of 1 Gy radiation for 14 days can result in significantly reduced number of spermatocytes (Rowley et al. 1974). However, the degree and persistence of damage in the gonads depend upon variable factors like dose, target volume, fraction size of radiation, and also the architecture and reserve capacity of specific target cell population (De Felice et al. 2016). When specifying the effect of radiation, two cell populations should be assumed as stated in the Oakberg-Hukins model of stem cell renewal and the Clermont and Bouton's two stem cell model:

(i) Stem cell spermatogonia: Occur as single isolated cells and are responsible for the repopulation of the germinal epithelium after radiation exposure
(ii) Differentiating spermatogonia: Occur in groups and signify the initial step in spermatogenesis (De Felice et al. 2019)

Stem cell spermatogonia are in continuous long cycle and hence are more resistant to radiation than the differentiating spermatogonia. These differentiating cells are randomly distributed over the tubules. After the RT or exposure, the fraction of re-populated seminiferous tubules is indicated by the re-population index (RI). This RI is directly proportional to the number of surviving stem cells (UNSCEAR 2008).

Although IR kills the cells immediately by necrosis and/or apoptosis during their prolifera-

tion, the decline in spermatogonial numbers to lower level does not happen at once but instead occurs in a progressive manner. The effects of radiation do not manifest until 18 weeks of irradiation when azoospermia is observed (Paulsen 1973). The exact reasons for this gradual decline are unknown, but it is conjectured that the expression of lethal damage by some of the non-cycling spermatogonia occurs only when they proceed into the cell cycle. The differentiation steps of spermatogonia into the spermatocytes are affected and get reduced. IR impairs spermatogenesis, spermatogonia being more radiosensitive than spermatocytes or spermatids. Radiation exposure leads to low sperm counts, decreased sperm motility, and increased rate of chromosomal abnormalities in some men. Sperm production is observed to remain >50% above control values during the first 50–60 days after low doses of irradiation (15–200 cGy). Also, multiple increments of a single dose of radiation lead to a dose-dependent reduction in semen volume and sperm count. It is evidenced that the time for spermatogenesis and semen volume to recover is directly proportional to the dose applied. This amounts to roughly 9–18 months for a radiation dose less than 1 Gy, 30 months for an exposure of 2–3 Gy, and 5 or more years after 4–6 Gy dose exposure (Ogilvy-Stuart and Shalet 1993).

5.3 Non-ionizing Radiation and Spermatogenesis

Non-ionizing radiation is of particular concern these days as the source of the radiations include commonly used devices like Wi-Fi, laptop, and cell phones in addition to base transceiver station (BTS) high-power electric lines. Continued exposure to low-frequency electromagnetic radiation (EMR) stimulates occurrences of damaged chromosomes and genomic instability and could potentially result in cancer development (Martin et al. 1986).

EMR impacts on different human organs but male testis is found to be most sensitive. The law of Bergonie and Tribondeau states "the radiosensitivity of tissue is directly proportional to its reproductive capacity and inversely proportional to the degree of differentiation of its cells." Accordingly, the spermatogonial stem cells with high mitotic activity tend to be more radio-sensitive than mature cells of testes (Vogin and Foray et al. 2013). EMR can significantly reduce sperm function like motility and vitality and may also impair DNA integrity (Fejes et al. 2005). Males experiencing subfertility, e.g., asthenozoospermia and oligozoospermia, show particular vulnerability to RF-EMR. It was found that such patients exhibit a marked decline in sperm motility following an exposure of their semen sample to a cellular device for just 60 minutes (Zalata et al. 2015).

5.4 Pathophysiology

Though the exact underlying mechanism is not completely known, some important mechanisms causing radiation-led DNA damage are discussed. It is believed to cause direct damage if the energy level is sufficient to break the intermolecular chemical bonds as commonly seen with ionizing radiation or cause the intracellular effects indirectly as seen mostly in non-ionizing radiation. The generation of free radicals is the commanding phenomenon among all the indirect methods.

Ionizing radiation directly attacks DNA structure by inducing DNA breaks, particularly double-stranded breaks (DSB). However, some other effects in DNA damage like generation of a basic sites and single-strand breaks (SSB) and oxidation of proteins and lipids can also occur. These effects occur as secondary complications through generation of ROS (Borrego-Soto et al. 2015).

Non-ionizing radiation interferes with the oxidative repair mechanisms within the cells resulting in an override of ROS concentration generating oxidative stress and damage to cellular components including DNA and also to cellular processes finally leading to cancer (Havas 2017).

5.4.1 Generation of Oxidative Stress

RF-EMR is well known to have the capacity to induce oxidative stress characterized by excessive generation of ROS. This increase of free radical in the cell occurs principally by Fenton reaction (Lai and Singh 2004). The reaction proceeds by the conversion of hydrogen peroxide, an oxidative respiratory product generated in the mitochondria, to free hydroxyl molecules via catalysis with iron (Bandyopadhyay et al. 1999).

Fenton reaction can be summarized as follows:

(i) The interaction of Fe^{++} salt with hydrogen peroxide results in the generation of free hydroxyl ions (OH).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^+ + OH^-$$

(ii) Any trace iron (Fe^{3+}) present further reacts with hydrogen peroxide forming hydrogen ion and superoxide given by the formula

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + O_2^- + H^+$$

(iii) Thereafter hydrogen peroxide interacts with superoxide ion leading to formation of OH.

$$O_2^- + H_2O_2 \rightarrow OH^- + OH^- + O_2$$

ROS cause cell injury and damage in three ways:

- Lipid peroxidation of membranes
- Oxidative damage of proteins
- DNA damage

ROS react with the double bond of free fatty acids of membrane lipids causing lipid peroxidation of plasma and organellar membranes, such as free hydroxyl molecules. This produces peroxides which are unstable and highly reactive. A chain reaction starts producing large amount of these products which cause extensive membrane damage. Oxidative damage of proteins are caused by ROS by oxidation of amino acid chains. This leads to damage in the active sites of enzymes, increased proteasomal degradation of misfolded proteins, and destroys formation of structural proteins. The formation of DNA adducts, breakage of single or double strands of DNA, and cross-linking of DNA eventually cause extensive DNA damage.

The unique, compact, and highly specialized structure of spermatozoa makes it more vulnerable to oxidative stress. Characteristically as sperm have low cytoplasmic volume, they possess limited protective antioxidant capacity than the other somatic cells. They also have relatively large substrate for free radical attack like DNA, thiol-rich proteins, and polyunsaturated fatty acids (PUFAs) (Aitken et al. 2012a). PUFAs are necessary for generating membrane fluidity which is essential for supporting both motility and fertilization.

Stress generators such as RF-EMR exposure to spermatozoa causes increased production of superoxide radical of mitochondrial and cytosolic origin (Agarwal et al. 2009; De Iuliiset al. 2009). This causes the peroxidation of PUFA and membrane lipids and elicits formation of electrophilic aldehyde like malondialdehyde, 4-hydroxynonenal (4HNE), and acrolein. These compounds further cause alkylation of sperm axonemal proteins particularly dynein heavy chain that is responsible to regulate sperm motility (Baker et al. 2015; Moazamian et al. 2015) and hence hamper sperm motility. 4HNE perpetuates a state of oxidative stress causing overproduction of mitochondrial superoxide radicals by adducting protein of electron transport chain (ETC) particularly succinate dehydrogenase (Aitken et al. 2012b). Hence a cascade of events shown in Fig. 5.2 following ROS attack on sperm substrates creates an override and imbalance in the cellular ROS concentration which finally leads to oxidative damage of DNA as the toxic hydrogen peroxide produced during the course moves to the sperm head and targets the guanine residues of the DNA.

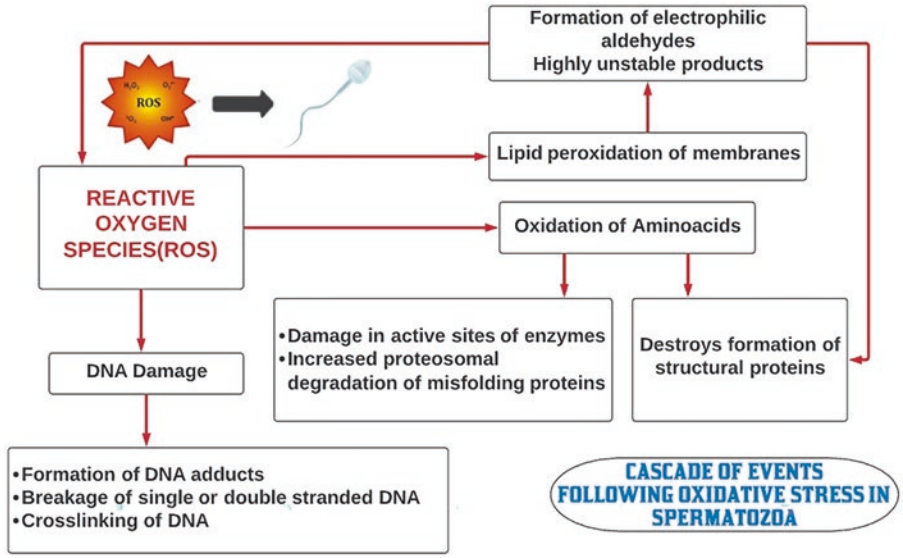

Fig. 5.2 Sequential molecular impacts of oxidative stress within the spermatozoon

5.4.2 Thermal Effect

The absorbed EMR when converted to heat causes thermal effect. Biological systems are affected by thermal effect when the heat generated exceeds 100 mW/ cm^2 (Habash 2011). While blood cells are capable of dissipating any excess heat, the sensitive organs like the eye, cornea, and testis do not have any temperature regulation mechanism.

Few studies have shown that use of laptop and exposure to low-frequency EMR may increase the temperature in the testis leading to impaired DNA integrity and apoptosis of germ cells (Durairajanayagam et al. 2015). Apart from several other lifestyle factors, use of cell phones has been vastly studied to induce DNA damage. It has been reported that if cell phone is kept in trouser pocket for a long time, it can cause DNA strand break in the sperm cells after exposure of only 2 hours/day for 60 days. The duration and power density of the exposure was found to be directly proportional to the magnitude of effect (Kumar et al. 2014). During the processing of repair mechanisms activated by radiation damage, heat is seemingly known to increase the levels of single stranded breaks (SSBs) and double stranded breaks (DSBs) of DNA by impairing the repair of corrupted bases.

Microwaves also operate by rotating the polar molecules assisted by generation of electromagnetic fields leading to hazardous effects on germ cells. Meena et al. have reported a significant increase in sperm DNA damage after a whole body microwave exposure of 2.45 GHz for 2 hours per day for 45 days. This was visually evaluated by a single cell gel electrophoresis also known as Comet assay. The undamaged DNA nucleotide was referred to as head, while trailing damaged DNA streak was referred to as tail. An increasing tail length and tail movement was demonstrated (Meena et al. 2014).

Studies have highlighted that the exposure to microwave EMR significantly suppresses histone kinase activity in the sperm as compared to the non-exposed counterparts (Kesari et al. 2011). During spermiogenesis, the germ cells undergo a distinct morphological change where to ease the chromatin compaction, the core histones are replaced by protamines. Defects in either the replacement or the modification of histones might cause male infertility with azoospermia,

oligozoospermia, or teratozoospermia. In the differentiating cells, a decrease in histone H1 activity, just before their entry into the M-phase from G2-phase, suggest the role of Cd2/CdK2 in regulating this phenomenon (Agarwal et al. 2009). Some studies have reported that depletion in the activity of both histone kinase and protein kinase may serve as enzymatic markers of microwave EMF's ability to affect spermatogenesis and sperm cell cycle (Shokri et al. 2015).

5.4.3 Calcium Ion Concentration

Calcium ion concentration affects vital events of fertilization and activities of sperm. The processes of sperm motility, chemotaxis, capacitation, and acrosomal reaction within the female reproductive tract are highly regulated by the calcium ions (Beigi Harchegani et al. 2019) together with many other factors. Seminiferous tubules and Leydig cells have pyruvate kinase enzyme complex (PKC) which modulate the ion conductance via calcium-dependent phosphorylation of membrane and ion exchange proteins. PKC, cAMP, and variations in calcium ion concentration have important function and affects sperm motility (Kimura et al. 1984). The coordinated sperm tail action requires energy obtained from ATP and signaling via cAMP and Ca^{2+} from the surroundings (Yan 2009). Hence factually reduced fertilization and male infertility can be coherently associated with deficiency in calcium ions and also disturbance in energy supply or signal transduction (Beigi Harchegani et al. 2019).

Animal studies conducted by Wang et al. proved significant disturbances in calcium ion homeostasis together with activation of endoplasmic reticulum stress (ERS) and apoptotic signaling molecules in the testicular cells of mice irradiated with low-dose radiation (LDR) ranging in 25–200 mGy. They proved a time- and dose-dependent decrease in Ca^{2+} ions and Ca^{2+}- ATPase activity and a similar increase in the ERS molecular markers and apoptotic signaling markers (Wang et al. 2013).

5.4.4 Endocrine Effects

Leydig cells are among the most susceptible cells to EMR. Radiation may disrupt Leydig cell population and thereby affect spermatogenesis. Leydig cells produce testosterone, and hence a decline in testosterone levels is observed. There may also be elevated luteinizing hormone (LH) levels along with reduced or even normal testosterone levels (Tsatsoulis et al. 1990). Meo and Al-Drees have proposed that the radiations can cause alterations in the polarization status of the cellular membranes of the Leydig cells which can evolve distinct changes in the composite biochemistry of testosterone synthesis and secretion (Meo and Al-Drees 2010). Further, several studies have reported that mobile phones can downregulate the production of melatonin which plays an important role in testosterone secretion. It is proved to exert an antigonadotropic effect by acting at the hypothalamo-pituitary axis (Yilmaz et al. 2000). Additionally, RT is known to cause damaging effects on the vessels and nerves of the pelvis which result in reduction in sexual function in males (Mahmood et al. 2016).

The cell function of Leydig and Sertoli cells was evaluated in a study conducted by Tsatsoulis et al. on male patients (ranging in age from 21–49 years old) who were subjected to orchidectomy followed by radiotherapy given in total dose of 30 Gy in 20 fractions. The results revealed that these patients had lower levels of testosterone but high LH as compared to the control group. This statistically significantly low testosterone/LH ratio clearly indicated Leydig cell damage (Tsatsoulis et al. 1990). Two relevantly similar studies were conducted on pubertal boys with acute lymphoblastic leukemia who were given direct testicular irradiation. Results showed total ablation and functional reduction of Leydig cells directly after radiotherapy without any observed reversal even after 5 years of the treatment. Androgen supplementation was suggested in most cases for normal sexual maturation (Brauner et al. 1983; Shalet et al. 1985).

LH plays the role of main hormone which controls the function of Leydig cells through its

receptors which are specific to it and are integrated with both phospholipase C and adenylate cyclase pathways (Cooke 1999). Hence radiation exposure would cause steroidogenic lesions seen as a reduction in the LH receptors of the Leydig cells (Payne and O'Shaughnessy 1996).

The downstream effects of LH and HCG on the Leydig cells occur via secondary messenger signaling molecule, cAMP. An estimation of the LH and basal triggered cAMP production in radiation exposed and normal Leydig cells showed that irradiation causes a dose-dependent decrease in the generation of basal and LH stimulated cAMP proving that down effects of HCG and LH on Leydig cells are employed mainly mediated through cAMP associated events (Sivakumar et al. 2006).

Even though the Leydig cells are much more radio-resistant to the germinal epithelial cells of testes and get affected by high doses of radiation, the Leydig cells in children are more sensitive to radiation than adults. Their function is usually preserved up to 20 Gy in pre-pubertal boys and 30 Gy in sexually mature men. Hence Leydig cell dysfunction due to RT can cause hypogonadism as they function to secrete testosterone (Izard 1995).

The most dramatic endocrine effect of irradiation of the testis is the increase in FSH levels. It is not the direct effect but results due to depletion of germ cells. FSH levels have been used as an index of radiation damage (Shapiro et al. 1985).

5.5 Radiation and Genotoxicity

Radiation is well known to induce genotoxicity. EMR induces genotoxic effects by forming SSBs, DSBs, micronuclei, chromosomal damage, alteration in gene expression, cell division, and apoptotic cell death (Meena et al. 2014). Even though it is evident that RT may damage DNA, the extent or significance of such effect on sperm chromatin integrity is unclear. A dose-dependent increase in DNA damage in testis cells has been reported after 14 days of RT (Stahl et al. 2004), and the overall results showed that DNA damage inducted in pre-meiotic germ cells is detectable in primary spermatocytes and is also found in mature spermatozoa. The damage can happen via one of two scenarios.

5.5.1 Direct Action

This refers to the direct impact of radiation on the DNA causing ionization of the atoms within the DNA helix. Such a "direct hit" on DNA is commonly possible due to the small; barely few nanometer diameter of DNA helix. It is advocated that the radiation must produce ionizing effects on DNA within only a few nanometers in order to advocate for the successful occurrence of such an action.

5.5.2 Indirect Action

Refers to the impact of radiation on rather non-critical target atoms, usually water creating reactive oxygen free radicals which damage DNA through successive events. This action does not necessarily require the occurrence of the initial ionization event very close to a DNA molecule, but at some other location from where it can act by initiating a signaling reaction to cause DNA damage at last. Indirect pathway is a more frequent phenomenon than a direct one. Either of the actions causes radiation to attack at specific location of DNA structure and damage it mostly by causing SSBs or nicks which is easily reported by the cell and is usually attended by the DNA damage control machinery of the cells where the opposite strands are used as template. However, if radiation causes DSBs in the DNA structure, the cells suffer difficulty in repairing it and can result in mutation and further lead to cancers or cell death. The ratio of occurrence of double-stranded to single-stranded breaks is about 1:25. Hence, at times, DNA damage due to radiation is repairable (Unknown authors 2012). Nonetheless DNA fragmentation index (DFI) is found to be significantly higher in men who are receiving RT (Lord 1999).

Genomic instability is emergence of genetic alterations during cell division. Radiation leads

to high frequency of mutations in the genome of a cellular lineage. Microtubule based structures may suffer alterations in their ultrastructure causing deviation in normal morphology of sperm tail. This causes defects in sperm motility and increase in sperm fatality (Sha et al. 2014). Kesari and Behari examined spermatozoa of RF-EMF radiation-exposed rat under transmission electron microscopy (TEM) and reported major changes in the axonemal microtubules, mid piece region, and outer dense fibers and membranes of mitochondria. They also found that the sperm nucleus showed distortion of the membrane head on the sagittal section. They concluded that the exposure of sperm to RF-EMF of cell phone in excess can cause disarray of sperm mitochondria and result in production of highly reactive free radicals. This hampers motility of sperm and also causes deformation of the acrosome which might lead to a lack of ability to penetrate oocytes resulting in infertility (Kesari and Behari 2012). Though studies have revealed that EMF exposure may lead to molecular irregularities, some have also shown that it may not cause direct DNA damage. The increase in autophagy can help in balancing homeostasis and apoptosis (Shen et al. 2016). A range of studies conducted on effects of exposure to extremely low-frequency EMF demonstrated alterations in important basic cell functions like protein and cell cycle regulation.

Luukkonen et al. found that exposing the human SH–SYSY neuroblastoma cell lines to extremely low-frequency EMF causes decreased p21 protein level after menadione treatment. p21 is a tumor suppressor gene. It induces tumor growth suppression through wild-type p53 activity. Its cleavage and inactivation in normal as well as cancerous human cells occurs by the action of caspase-3 (Fig. 5.3). p21 expression is a poor prognostic marker linked to poor survival rate and resistance to chemotherapy. Also, post menadione treatment and EMF exposure conditions are accompanied by an increase in number of cells in the G1 phase and reduction in number of cells in the S phase. EMR displaces electrons in DNA, which is accompanied by electron transfer (Luukkonen et al. 2017). These displaced electrons break hydrogen bonds causing separation of DNA strands followed by transcription.

Very risky situation can also be encountered in case of assisted reproductive techniques (ART) using irradiated sperm. The specific selection processes of a sperm which occur during natural conception are circumvented in ART. This may result in fertilization of oocyte with a sperm containing damaged DNA. Such occurrences may however result in successive transfer of genetic aberrations into the dividing embryo and also lead to future complications and reproductive failure (Fatehi et al. 2006). Hence fertility is negatively affected by injury to nuclear DNA of sperm. Compliantly Kamiguchi and Tateno have also shown that despite the fact that human spermatozoa are extremely radiosensitive, they retained the fertilization capacity even after a high dose (4.23 Gy of γ rays) of irradiation. They also inferred that the sperm having damages may escape selection process during fertilization and cause the damage of DNA to pass into the next generation (Kamiguchi and Tateno 2002).

5.6 Effects on Semen Parameters

Highlighting effects on semen parameters, a study done by Vakalopoulos et al. showed a statistically significant reduction in semen volume, sperm concentration per ml and total sperm count, as well as forward motility with a statistically significant increase in occurrence of abnormal forms of sperm in the semen continually up to 12 months following the therapy (Vakalopoulos et al. 2015). Another study revealed no differences at the beginning and 24-month post therapy for any semen parameters except in volume, which could indicate a return of sperm quality to pre-radiotherapy conditions (Stahl et al. 2004).

Radiation effects on sperm counts may be subdivided into three phases. Phase 1 is the 8-week period post radiation when sperm production is still maintained at normal levels, especially after low doses of irradiation. Phase 2 is represented by the gradual reduction of sperm production reaching its lowest 3–8 months after irradiation as a possibility associated with

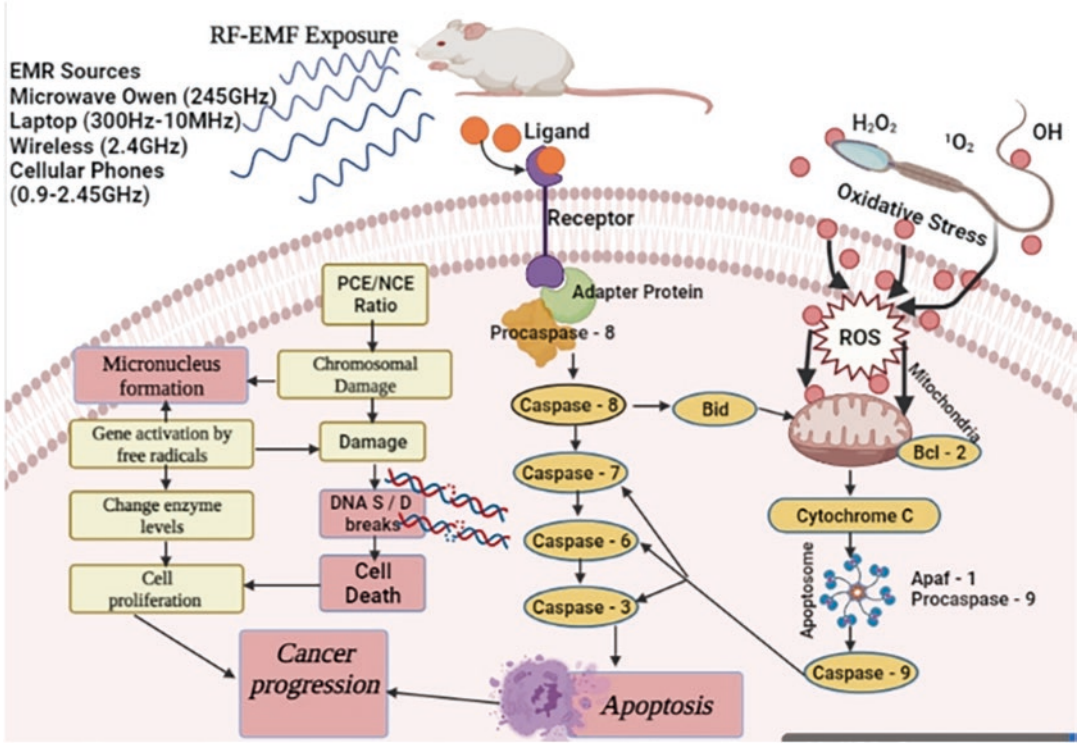

Fig. 5.3 Diagrammatic representation of the effects of exposure from EMF from various sources (mobile phone, microwave ovens, wireless devices, computers) causing genotoxicity. (Reproduced from Kesari et al. 2018)

azoospermia. Phase 3 is signified by the initiation of recovery from oligospermia or azoospermia. The final phase is marked by recovery of sperm production to control levels.

5.7 Conclusion

Several studies advocate that the direct or diffused exposure of human testes to ionizing and non-ionizing radiations emitted from sources like cell phones, microwave oven, laptops, X rays, γ-rays, etc. exerts damaging effects on the male reproductive system resulting in serious defects in sperm morphology, sperm count, and functions (mobility and fertilization). These effects are predominantly caused due to damage in sperm DNA which attenuates micronucleus formations and genomic instability. Disturbed functions of protein kinases, hormones, and antioxidant enzymes are also evident and participate in causing such abnormalities. On the one hand where direct ionization of DNA may result in mutations in chromosome, injury to DNA ultimately leading to cell cycle arrest, apoptosis, and cancer, the indirect effects are demarcated by excess accumulation of mitochondrial and cytoplasmic ROS by over-powering the cellular antioxidant machineries. It is the ROS that are considered the prime initiators for activating the intracellular signaling pathways ultimately resulting in severe DNA damages and apoptotic changes in the testicular cells.

Most notably, there exists a range of response to radiation exposure, and it is invariably dependent on the type of source and effective irradiation dosages. This is further dependent upon the duration of exposure and most importantly on the genetic and epigenetic makeup of the exposed individual. Also these observations give us a reasonable shift from assumptions like only a direct cellular interaction or a long-standing exposure

to radiation can lead to significant damage to the fact that indirect damage and a conglomerated effect of short-term exposures can also lead to significant impacts resulting in male infertility.

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6 Arsenic-Induced Sex Hormone Disruption: An Insight into Male Infertility

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Abstract

Arsenic (As) is one of the most potent natural as well as anthropogenic metalloid toxicants that have various implications in the everyday life of humans. It is found in several chemical forms such as inorganic salt, organic salt, and arsine (gaseous form). Although it is mostly released via natural causes, there are many ways through which humans come in contact with As. Drinking water contamination by As is one of the major health concerns in various parts of the world. Arsenic exposure has the ability to induce adverse health effects including reproductive problems. Globally, around 15% of the couples are affected with infertility, of which about 20–30% are attributed to the male factor. Arsenic affects the normal development and function of sperm cells, tissue organization of the gonads, and also the sex hormone parameters. Stress induction is one of the implications of As exposure. Excessive stress leads to the release of glucocorticoids, which impact the oxidative balance in the body leading to overproduction of reactive oxygen species (ROS). This may in turn result in oxidative stress (OS) ultimately interfering with normal sperm and hormonal parameters. This study deals with As-induced OS and its association with sex hormone disruption as well as its effect on sperm and semen quality.

Keywords

Arsenic · Health issues · Hormonal disorders · ROS · Oxidative stress · Male infertility

6.1 Introduction

Arsenic (As) is one of the most widely distributed environmental toxicants, chiefly occurring in the earth's crust and ground water (Garelick

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et al. 2008). In the earth's crust, the average abundance of As is about 5 mg/kg, and it occurs naturally in about 200 mineral forms (Garelick et al. 2008). Under natural conditions, it is rarely found as a free element (National Research Council 1977) and rather occurs as a metalloid as inorganic salt, organic salt, and gaseous forms (arsine) (Hughes et al. 2011; Kuivenhoven and Mason 2022). It also exists in some valence states such as arsenic element (0), arsenite (trivalent +3), and arsenate (pentavalent +5) (Kuivenhoven and Mason 2022). The Agency for Toxic Substances and Disease Registry (ATSDR) has designated inorganic As as the 'most hazardous compound' in the environment (Ferrario et al. 2016). According to the World Health Organization (WHO), the preferred acceptable level of As in water is less than or equal to 10 μg/L, and the maximum permissible limit is 50 μg/L. However, in certain regions, water sources have As concentration much above this range (Ferrario et al. 2016; Basu et al. 2001). Due to commercial uses and production of inorganic and organic As compounds, the environmental level of As has risen beyond the acceptable limits rendering it as a toxicant (National Research Council 1977). It can dissolve in water bodies through rain and hence contaminate water bodies along with groundwater sources (Chung et al. 2014).

Like most other environmental and anthropogenic pollutants, As has been reported to affect living systems via a wide array of processes interfering with almost all physiological activities including reproduction and fertility, mostly by the generation of oxidative stress (OS) (Sengupta and Dutta 2018; Sengupta 2013). Arsenic has been reported to induce spermatotoxicity, inhibit testicular steroidogenesis, and impact the overall gonadal organization in exposed individuals (Kim and Kim 2015). It can also disrupt gene regulation of androgens and progesterone receptors (Ahmad et al. 2021). One of the ways by which As renders toxicity is via inducing OS in affected individuals, which leads to a serious imbalance between the free radicals and cellular antioxidant defence (Flora and Pachauri 2013). On the other hand, there are many reports that suggest negative effects of OS on sperm cells including sperm functions, and sperm genome; mostly attributed to the lack of antioxidant capacity, cell repair mechanisms, and structural peculiarity of sperm cells (Agarwal et al. 2020; Bisht et al. 2017; Sengupta and Banerjee 2014). In a different perspective, OS-induced testicular ageing has been proposed to be the key contributor towards male infertility and cancer (Bisht et al. 2017). Interestingly, OS has been reported to be associated with disruption of the hypothalamic-pituitary-gonadal (HPG) axis, thereby impacting the reproductive hormones and fertility (Roychoudhury et al. 2021). Hence, As-induced OS could be a probable route through which As can affect the reproductive system apart from impacting other physiological systems. Therefore, As is a toxin of great concern to male reproduction and fertility besides other health issues.

6.2 Sources of Exposure and Geographical Distribution

Naturally, As is released into the environment via weathering, leaching, and subsequent runoff as well as volcanic eruptions (which is one of the major natural causes of As emission) or by anthropogenic activities such as mining, burning of fossil fuels, etc. (Kuivenhoven and Mason 2022; IARC 2012) (Fig. 6.1).

However, As contamination of drinking water is mainly due to the geological sources rather than anthropogenic activities (Ratnaike 2003). Although it is toxic to living organisms, As is employed in a number of industrial uses such as in distillery plants, electronics (as gallium arsenide), medicine, chemical industries, agriculture, and wood preservatives (Kuivenhoven and Mason 2022; Chung et al. 2014; IARC 2012). There are specific geographical regions where relatively high concentrations of As have been reported in groundwater and drinking water sources. These regions include parts of Bangladesh, India, China, Cambodia, Argentina, Australia, Chile, Mexico, Thailand, the USA, Nepal, Taiwan, Myanmar, and Vietnam (IARC 2012; Mazumder

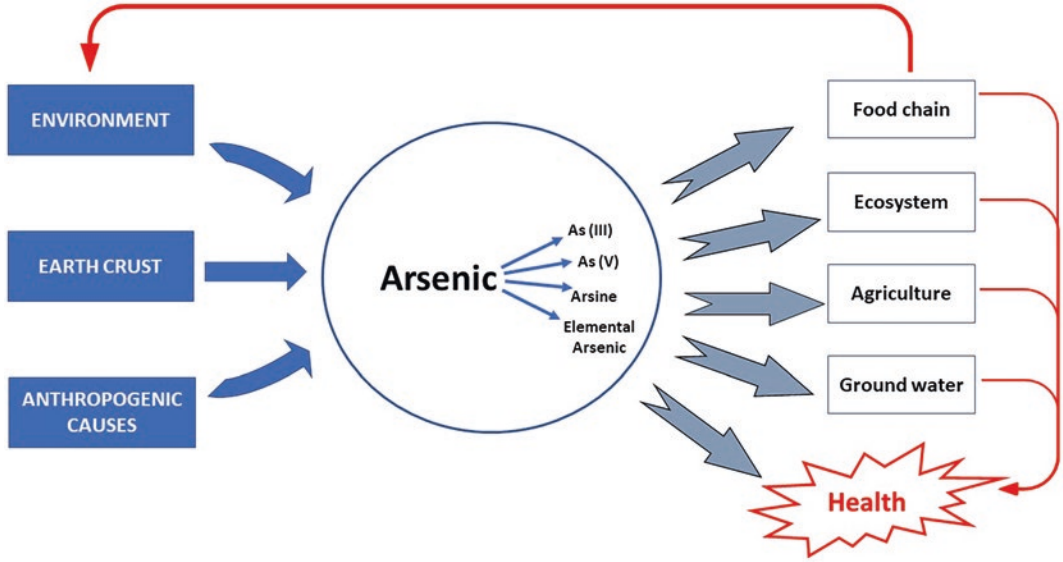

Fig. 6.1 Origin and exposure routes of arsenic (As) in the environment. It is released form natural as well as man-made sources which leads to the various components of the environment such as the food chain or agriculture and may affect the ecosystems, ultimately impacting human health

et al. 2010). However, West Bengal (in India) and Bangladesh remain two of the worst affected regions in the world (Ratnaike 2003), and the source of As is geological in origin which contaminates the aquifers that supply water to about one million tube wells (Ratnaike 2003). Around 2.5 billion people worldwide depend on ground water for drinking purpose (Shaji et al. 2020) and hence, contamination with As is a matter of great concern for the population who depend on these aquatic sources. According to a recent report, around 27 million people are believed to have been exposed to As in drinking water with levels beyond the permissible limits in Bangladesh alone (Kuivenhoven and Mason 2022). However, about 50 million people are at a risk of groundwater As contamination in India (Shaji et al. 2020). Apart from the abovementioned regions, North America is one of the hotspots of As contamination, with food products such as rice, fruits, even animal products, and drinking water being contaminated with As (Janković 2020). The severity of As contamination has also been reported from European countries, with groundwater contamination being a common matter of concern (Katsoyiannis et al. 2015; Medunić et al. 2020). Agricultural products grown in As-contaminated soils are another cause of As exposure. Seafood, fishes, and some forms of algae are the richest organic sources of As (Ratnaike 2003).

6.3 Arsenic: Pathways to Disrupt Physiological Functions

Arsenite and arsenate are two of the most common and toxic forms of As (IARC 2012; Druwe and Vaillancourt 2010; Kuivenhoven and Mason 2022), with arsenite being more toxic than the latter (Kuivenhoven and Mason 2022). There are various routes through which As affects the physiology, while simultaneously interfering with various organs and biochemical pathways, as discussed below.

Arsenate blocks the normal glycolytic pathway by forming glucose-6-arsenate which resembles glucose-6-phosphate and therefore hinders the downstream processes of this biochemical pathway (Kulshrestha et al. 2014). Because of its high affinity to sulphhydryl groups, arsenite dysregulates the proteins and enzymes of the living

system by changing their conformation, resulting in loss of function, and affecting its ability to interact with other proteins or genetic material (Shen et al. 2013). Arsenite has also been found to inhibit the pyruvate dehydrogenase enzyme complex by binding to the lipoic acid moiety, ultimately affecting the citric acid cycle and subsequent production of adenosine triphosphate (ATP) (Shen et al. 2013). Arsine, a colourless and odourless gas, known to be the most toxic form of As, is a lethal haemolytic agent. The haemolytic activity has been attributed to OS and further denaturation of protein (Kuivenhoven and Mason 2022). Lifestyle habits such as smoking have been found to exaggerate As toxicity when already exposed to it (Chung et al. 2014). Electrothermal atomic absorption spectrometry studies have shown that the highest concentration of As is present in the kidney and liver following acute poisoning. Chronic exposure to As leads to its accumulation in the liver, kidneys, heart, lungs, muscles, nervous system, gastrointestinal tract, and spleen. This may lead to dermatological issues, hypertension, respiratory diseases, cancer, and arsenicosis (which is characterized by melanosis, keratosis, and leucomelanosis) (Ratnaike 2003; Shaji et al. 2020). Acute toxicity of As results in ailments such as gastroenteritis, diarrhoea, cough, chest pain, renal failure (Kuivenhoven and Mason 2022), intravascular coagulation, and peripheral neuropathy (Ratnaike 2003). Inorganic As can affect the intestinal tract by disrupting intestinal cellular barrier, as shown in an in vitro study using human intestinal cell model (Chiocchetti et al. 2019). Arsenic exposure has been found to be associated with chronic kidney disease (Zheng et al. 2014). Exposure to arsine gas may also lead to headache, nausea, and fever within 1–12 hours of exposure (Kuivenhoven and Mason 2022) (Fig. 6.2).

One of the disrupting pathways through which As affects health is by interfering with the nervous system, thereby dysregulating neuronal and hormonal physiology. Arsenic species accumulates in various parts of the brain, with the highest accumulation being recorded in the pituitary (Sánchez-Peña et al. 2010). One of the deadliest properties of As is that it can cross the blood-brain barrier (BBB) by affecting the tight junction proteins present in the BBB, thereby increasing its permeability (Medda et al. 2020). It has a notorious ability to alter the functions of the hypothalamic-pituitary-adrenal (HPA) axis, which not only induces depressive behaviour (Tyler and Allan 2014) but also leads to the suppression of the HPG axis (Joseph and Whirledge 2017).

6.4 Effects of Arsenic on Male Reproduction

As already mentioned, disturbances in the HPG axis leads to the abnormalities in reproductive hormone secretion, thereby impacting the reproductive functions. Another pathway through which As impacts the reproductive hormones is by disrupting their overall biosynthetic and metabolic pathway. This is because As is also a potential endocrine-disrupting chemical (EDC) which can interfere with the synthesis, metabolism, and transport of hormones in the body (Sun et al. 2016). In reference to reproductive hormones, HPG axis is responsible for proper regulation of the reproductive hormones, which is mainly brought about by the dual actions of gonadotropin-releasing hormone (GnRH) and gonadotropins (Sun et al. 2016). Arsenic has been reported to interfere with the reproductive hormones by affecting the gonads and reducing the sperm quality (Sun et al. 2016). Like other environmental toxicants which affect both sperm and hormonal parameters, in exposed men, As can affect male reproductive physiology and fertility probably by OS-induced sex hormone disruption. Gonadotropins such as luteinizing hormone (LH) act as a major regulator of sperm maturation (Zhao et al. 2020). It has been asserted that any disturbance in the sex hormone synthesis or action results in abnormal reproductive functions (Rehman et al. 2018). Reprotoxicity of As, such as disruption of spermatogenesis, has been attributed to the activation of ERK/AKT/NF-κB signalling pathway (Lovaković 2020). An in vitro study has shown that As can induce the subexpression of genes which encode claudin 11 and occludin proteins that maintain the functions of

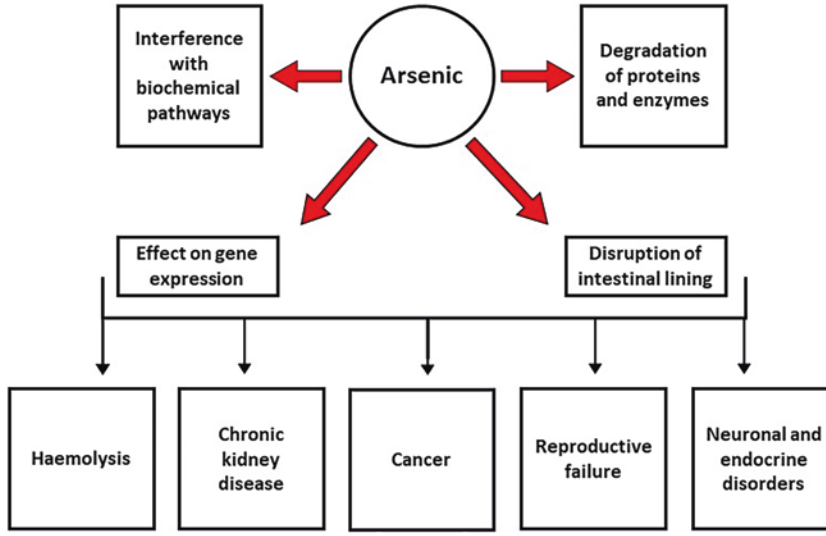

Fig. 6.2 Multifaceted effects of arsenic (As) on physiological parameters. It can impact most of the organs or biochemical pathways by interfering with the molecular setup and the vital pathways, altering the normal physiology

tight junctions in the blood-testis barrier, thereby disrupting the structural integrity (Ramos-Trevino et al. 2018). The various effects of As in the reproductive system has been discussed in the following sections.

6.4.1 Effects of Arsenic on Male Gonadal Tissue and Sperm Quality

There is a considerable amount of evidence asserting the harmful effects of As in the male reproductive system, both in humans and animals (Pant et al. 2001; Wang et al. 2006; Kim and Kim 2015), although most of the findings of As toxicity in reproductive health has been recorded in animal models (Ahmad et al. 2008; Mukherjee and Mukhopadhyay 2009). In rats, As treatment resulted in the disruption of testicular cells and overall tissue arrangement (Ahmad et al. 2008), reduced sperm count, sperm motility, and necrotic effects in the gonads (Mukherjee and Mukhopadhyay 2009). Seminiferous tubules in the As-treated mice had reduced diameter (Hazra et al. 2008), lower testicular weight, and low sperm count in mice (Nath et al. 2017). Lower epididymal sperm count and motility was also noted following 5 weeks As treatment in mice (Im Chang et al. 2007). Arsenic treatment also reduced gonadal weight and sperm count in rabbits (Zubair et al. 2014). It affected the epididymis and seminiferous tubules of goat testes, by increasing the thickness of epididymal covering and trabeculae and narrowing the diameter of seminiferous tubules (Wares et al. 2015). Sodium arsenate toxicity also leads to the deterioration of sperm motility, by the binding of thiol proteins by As. Also, peroxidation of the polyunsaturated fatty acids on the sperm membrane is suspected to be behind the reduction of sperm motility by As (Zubair et al. 2017). Production reactive oxygen species (ROS) has been considered as a cause of distortion of sperm morphology in rats (Kumar et al. 2002), thus revealing a pathway for the oxidizing actions of As. Also, As degrades the mitochondrial membrane potential of sperm, rendering altered sperm morphology (Zubair et al. 2017). From a different perspective, As was shown to interfere with the expression of Y-linked gene Ddx3y, which is associated with spermatogenesis and sperm maturation. Treatment of adult male mice with As for 2 months resulted into down-regulation of Ddx3y expression, altered spermatogenesis, and sperm development (Li et al. 2012). Mouse Ddx3y gene shares high degree of

similarity with the human DDX3Y gene, whose deletion is associated with male infertility (Li et al. 2012). In men exposed to inorganic As, poor semen quality (such as semen volume, sperm concentration, motility, acrosome integrity, sperm vitality, etc.) and erectile dysfunction (ED) were reported (Nie et al. 2006; Kim and Kim 2015; Xu et al. 2012; Hsieh et al. 2008). Men living in areas with high As concentration have been found to be oligozoospermic or azoospermic (Sengupta et al. 2013). Unexplained male infertility has also been related to As exposure (Wang et al. 2016). In another study, inverse dose-response relationship was observed between human seminal plasma As and computer-aided sperm analysis (CASA) parameters such as straight-line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP) (Wan et al. 2019).

6.4.2 Effects of Arsenic on Male Reproductive Hormones

Both As (trivalent +3) and As (pentavalent +5) are capable of disrupting the gonadal endocrine system (Sun et al. 2016). Arsenic-induced methylation and ROS production have been reported to affect the gonadal receptor genes particularly the gene expression of the enzymes P450scc and CYP17 in testosterone synthesis pathway (Sun et al. 2016). As mentioned earlier, As can cross the BBB and interfere with the hormonal and neurotransmitter regulatory pathways, mainly due to its endocrine disruptive properties (Medda et al. 2020). Exposure to >50 ppb As has been found to be associated with high incidence of endocrine disruption which can be attributed to the reduction in levels of testosterone and/or nitric oxide synthase (NOS) activity – a mediator of penile smooth muscle relaxation (Hsieh et al. 2008). In experimental rat model, As treatment resulted in the decrease of paired testicular weight, epididymal sperm count, and plasma level of hormones such as LH, follicle-stimulating hormone (FSH), and testosterone as well as the testicular concentration of testosterone (Jana et al. 2006). Low levels of gonadotropins, i.e. LH and FSH in As-treated rats, have been attributed to the increased plasma concentration of corticosterone (Jana et al. 2006). In another study on male rats, As reduced the level of dopamine and probably acted via estrogenic mode of action (Jana et al. 2006). It affected testosterone synthesis by impacting the LH concentration in treated mice (Soleymani and Hemadi 2007). Testicular steroidogenesis is assisted by the enzymes 3 beta hydroxysteroid dehydrogenase (3β-HSD) and 17 beta hydroxysteroid dehydrogenase (17β-HSD), and As was reported to lower the level of 17β-HSD in mice (Im Chang et al. 2007), thereby impacting the synthesis of testosterone. Another suggested reason behind decreased testosterone secretion in As-treated animals is the Leydig cell atrophy (Zubair et al. 2017), as reported in an experiment conducted on experimental mice (Hazra et al. 2008). Plasma levels of both FSH and LH and testosterone were reduced in As-treated rabbits, too (Zubair et al. 2014). Under the condition of stress, glucocorticoids are released by the adrenal gland due to the stimulatory action of adrenocorticotropic hormone (ACTH) released from anterior pituitary (Vyas et al. 2016). Glucocorticoids such as corticosterone have the ability to regulate testosterone production by inhibiting the testicular LH receptor (Bambino and Hsueh 1981). This could be a probable link behind the deterioration of testosterone synthesis via As-induced stress in animals. It has been shown that low doses of As exposure in humans disrupts sex hormones by stimulating Leydig cell steroidogenesis and inducing urinary steroid secretion (Tian et al. 2021). Using meet-in-metabolite analysis (MIMA) for male infertility, testosterone was found to be a potential biomarker for determining the effects of As exposure on male infertility (Wu et al. 2021).

6.5 Arsenic, Oxidative Stress, and Male Reproduction

Adverse effects of As on male reproduction have largely been attributed to the generation of OS (Im Chang et al. 2007; Dutta et al. 2021). The idea that OS could be a pathological consequence of As toxicity evolved by the 1990s (Flora et al.

2007) – an effect based on the generation of ROS and reactive nitrogen species (RNS) by As (Jomova et al. 2011). It is now known that As induces the production of ROS through the complex I and III of mitochondrial electron transport chain. As-induced ROS include superoxide anion ($O^{2}\bullet-$), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O^2$), and peroxyl radicals. Arsenic disrupts the mitochondrial organization through free radical generation, especially RNS and peroxynitrites (Jomova et al. 2011; Muthumani and Miltonprabu 2012). Another possible route of H_2O_2 production under physiological conditions is the oxidation of As (trivalent +3) to As (pentavalent +5) (Jomova et al. 2011). Furthermore, As can display mitochondrial toxicity by inhibiting the succinate dehydrogenase activity (Hu et al. 2020). In living systems, inorganic As is reduced from As (pentavalent +5) to As (trivalent +3), which are then taken up by hepatocytes, and oxidatively methylated to form monomethyl arsenic acid (MMA) and dimethyl arsenic acid (DMA) (Kim and Kim 2015). Monomethylated and dimethylated arsenicals have numerous cellular as well as genetic toxicities which include elevated OS and oxidative DNA damage (Kim and Kim 2015). Dimethylarsine (an in vivo metabolite of DMA) reacts with molecular oxygen to form radicals and superoxide anions, exposure to which may lead to DNA damage, lipid peroxidation (LPO), and cancer (Flora et al. 2007). In an experimental study, treatment with As for 10 weeks resulted in the generation of ROS in male rats (Flora et al. 2005), whereas treatment of male rats with As for 5 weeks resulted in increased LPO in tissues, indicating the development of OS (Im Chang et al. 2007). In a cross-sectional study, it was reported that subjects who were highly exposed to inorganic As in drinking water for 18 years had increased levels of serum lipid peroxides, also indicating the development of OS (Pi et al. 2002). Moreover, it is postulated that As induces the accumulation of free radicals by interacting with antioxidants and increasing inflammation (Muthumani and Miltonprabu 2012). In vitro studies have also confirmed the generation of free radicals in cell lines exposed to As (Muthumani and Miltonprabu 2012; Lynn et al. 2000) (Table 6.1). It was shown that As treatment for 30 days reduced sperm concentration, motility, morphology, and vitality; decreased testosterone, LH and FSH; and increased malondialdehyde (MDA) in male rats, suggesting the role of OS in deteriorating the reproductive health (Daramola et al. 2018). In another study, As administration to male mice for 35 days resulted in decreased testicular weight, epididymal sperm count, sperm motility, viability, reduced serum testosterone levels, reduced activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase, and increased LPO, indicating the possible role of OS in inducing reprotoxicity (Reddy et al. 2011). It has been reported that treatment of male mice with As for 40 days results in reduction of sperm motility, viability, mitochondrial membrane potential, sperm functional membrane integrity, total antioxidant capacity (TAC), and increased LPO in testicular homogenates, indicating the role of OS in deteriorating the reproductive abilities of the animals (Guvvala et al. 2019). It was found that administration of inorganic As for 40 days in male mice resulted in dose-dependent decrease in the sperm cell motility, viability, plasma membrane functional integrity, mitochondrial membrane potential, and serum testosterone levels. It was observed that doses of As above 50 ppm were testicular toxicant, suggesting its reprotoxicity through the generation of OS (Guvvala et al. 2016). These evidence clearly indicate the possible role of OS in aggravating As-induced reproductive toxicity.

6.6 Oxidative Stress and Sex Hormones: Connecting Link in Male Infertility

The role of OS is well established in the deterioration of sperm quality (Cocuzza et al. 2007). It was reported that the TAC has negative correlation with sperm concentration, affirming the role of OS in deteriorating sperm quality (Appasamy et al. 2007). OS has also been reported to cause LPO, which leads to oxidation of membrane lipid components, disruption of mitochondrial mem-

Table 6.1 Experimental evidence highlighting the role of arsenic (As) in inducing oxidative stress in various biological samples

Type of study	Experimental model	Species of arsenic	Dosage	Duration	Effect(s)	References
In vivo	Male mice	Sodium arsenite	20, 40 mg/L (drinking water)	35 days	Lipid peroxidation in testicular tissues, indicating OS	Im Chang et al. (2007)
In vivo	Male mice	Sodium arsenite	4 ppm (drinking water)	35 days	Decreased testicular weight, sperm quality, serum testosterone levels, reduced activities of SOD, CAT, increased LPO indicating OS	Reddy et al. (2011)
In vivo	Male mice	As(V)	10, 25, 50, 100, and 200 ppm (drinking water)	40 days	Reduced sperm quality, TAC; increased testicular LPO	Guvvala et al. (2019)
In vivo	Male rats	Arsenic trioxide	3 mg/kg body weight (oral)	30 days	Reduced sperm quality, decreased testosterone LH and FSH, increased MDA suggesting generation of OS	Daramola et al. (2018)
In vivo	Pregnant rats	Sodium arsenite	2 mg, 4 mg/kg body weight (Oral)	–	Generation of ROS	Chandravanshi et al. (2018)
In vitro	Mice zygotes	Arsenite	8 μg/mL	2 hours	Generation of ROS	Liu et al. (2003)
Cross-sectional	Human	Inorganic arsenic	0.41 mg/L (mean value) (drinking water)	18 years	Higher levels of lipid peroxides	Pi et al. (2002)

Experimental evidence showing the effects of As in the generation of ROS or OS in the body. Most of the animal model studies have affirmed the potential role of As as an inducer of cellular stress.
CAT catalase, *DMA* dimethylarsinic acid, *GSH* glutathione, *SOD* superoxide dismutase, *LPO* lipid peroxidation, *OS* oxidative stress, *ROS* reactive oxygen species, *TAC* total antioxidant capacity, *MDA* malondialdehyde, *OS* oxidative stress, *ROS* reactive oxygen species

brane potential, protein phosphorylation, impairment of acrosome reaction, and apoptosis (Sharma et al. 2017; Barati et al. 2020). Environmental toxicants have the ability to affect male infertility by inducing OS (Roychoudhury et al. 2019), and OS has been related with the imbalance in sex hormones (Roychoudhury et al. 2021). In a prospective case-control study, men suffering from hypertension showed higher incidence of OS and lower level of testosterone (Onwubuya et al. 2018). Under stressful conditions, human body produces cortisol and norepinephrine which are responsible for the increased ROS in the body (Flaherty et al. 2017). This may also affect the action of glucocorticoids on the Leydig cells of testes, ultimately decreasing the concentration of testosterone (Darbandi et al. 2018). Corticosterone has been asserted to supress the sensitivity of gonadotroph cells (which secrete LH and FSH) to GnRH, thereby lowering the secretion of these hormones (Jana et al. 2006). Similar results were obtained in goats treated with As. The serum concentration of cortisol was found to be enhanced post treatment with As while there was a reduced concentration of testosterone, LH, and FSH – carving out the effect of stress-induced release of glucocorticoids and subsequent reduction of sex hormones in As-treated animals (Zubair et al. 2016; Zubair et al. 2020). Cortisol also enhances the apoptotic potential of the Leydig cells and negatively affects the production of LH through a crosstalk between HPA and HPG hormonal axes. Reduction of LH also contributes towards the failure of the Leydig cells to produce testosterone (Darbandi et al. 2018). In another experiment with male rats, it was found that administration of 40 ppm of As for 28 days affects reproductive

organs causing a decline in sperm quality and testosterone level – OS being one of the causative phenomena in this case (Khan et al. 2013). It was also shown that exposure of male rats to As at 5 mg/kg body weight for 28 days results in reduced testicular weight, deterioration in sperm quality, and reduction in plasma levels of LS, FSH, and testosterone, while increasing the OS markers (Sumedha and Miltonprabu 2014), suggesting a role of As in reducing sex hormones by inducing OS. In another experiment with male rats, it was shown that treatment with 8 mg/kg sodium arsenite for 8 weeks caused a reduction in testicular organization, LH, and testosterone level in the animals (Soleymani and Hemadi 2007), probably through the generation of OS-induced cortisol (Zubair et al. 2017). Thus, accumulating evidence suggest that As can aggravate sex hormone imbalance and associated male reproductive disorders by the induction of OS in the exposed men. The most probable pathway of As action on sex hormones is via stress-induced release of cortisol in humans, followed by its effect on the hormonal axes which leads to diminished testosterone synthesis apart from direct disturbance to sperm and semen parameters (Fig. 6.3).

6.7 Conclusions and Future Perspectives

Arsenic is a common environmental toxicant which leads to various hazardous changes in the physiological parameters of living organisms. Since the concentration of As exposure in some

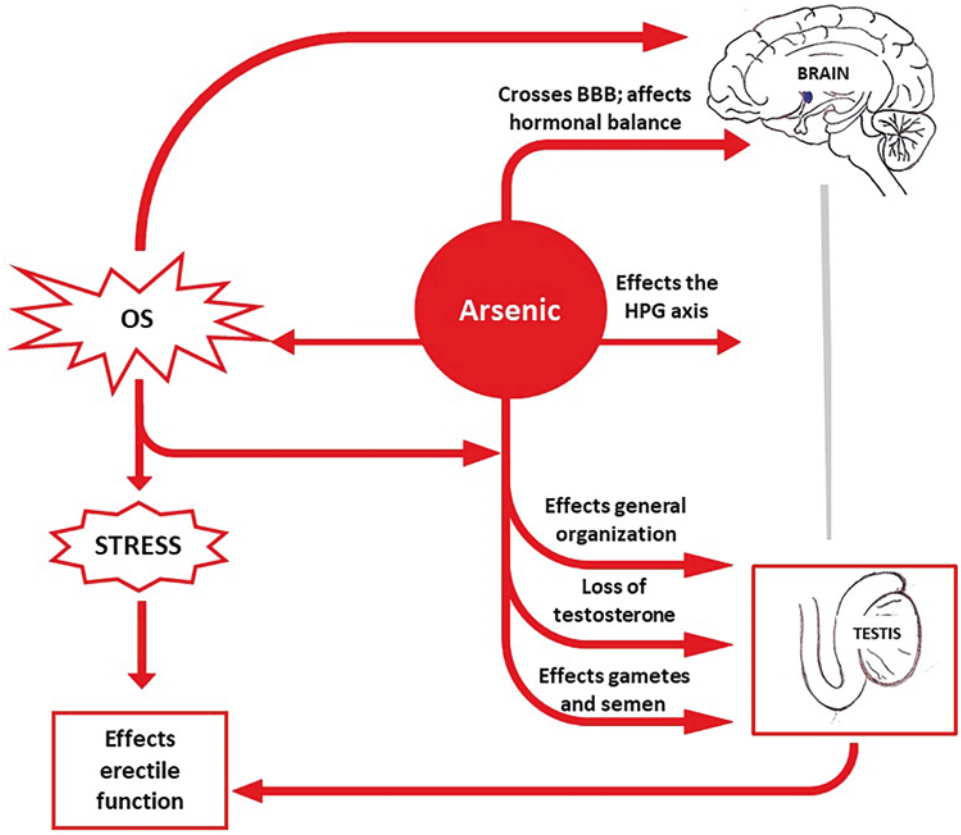

Fig. 6.3 Effect of arsenic (As) on male reproduction. As interferes with the hypothalamic-pituitary-gonadal (HPG) axis, thereby affecting the sex hormones. Oxidative stress (OS)-induced effects on sexual functions apart from the modulation of sperm and semen quality parameters are shown. BBB, blood-brain barrier

of the geographical regions is higher than the normal range, proper assessment and removal techniques for As is of utmost necessity. Arsenic affects the reproductive system via different pathways, and hence, it is important for the andrology clinics to diagnose the effects of As in the reproductive system. Apart from these, As can alter the antioxidant defence system of the body, exaggerating the effects of ROS or OS in the system. Owing to its multiple side effects, proper diagnosis of As contamination is a necessity. It is metabolized from blood within few hours, and hence, analysis of blood As levels is not a reliable indicator of As exposure. In contrary, most of the As is excreted in the urine, and hence, measurement of urinary As levels is considered as a reliable marker for assessing As exposure. Sex hormone evaluation in these subjects may even throw light upon the impact of As-induced OS. Also, certain urinary metabolomic biomarkers which have a significant association with As exposure have been identified from human subjects such as testosterone, guanine, hippurate, acetyl-N-formyl-5-methoxy kynurenamine (AFMK), and serine. Coupling these techniques may assist in the proper diagnosis and risk assessment policies for preventing As contamination in men.

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A Perspective on Reproductive Toxicity of Metallic Nanomaterials

7

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Abstract

Nanotechnological tools have been greatly exploited in all possible fields. However, advancement of nanotechnology has raised concern about their adverse effects on human and environment. These deleterious effects cannot be ignored and need to be explored due to safety purpose. Several recent studies have demonstrated possible health hazard of nanoparticles on organism. Moreover, studies showed that toxicity of metallic nanomaterial could also lead to reproductive toxicity. Various deleterious effects have demonstrated decreased sperm motility, increased abnormal spermatozoa, altered sperm count, and altered sperm morphology. Morphological and ultrastructural changes also have been reported due to the accumulation of these nanomaterials in reproductive organs. Nonetheless, studies also suggest crossing of metallic nanoparticles through blood testes barrier and generation of oxidative stress which plays major role in reproductive toxicity. In the present study, we have incorporated updated information by gathering all available literature about various metallic nanomaterials and risk related to reproductive system.

Keywords

Histopathological changes · Reactive oxygen species · Teratogenicity · Fertility · Iron oxide nanoparticles · Gold nanoparticles

7.1 Introduction

Nanotechnology deals with the particles of size less than 100 nanometers (nm) at least in one dimension. Nanotechnology includes synthesis, exploitation, and handling of nanomaterials (NMs) (Hoyt and Mason 2008) due to their unique features. Nanoparticles are being employed in various fields such as medicine, cosmetics, and industrial purposes (Gaharwar et al. 2019).

However, the huge concern that has arisen is because of the extraordinary physiological properties of the nanomaterials which resulted in their increased used in different applications. Nanoparticles are highly reactive as they possess high surface area-to-volume ratio which ultimately results in adverse effects in organism (Oberdorster et al. 2005). There are several means through which nanoparticles may come in contact with human lives and ecosystems such as water, food products, commercial applications, and medicinal uses (Gaharwar et al. 2019). Medicinal and therapeutic uses of nanomaterials lead to their distribution in different organs

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through blood circulation. Apart from these, during production, recycling, and disposal, nanoparticles may get released into the environment (Roy et al. 2014).

In addition, nanomaterials have been reported to induce reproductive toxicity (Liu et al. 2007; Srám et al. 1999; Yokota et al. 2013). Reproduction and fertility are related to the species sustainability. Reproductive toxicity has drawn the public attention toward engineered nanoparticles and their adverse effects on animals (Hougaard et al. 2015). Metallic nanomaterial is known for the generation of oxidative stress and inflammation to which reproductive organs are sensitive (Azenabor et al. 2015; Das et al. 2016; Walczak-Jedrzejowska et al. 2013). Increased oxidative stress has been recognized as major source of damage in reproductive organs and resulted in infertility due to reduced spermatogenesis (Bisht et al. 2017; Han et al. 2016). Previous studies have shown that various metallic nanoparticles, such as silver nanoparticles (AgNPs) (Ema et al. 2016 & Opris et al. 2019), titanium dioxide nanoparticles (TiO2NPs) (Meena et al. 2014), and cerium oxide nanoparticles (CeNPs) (Qin et al. 2019) induce oxidative stress in male reproductive organs. CeO_2 NPs treatment reduced synthesis of testosterone hormone and impaired maturation of sperm (Qin et al. 2019). CeO_2 NPs were also reported to be found in the testicles and epididymis in rats after inhalation, but the reproductive outcomes associated with CeO_2 NPs accumulation in the testis were not assessed (Geraets et al. 2012). Now, infertility has become a major issue among young individuals; however, possible reason behind this is unclear till date (Lan and Yang 2012). Moreover, exposure to engineered nanomaterial could pose threat as their release in the environment increased the availability. Nanomaterials have been reported to cross blood testes barriers too which raises the issue about their safety (Gaharwar et al. 2019; Shittu et al. 2018). Therefore, reproductive toxicity has raised concern among researchers to demonstrate the potential adverse effects of metallic nanomaterials (Campagnolo et al. 2012; El-Sayed et al. 2015; Hougaard et al. 2015; Kadar et al. 2013). However, little information is still available about them, especially in mammals (Ema et al. 2010), and mechanisms of toxicity in reproductive system is yet to be understood. In this study, we have incorporated probable toxicity of metallic nanoparticles along with their possible mechanism.

7.2 Manganese Nanoparticles (Mn NPs)

Manganese (Mn) is naturally present in many foods and is an essential trace element. It is important for the development and normal functioning of the brain (Elder et al. 2006) but affects its function when present in excess. Tissues rich in mitochondria like the liver, muscles, brain, etc. attract Mn. It also functions as a co-factor for metalloenzymes such as superoxide dismutase (SOD), glutamine synthase, and pyruvate carboxylase (Erikson and Aschner 2003). These enzymes require Mn for their function, but its excess quantity has been reported to inhibit their function. For example, in the central nervous system, glutamic acid is converted to glutamine by the enzyme glutamine synthetase, but in the presence of excess Mn, the enzyme is inhibited (Normandin and Hazell 2002).

Manganese oxide has attracted widespread research interest due to its distinct chemical, physical, catalytic, electrical, and magnetic properties that is different from its bulky counterpart (Wei et al. 2011; Song et al. 2013). Manganese oxide nanoparticles represent a prospective nanomaterial that has shown significant potential in the areas of ion exchange, molecular adsorption, biological and chemical sensing, energy storage, catalysis, magnetic data storage, magnetic resonance imaging, targeted drug delivery, and as an antimicrobial agent (Deng et al. 2013; Miyamoto et al. 2015; Kumara et al. 2014; El-Deab and Ohsaka 2006; Li et al. 2006; Frey et al. 2009; Estelrich et al. 2015; Haneefa et al. 2017). Despite the potential benefits of manganese nanoparticles (MNPs), its adverse effect on animal health is a cause for concern (Pardhiya et al. 2020; Singh et al. 2013a). Thus, understanding the toxicological characteristics of MNPs is cru-

cial prior to its clinical translation. Discharge from factories that produce steel and non-steel alloy, colorants, battery, catalysts, and metal coatings contain high concentration of Mn. Airborne particles containing Mn is also an occupational and environmental problem. The Mn content of the NPs could reach the brain through the airways from breathing and may result in brain damage (Sárközi et al. 2009). Many studies have reported that MNPs have the potential to induce neurotoxicity that could cause neurological syndromes like Parkinson's disease (Karmakar et al. 2014; Singh et al. 2013a; Máté et al. 2016). The gastrointestinal tract is another important portal of MNPs entry. MNPs have also been shown to cause serious pathological risks to hepatic, renal, spleen, blood, reproductive, and developmental systems (Singh et al. 2013a, b; Hussain et al. 2005; Pardhiya et al. 2020; Yousefalizadegan et al. 2019). MNPs have also been reported to induce toxicity to intestinal epithelial cells (Fredericks et al. 2020). In the last decade, many researchers have studied the reproductive toxicity of MNPs.

7.2.1 Effect of Mn NPs on Male Reproductive System

In another study, gonadotoxicity of MnO_2 nanoparticles was examined upon oral administration via gastric tube in male Wistar rats (Zaitseva and Zemlyanova 2019). In this study, rats were divided into five groups, where the first group received 257.7 mg/kg, group 2 received 51.54 mg/kg, group 3 received 10.3 mg/kg, group 4 received 5.15 mg/kg, and group 5 was the control group that received distilled water. The nanosuspension of MnO_2 was administered once a day, for 90 days. After the treatment period, rats were euthanized and their sperm were examined for any alterations. Sperm from rats of group 1 and 2 showed 2.18 and 1.7 times decrease in quantity, respectively, as compared to the control rats, while no significant alterations in sperm count was found for the rats in groups 3 and 4. They also found that sperm from rats of groups 1 and 2 showed a significant decrease in osmotic and acidic resistance (1.2–1.3 times) as compared to the control group. Changes in head, neck, and tail morphology in groups 1 and 2 were 1.7–10.3 times more frequent than those from control rats. Thus, only high doses of 51.54 mg/kg and 257.7 mg/kg MnO_2 nanosuspension exerted significant changes in sperm parameters of rats.

Negahdary et al. (2015) administered Mn_2O_3 nanoparticles orally to rats at different doses (100, 200, and 400 ppm) for 14 days. He found a significant decrease of LH, FSH, and testosterone in the serum of 400 ppm administered rats. They also reported a significant decrease of spermatogonial cells, primary spermatocytes, and spermatid cells in the testis of the 400 ppm group of rats. The histopathological evaluation of the rat testis showed that the 400 ppm dosage of the nanoparticles led to an increase in cellular disruption, interstitial edema, and vacuole formation in the seminiferous tubules. They also observed increased interstitial space between the seminiferous tubules and decrease in the epithelium diameter.

To study the plausible reproductive toxicity of MnO_2 micro- and nanoparticles, Yousefalizadegan et al. (2019) exposed adult male Wistar rats to the MnO_2 particles via subcutaneous injection. They injected the micro- and nanoparticles at 100 mg/kg dose to rats once a week, for 4 weeks. After the experimental duration, they found that the weight of reproductive organs such as testis, epididymis, and prostrate did not alter significantly in the treated rats as compared to the control rats. There were also no significant alterations in the diameter of left testis. However, they reported that the sperm count decrease was highly significant in both the micro- and nano-MnO_2 treated groups. The treated groups also showed 100% immobile sperm. The decrease in the number of spermatocytes and spermatogonia in treated groups was highly significant as compared to control group. Serum concentrations of testosterone, estradiol, and follicle-stimulating hormone did not alter significantly among the groups.

Zhang et al. (2020) examined the reproductive toxicity of Mn_3O_4 on 6- to 7-week-old Sprague-Dawley rats. They administered 10 mg/kg

nanoparticles intravenously to the rats once every week for 0, 60, and 120 days. After the experimental duration, they found that the Mn content in the serum and testis increased significantly after 60 and 120 days of the NP administration. Serum testosterone levels decreased significantly in 60 and 120 days groups, whereas follicle-stimulating hormone decreased significantly only in 120 days exposure group as compared to the 0 day group. There was no significant alteration in the luteinizing hormone among the groups. Sperm count and percentage motility decreased significantly and percentage sperm abnormality significantly increased only in 120 days exposure group. They also found significant reduction of the antioxidant enzyme SOD and significant increase in the lipid peroxidation parameter, i.e., malondialdehyde (MDA) after 120 days exposure period. Histopathological assessment of testis from rats of 60 days exposure period revealed that the germinal layer in seminiferous tubules decreased and some Sertoli cells and spermatogenic cells were separated from basal membrane. The 120 days nanoparticle exposure revealed severe damage to the testis. They observed significant decrease in germinal layer with degeneration of seminiferous tubule such as germ cell degeneration, sloughing, atrophy, structural shrinkage, and increased interstitial space between the tubules.

Our group has also studied the toxic effects of MnO_2 nanoparticle on the reproductive organ, liver, and kidney and their function on rats (Pardhiya et al. 2020). The adult male Wistar rats were administered with the NPs (30 mg/kg) every alternative day for 45 days. At the end of the experiment, morphometric analysis of the testes showed that its seminiferous tubule height decreased insignificantly and germinal epithelial height decreased significantly. Various alterations like formation of vacuoles, desquamation of epithelial cells, and loss of tubular morphology of seminiferous tubules was observed. Sperm count in the NP-treated rats decreased significantly and sperm damage increased significantly. Treated rats also showed different abnormal sperm head morphology like amorphous head, banana-shaped head, and bent head. The NP-treated rats also showed significant variations in liver function tests and histopathological observations of liver and kidney. Additionally, in the presence of a physical stressor, radiofrequency radiation (RFR), there was a synergistic response in sperm damage.

7.2.2 Effect of Mn NPs on Embryotoxicity and Teratogenicity

Effect of MnO_2 NPs on embryotoxicity and teratogenicity was examined by the oral administration of the NPs dispersed in water to male and female Wistar rats (Zaitseva and Zemlyanova 2019). Male and female rats were made to mate during two estrous cycles. Nanosuspension of MnO_2 was administered via gastric tube to female rats from the first day of pregnancy to the twenty-first day, once daily in two doses, 0.25 and 2.5 mg/kg, while the control group of rats received distilled water. During the experimental period, the female rats had normal motion activity, reaction to external irritants and feeding of forage was normal, visible mucous tunic was physiologically colored with no discharge, and they had normal body weight, appearance, and behavior as those of control group. The pregnant female rats were euthanized on the twenty-first day of pregnancy, and embryotoxicity and teratogenicity were examined. The embryotoxicity parameters like the number of implantation points, viable fetuses, and number of resorptions of the pregnant rats did not alter significantly from those of the control group. Teratogenic effects of the MnO_2 nanoparticles were examined. Fetuses did not show any external congenital malformations, and no discrepancies in the body weight and cranio-caudal body dimensions were observed in the treated and control fetuses. No changes were observed in the morphology of fetus internal organs or skeletal system in the treated groups. Thus, nanodispersed MnO_2 did not show any embryotoxic or teratogenic effects upon intragastric administration at 0.25 and 2.5 mg/kg doses.

7.2.3 In Vitro Toxicity of Mn NPs

Upon in vitro assessment of Mn_3O_4 NP on TM4 cells, reactive oxygen species (ROS) increased with the increasing concentration of the NPs from 0 to 20 μg/ml (Zhang et al. 2020). The authors also reported alterations in the integrity of mitochondrial membrane of TM4 cells when exposed to the nanoparticles, suggesting that Mn_3O_4 nanoparticles may induce collapse of the mitochondrial membrane potential. Furthermore, they reported increased apoptotic rate in TM4 cells with increase in the nanoparticle concentration.

7.2.4 Mechanism of Mn NPs Toxicity

In order to assess the mechanism of Mn_3O_4 NPs toxicity to the testis of rats, transcription profiling analysis of the testis from the control and treated rats was done (Zhang et al. 2020). Mn_3O_4 NPs treatment resulted in upregulation of few genes, which was further enhanced upon 120 days of treatment. Based on Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, they reported that after 120 days of treatment, four pathways were activated based on the upregulated genes, i.e., PPAR (peroxisome proliferator-activated receptors) signalling pathway (genes Fabp1, Apoa2, Apoa3, and Pck1 upregulated), steroid hormone biosynthetic pathway (genes LOC100361547, Cyp2c12, Cyp2c6v1, and Ugt2b37 upregulated), xenobiotic metabolism by cytochrome P450 (genes Gsta4, LOC102550391, Ugt2b37 and Sult 2a2 upregulated), and drug metabolism by cytochrome P450 (genes Gsta4, LOC102550391, Ugt2b37 upregulated). The authors have proposed a mechanism for the toxicity induced by Mn_3O_4 NPs based on these findings (Fig. 7.1).

7.2.5 Effect of Mn NPs on Fertility

Effect of Mn_3O_4 NPs on the fertility of rats was studied (Zhang et al. 2020). The authors reported that the fertility rates, fetus numbers, and rate of live fetus did not differ significantly in the 60 day exposure group of rats as compared to the 0 day exposure. However, after 120 days of nanoparticle exposure, these parameters decreased significantly as compared to 0 day exposure.

7.3 Silver Nanoparticles (AgNPs)

For thousands of years, silver and its compounds have been used as antibacterial and therapeutic agents (Alexander 2009; Barillo and Marx 2014). Silverwares were used to store food, water, and wine to prevent spoilage by the ancient Romans and Greeks. Silver preparations were used to treat ulcer and for wound healing by Hippocrates. Silver nitrate was used for wound care and as an instrument disinfectant. Sims, in 1852, used fine wires of silver to suture the vesicovaginal fistulas caused by delivery and found that it decreased infection significantly. Silver preparations to treat wound infection and burn care were developed at the beginning of nineteenth century. Silver nanoparticles (AgNPs) thus have several medical applications like antimicrobial (Salomoni et al. 2015, 2017; Paredes et al. 2014; Rónavári et al. 2018; Kumar et al. 2017; Sun et al. 2005) and anticancer agents (Gurunathan et al. 2015, 2018; Al-Sheddi et al. 2018; Yuan et al. 2017; Zielinska et al. 2018; Fard et al. 2018; Ahmadian et al. 2018; Tavakoli et al. 2018; Kovács et al. 2016), dental applications (Oei et al. 2012), bone healing and wound repair promoter, enhancement of vaccine immunogenicity (Asgary et al. 2016), anti-diabetic effects (Saratale et al. 2017), and biosensors (Anderson et al. 2017). In spite of these applications, various in vivo studies suggest that AgNPs may be toxic in nature. AgNPs can be distributed in the body through various routes of exposure mainly through ingestion, inhalation, subcutaneous or intravenous injections, and skin contact (Xu et al. 2020). They are absorbed and distributed in systems like the dermis, spleen, digestive, respiratory, nervous, immune, urinary, and reproductive systems (Singh et al. 2017b; Lansdown 2006). Thus, its non-specific distribution may cause toxicities like dermal toxicity, hepatobiliary toxicity, respiratory toxicity,

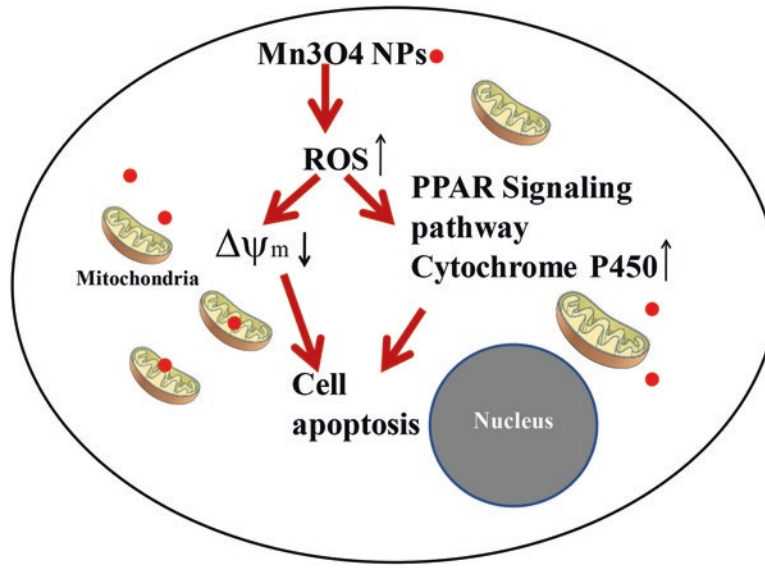

Fig. 7.1 Proposed mechanism of the toxicity induced by Mn_3O_4 nanoparticles

neurotoxicity, ocular toxicity, and reproductive toxicity. The potential of the toxicity depends on route of administration and NP properties like its size, shape, and concentration. AgNPs can cross biological barriers like blood-testis, placental barriers, epithelial barrier and deposit in the testis, epididymis, ovary and uterus. Thus, cells of the reproductive system such as germ cells and related cells like primary follicle, secondary follicle, germline stem cells, and Leydig and Sertoli cells (Ong et al. 2016; Zhang et al. 2015) may be at the risk of damage by these NPs. It may also cause changes in sexual behavior by altering the secretion of reproductive hormones. Studies have reported that the NPs cause reproductive toxicity by increasing inflammation, decreasing the function of mitochondria, downregulating gene expression, inducing the production of ROS, and disrupting DNA structure and apoptosis, and these toxicities depend on size of NPs, dose, and duration of exposure (Zhang et al. 2015; Fathi et al. 2019).

7.3.1 In Vitro Effect of AgNPs

Zhang et al. (2015) reported that 10 nm AgNPs were more toxic to male somatic Leydig (TM3 cells) and Sertoli (TM4) cells than 20 nm sized NPs. The cell proliferation decreased in a concentration-dependent manner from 0 to 100 μg/ml of NPs. The Sertoli cells treated with the NPs showed decreased expression of ZO-1 and Cx43 which encodes tight junction proteins involved in the formation of the blood-testis barrier. However, NP-treated Leydig cells showed decreased expression of StAR, 3β-Hsd, and 17β-Hsd which are involved in testosterone production. Testosterone in turn is needed for induction of spermatogenesis and normal functioning of Sertoli cells. Sertoli cells secrete cytokines that are involved in the proliferation and renewal of spermatogonial stem cells (SSCs). SSCs produce sperm throughout the male's postnatal life. Thus, the study suggested that AgNPs can affect Leydig and Sertoli cell function, decrease SSCs function, and eventually decrease male fertility.

7.3.2 Effect of AgNPs on Male Reproductive System

Lafuente et al. (2016) investigated the effect of oral administration of polyvinylpyrrolidone-coated AgNPs (50, 100, and 200 mg/kg) per day for 90 days on the sperm of rats. They reported abnormality in sperm morphology in a dose-dependent manner. The various abnormalities in

sperm morphology like banana-headed sperm, sperms with bent tail, headless, and neck abnormalities were significantly increased in 100 mg/kg treated rats. Higher doses of NPs showed a progressive but not significant adverse effect on the viability and motility of sperm. Shehata et al. (2021) also showed that 50 mg/kg AgNPs oral administration to rats for 90 days adversely affect sperm count, morphology, mobility, and viability, decrease the levels of reproductive hormones (luteinizing hormone, follicle-stimulating hormone, and testosterone), and induce oxidative stress and lipid peroxidation in testis. The NPs also resulted in DNA degeneration in the testis, and testicular histopathological alterations like thickened testicular capsule, congested blood vessels in capsules and edema of walls, layers of disorganized spermatogonial cells, detachment of germinal epithelium from the basement membrane of seminiferous tubules and mild interstitial edema, degeneration of spermatogonial cells with necrosis, and nuclear pyknosis were observed. The presence of multinucleated giant spermatid cells, defective spermatogenesis, and irregular contour of seminiferous tubules with wide interstitial spaces was also observed. Various other studies have reported the toxic effect of AgNPs on Leydig cells, reproductive hormones, sperm morphology, and mobility in rats (Baki et al. 2014; de Brito et al. 2020). Dziendzikowska et al. (2016) showed that AgNPs had an adverse effect on the hormonal regulation of male rat reproductive system. The NPs altered the sex steroid balance and expression of genes that are involved in steroid metabolism and steroidogenesis. AgNPs have also been reported to alter gene expression in the testis. Intraperitoneal treatment with 50 mg/kg/day AgNPs for 79 days showed alterations in the testicular nuclear transcription factors that are involved in biogenesis and function of mitochondria (Younus et al. 2020). AgNPs treatment resulted in a two-fold increase in the expression of uncoupling protein 2 (UCP2) gene as compared to control. While combined treatment of AgNPs with iron oxide NPs (Fe_2O_3 NPs) increased the expression of UCP 2 to more than five-folds as compared to control. They found a decreased expression of mitochondrial transcription factor A (mtTFA). Its decreased expression may indicate a reduced mitochondria biogenesis and decreased mitochondrial DNA replication and transcription which may lead to mitochondrial dysfunction. Wang et al. (2016a, b) showed alteration in reproductive parameters and gene expression in Balb/c mice exposed to 125 mg/kg AgNPs. The NPs showed accumulation in the testis and alteration in sperm count, histology, and apoptosis in the testis of treated rats. They showed that 383 genes were altered in mice testis significantly after the treatment. These genes were associated with oxidative stress, apoptosis, and other signalling pathways. Apoptosis-related gene (caspase 3, Myc, and Mdm2) expression was increased, thereby explaining high cellular apoptosis in the testis of 125 mg/kg AgNP-treated mice.

7.3.3 Effect of AgNPs on Female Reproductive System

Studies have also reported the effect of AgNPs on female reproductive system. Intravenous administration of AgNPs at 2 and 4 mg/kg once a day, 10 times, showed the inhibition of oocyte maturation in mice (Lytvynenko et al. 2017). A 1 and 5 times administration of the doses resulted in an increased number of apoptotic cells, whereas 10 times administration increased apoptosis and necrosis of follicular cells surrounding oocytes.

7.3.4 Effect of AgNPs on Fertility and Development

AgNPs have been reported to show fetal developmental toxicity (Mozafari et al. 2020). 1 mg/kg AgNPs was orally administered to pregnant mice at gestation days (GD) 1–7, GD 8–14, and GD 1–14 (Mozafari et al. 2020). On GD 15 the uterus was excised. The NPs decreased the fetal body weight and crown-rump length significantly. Various disorders like exencephaly, scoliosis, small head, lordosis, short thorax, short trunk, and fused digits were found in the treated groups. The fetal midbrain showed fibrosis, necrosis, and

apoptosis in groups GD 8–14 and GD 1–14. These results suggest adverse effect of AgNPs in the development of the fetus. A study on the fertility and development of *Drosophila* was done by Ong et al. (2016) at different AgNP concentrations (0–5 μg/ml). The NPs were fed to *Drosophila*, absorbed through ingestion, and accumulated in a dose-dependent manner. The treatment resulted in a decreased viability and delayed development in a dose-dependent manner. The germline stem cells and early germ cells were observed to be concentrated at the apical tip of testis, and a significant level of ROS was reported at this tip in the 5 μg/ml AgNP-treated group. They also showed that AgNP treatment promoted precocious differentiation of germline stem cells which might disrupt its maintenance, thereby resulting in a decreased sperm cell count. They also reported a delayed eclosion or hatching of *Drosophila* and a decreased number of male offsprings in the groups treated with higher concentrations of the NP. Further, they reported a decreased success in mating and number of second and third generations in NP-treated groups, suggesting the possible transfer of AgNPs accumulated in germline stem cells to offsprings which might have affected the development and fertility of the offsprings.

7.3.5 Reproductive Toxicity of AgNPs on Zebrafish

Chen et al. (2017) used zebrafish ovarian follicle as an in vitro model to assess the toxicity of AgNPs and Ag+ on the oocyte maturation. The follicular cells showed vacuolation, swollen mitochondria, and condensed nucleus in the treated follicular cells. The oocytes showed decreased cyclic adenosine monophosphate (cAMP) concentration resulting in resumption of meiosis. Caspases 3 and 9 were upregulated in AgNPs- and Ag+ treated groups, respectively, leading to apoptosis of ovarian follicle cells. Ma et al. (2018) exposed zebrafish to 0, 10, 33, and 100 μg/L AgNPs for 5 weeks and assessed their effect in the reproductive system. Exposure at 33 and 100 μg/L for 5 weeks was reported to decrease the fecundity in female zebrafish significantly. The number of apoptotic cells also increased in ovarian and testicular tissues. The NPs were biodistributed in both the ovary and testis, and increasing ROS levels were reported. The expression patterns of genes involved in mitochondria-mediated apoptotic pathway (bax, bcl2, caspases 3 and 9) were altered to some extent.

7.4 Gold Nanoparticles (AuNPs)

Gold nanoparticles (AuNPs) have optical and electrical properties owing to which it has gained increasing attention in optical, chemical, and biochemical fields. They have been functionalized with different molecules such as peptides, drugs, genes, and other targeting ligands to achieve an improved antiviral and antibacterial function (Burygin et al. 2009). They also have potential application in drug delivery where it could be used to deliver proteins, vaccines, drugs, or nucleotides to their target. They have been studied to overcome bacterial drug resistance by conjugating the antibiotics with AuNPs (Singh et al. 2017a). AuNPs are usually considered safe and thus have been used extensively in cosmetic materials, antimicrobial, and in medical filling material and as drug carriers (Selvaraj et al. 2010). However, studies have shown in vitro and in vivo cytotoxic effects of AuNPs (Jia et al. 2017). Studies in rodents showed toxicity in the liver, kidney, spleen, and sperm (Chen et al. 2013; Fraga et al. 2014; Wiwanitkit et al. 2009). Yahyaei et al. (2019) assessed the toxicity of AuNPs in male Wistar rats. They synthesized 50 nm AuNPs and decided the toxic and non-toxic dose based on in vitro study. They exposed the rats with both doses for 3 days after which they assessed histopathological alterations in the liver, kidney, and testis. They found that at non-toxic dose, there were no alterations in testis and mild alterations in some parts of the liver and kidney. At the toxic dose, they observed mild alterations in all the three organs. They concluded that the toxic response of AuNPs in vitro and in vivo are different and that mild changes even at the toxic dose may be due to shorter duration of exposure and/or the fact that AuNPs used were biologi-

cally synthesized from a fungal strain *Fusarium oxysporum.*

7.4.1 Effect of AuNPs on Male Reproductive System

Wiwanitkit et al. (2009) showed that upon 15 minutes of incubation of human semen with AuNPs, the motility of the sperm decreased (75% motility) as compared to control (95% motility). The NPs were found to penetrate the sperm head and tail regions. AuNPs (2.5–15 nm) also caused swelling and chromatin unpacking in mouse sperm DNA (Skuridin et al. 2010). Balb/c mice were exposed to AuNPs (10–30 nm) at 40 and 200 μg/kg/day for 7 and 35 days, respectively (Nazar et al. 2016). The results showed decreased motility, increased abnormal spermatozoa, and altered sperm morphology in the exposed groups especially in the 35 day exposure group. Other studies also suggest adverse effects of AuNPs on spermatozoa (Taylor et al. 2010, 2012). In contrast, the intramuscular administration of gold core silica shell NPs (70 nm) did not show the presence of NPs in the testis with no morphological alterations in the testis (Leclerc et al. 2015). AuNPs at 1 mg/kg dose for 10 days was reported to cause more alteration in the epididymis of adult rats (6 months old) than in the young rats (1 month old) upon intraperitoneal administration (Kalynovskyi et al. 2016). The mechanism of cellular toxicity was shown by Liu et al. (2020). They observed that the 5 nm AuNPs could enter Leydig cells (TM3 cell line) and induce formation of autophagosomes, thereby increasing ROS production and disrupting S phase of the cell cycle. This resulted in concentration-dependent toxicity of NPs in cells and DNA damage. The AuNPs also inhibited expression of androgen synthesizing enzyme, 17α-hydroxylase, due to which the testosterone production in the cells reduced significantly. Repeated administration of 0.17 and 0.5 mg/kg AuNPs intravenously to Balb/c mice caused retained accumulation of NPs in mice testis. The testosterone level in 0.5 mg/kg group was reduced significantly with a decreased expression of 17α-hydroxylase enzyme in testis. The AuNP treatment also significantly increased the rate of sperm malfunction in the epididymis, but the treatment did not affect mice fertility. AuNPs also alter the levels of reproductive hormones. Behnammorshedi and Nazem (2015) showed that intraperitoneal injection of 25 ppm, 50 ppm, and 100 ppm AuNPs for 10 days increased the levels of luteinizing and follicle-stimulating hormones and decreased the level of testosterone with increase in the NP dose. The testis histopathological analysis showed degeneration of seminiferous tubules at 100 ppm dose. Gupta et al. (2018) studied the biodistribution of AuNPs after the oral treatment of male rats with 20 μg/g for 90 days. They observed the NPs to be accumulated in Leydig cells, interstitial space in testis, and in germ cells. AuNPs were also detected in the cytoplasm of Sertoli cells, and AuNPs entrapped in lysosomes were observed near developing spermatids and cytoplasm of germ cells. The testis histopathology showed mild sloughing of germ cells from basement membrane of seminiferous tubules. A report suggested that laser-synthesized dextran-coated AuNPs are safe for biological use (Bailly et al. 2019). Upon intravenous administration of AuNPs (1 mg/kg) in mice, they accumulated preferentially in the liver and spleen without causing histopathological alterations or inflammation toxicity in the tissues. There was no acute or chronic toxicity in the liver, kidney, and spleen of the mice. However, in this study the dose of AuNPs taken was less, and it was a one-time exposure to the NPs.

7.4.2 Effect of AuNPs on Female Reproductive System

AuNPs have also been reported to cause adverse effects in female reproductive system. Biodistribution of PEGylated AuNPs in the ovary and uterus is size dependent (Poley et al. 2020). When 20 nm, 50 nm, 100 nm, and 200 nm sized PEGylate AuNPs were intravenously injected during estrus stage of female mice, accumulation of 100 nm and 200 nm NPs in ovary was ~two-fold and ~five-fold less, respectively, as compared to 10 nm and 20 nm sized NPs. Similarly, in the uterus, accumulation of 100 nm was

~3.5-fold less and 200 nm was ~12.5-fold less as compared to smaller sized NPs. Granulosa cells in the ovary are involved in steroid synthesis. AuNPs can traverse granulosa cell membrane and certain organelles like lipid droplets and mitochondria.

7.4.3 Mechanism of AuNPs Toxicity on Ovarian Follicle

AuNPs of smaller size can accumulate in granulosa cells of the ovary (Fig. 7.2) and affect hormone secretion. Figure 7.2 shows accumulation of AuNPs in theca and granule cells of follicle. Their accumulation may result in apoptosis of ovarian cell and acceleration of antrum formation. Major NPs are accumulated in the cumulus cell layer that surrounds the oocyte. No NPs enter the oocyte as they are trapped in the zona pellucida layer. AuNP toxicity was shown to induce imbalance in steroid hormone synthesis and ovum dysplasia when the 10 nm AuNPs were engulfed by granulosa cells (Stelzer and Hutz 2009).

The level of estradiol-17 beta was shown to significantly alter after 24 h. Oral administration of AuNPs (20 μg/g/day) for 28 days to zebrafish caused accumulation of the NPs in ovary, histopathological alteration of ovarian tissue, and DNA strand breaks of ovarian cells (Dayal et al. 2017). Mammalian oocytes show different toxicological response to different NPs (AuNPs, AgNPs, and Au and Ag alloy NPs) (Tiedemann et al. 2014). Tiedemann et al. (2014) showed that both AuNPs and AgNPs accumulate in cumulus layers and oocytes, but toxicity of NPs to oocytes increased with increase in the silver molar fraction.

7.4.4 Toxicity of AuNPs to Placental Barriers and Embryonic Development

Transplacental crossing of AuNPs have been reported in experimental animals (Semmler-Behnke et al. 2007; Hougaard et al. 2015). A study showed that intravenously administered AuNPs could enter placental cells via endocytosis (Rattanapinyopituk et al. 2013). However, permeability of NPs also depends on its coating. Rather than crossing the placental barrier, PEGylated AuNPs were found to be aggregated in the syncytiotrophoblast cell layer in human placenta (Myllynen et al. 2008). Fetal exposure to NPs also depends upon the stage of gestational maturation. Yang et al. (2012) showed that three types of AuNP coatings, viz., ferritin, PEG, and citrate, were administered from GD 5.5 to GD 15.5 to the pregnant CD1 mice. Before GD 11.5, all the coating types of AuNPs were detected in the fetal tissue. Thereafter their levels declined in the tissue. This may be due to the maturation of the placental barrier. Overall, out of the three coatings, PEG- and ferritin-coated AuNPs were found to be accumulated to a higher degree than the citrate-coated NPs. AuNPs have generational impact as well where it can disrupt embryonic development. Exposure to AuNPs altered the expression of 19 genes in the human fetal lung fibrolasts (Ng et al. 2011). AuNPs show comparatively lower toxicity than AgNPs (Asharani et al. 2011; Bar-Ilan et al. 2009). Exposure to AuNPs and AgNPs at 3, 10, 50, and 100 nm sizes caused 3% and 100% mortality, respectively, to zebrafish embryo post 120 h fertilization (Bar-Ilan et al. 2009). While AuNPs induced a minimal toxic effect, AgNPs produced a variety of embryonic morphological malformations. Parallel sized AgNPs and AuNPs showed very different toxicity profiles, with AgNPs showing size-dependent toxicity while AuNPs were inert in all the sizes. AgNPs showed concentration-dependent mortality, whereas AuNPs did not increase mortality at higher doses. As both the NPs were accumulated in the embryo, the reason for AgNP toxicity may have been caused by the NP itself or Ag+ formed due to in vivo NP destabilization. On the other hand, Browning et al. (2009) showed that the bioaccumulation of AuNPs in zebrafish embryo increased with increasing concentration but the effect of the NPs in embryo development was not proportional to its concentration. Furthermore, AuNPs synthesized using polyvinyl alcohol as the capping agent did not show any embryonic toxicity (Asharani et al. 2011). The extent of toxicity of AuNPs has been related to its morphology. Spherical AuNPs

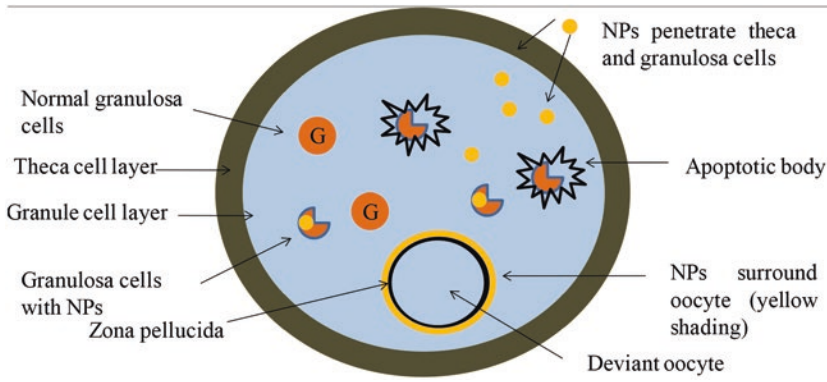

Fig. 7.2 AuNP penetration in developing follicles induces granulosa cell apoptosis and interferes with oocyte maturation

have been reported to be more toxic to zebrafish embryos than the rod- or polyhedron-shaped AuNPs (Wang et al. 2016a, b).

7.4.5 Mechanism of AuNPs Toxicity

AuNPs enter cells through endocytosis and accumulate. These NPs are phagocytosed by lysosomes which result in accumulation of AuNPs in lysosomes. This increases the lysosomal pH and makes it alkaline, impairing its degradation capacity. This process can induce autophagy. Previous studies have shown that AuNPs induce autophagy in germ cells. There was an upregulation of LC3, a autophagy involved protein and downregulation of P62 indicating the impediment of autophagosome degradation (Ma et al. 2011).

7.5 Titanium Nanoparticles (TiNPs)

TiNPs have tremendous application such as white pigment in paint, in ceramics, in sunscreens, as food additive and packaging material, in cosmetic creams, and in surgical implants. Due to its radical generating property, TiO_2 NPs are being used as an antimicrobial agent in paints (Kaiser et al. 2013). They are also used in environmental decontamination of water, air, and soil (Choi et al. 2006; Tran and Webster, 2009; Besov et al. 2010; Shi et al. 2013), anti-fogging materials, as well as in sanitization and disinfectant products in hospitals (Krystek et al. 2014). It has also been studied for therapy in dermatologic diseases such as acne vulgaris, condyloma acuminata, hyperpigmented skin lesions, and atopic dermatitis (Wiesenthal et al. 2011). However, toxicological reports of TiNPs in in vitro and in vivo models have raised concerns on human impact of TiNPs. Several studies suggest its adverse effects on the brain, liver, bone marrow, RBC, sperm, testis, ovary, and embryo development (Bakare et al. 2016; Jia et al. 2017; Morgan et al. 2017; Ma et al. 2010; Li et al. 2009; Ze et al. 2014; Hu et al. 2010; Solaiman et al. 2020; Ali et al. 2019).

7.5.1 Effect of TiNPs on Male Reproductive System

The effect of different concentrations of TiO_2 NPs (1, 20, and 100 μg) on buffalo spermatozoa was observed (Pawar and Kaul 2014). The NPs were internalized by the sperm head and cytoplasm. They found that after 6 h of exposure, there was a significant decrease in cell viability and membrane integrity of sperm. At higher doses, a significant increase in sperm capacitation was observed. There was a dose-dependent increase in the DNA fragmentation of sperm. In vivo study of the effect of intraperitoneally administered anatase TiO_2 NPs (2.5 and 5 mg/kg) for 3 days in mice showed accumulation of nanomaterial in mice scrotum (Smith et al.

2015). This led to testicular histopathology and affected the sperm maturation and function in the epididymis after 4–8 days (but not 10 days to 5 weeks) postinjection. In the treated rats, there were several sperm abnormalities like flagellar abnormality, inability of sperm acrosome to react, excess residual cytoplasm, reduced motility, and increased ROS levels. Another study was done by Bakare et al. (2016) in mice at 9.38, 18.75, 37.5, 75, and 160 mg/kg body weight of animal administered intraperitoneally for 5 consecutive days. They found increase in number of abnormal sperm. The testes showed histopathological alterations such as vacuolation, necrosis, and congestion of interstitial edema. Gao et al. (2013) also reported lesions in the testis and sperm, decrease in sperm count and motility, imbalance of sex hormones, and alteration of 254 genes (153 genes upregulated and 101 downregulated) in the testicular tissue of mice after 90-day intragastric administration of TiNPs.

7.5.2 Biphasic Effect of TiNPs on the Sperm and Testis

A study showed a decrease in sperm motility after intravenous administration of TiNP (10 and 50 mg/kg) to male C57BL/6 J mice (Miura et al. 2019). They showed the adverse effect of short-term exposure of TiNPs to mice. They also administered the NPs orally at 20 or 100 mg/kg to mice. However, in both cases (intravenous and oral administration), they did not find significant reduction in sperm count in the testis and cauda, and thus they hypothesized that the NPs targeted mature spermatozoa. In an in vitro study, they found that TiNP reduced sperm motility, incorporation of [^{3}H]-thymidine, and ATP levels. Since long-term exposure studies of TiNPs indicate testicular impairment, they concluded that the effect of TiNPs on the sperm and testis is biphasic, i.e., short-term exposure to TiNPs affects mature sperm by attacking the blood-epididymis barrier and long-term exposure affects the testis by attacking the blood-testis barrier (Fig. 7.3).

Morgan et al. (2017) showed the long-term oral exposure effect of TiNPs on rats. They exposed rats with 100 mg/kg TiNPs for 8 weeks daily. The results showed apoptosis in rat testis with edema, sloughing of germ cells, and pyknosis of spermatogonial layer. NPs also decreased the testosterone level significantly, decreased viability, and increased the morphological abnormalities of sperm like presence of deformed and detached heads and curved and coiled tails. The TiNPs significantly increased lipid peroxidation and decreased antioxidant glutathione in testis. This provide insights into oxidative stress induction by TiNPs. The expression of testin gene increased 27.47-folds as compared to untreated rats. Concentration of testin, secreted by the Sertoli cells, is inversely correlated with the cell

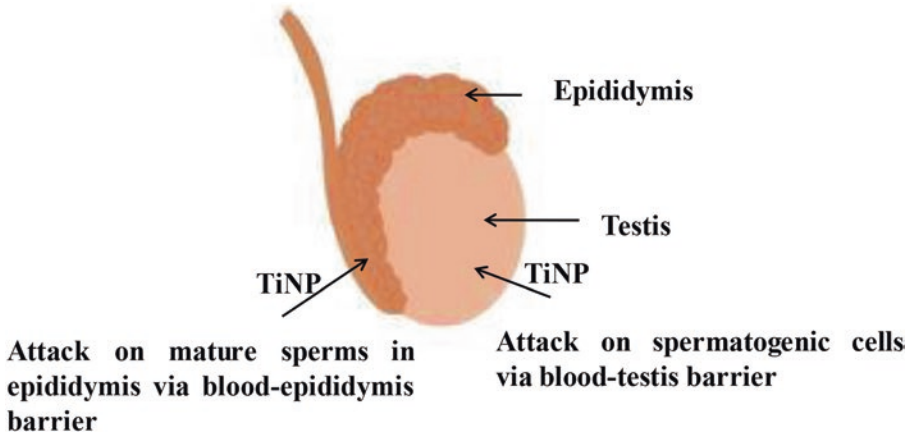

Fig. 7.3 Schematic showing biphasic behavior of TiNPs

junctions in the testis. Thus, increase in the gene expression of the testis may be due to disruption of the testicular cell junction. Moreover, 90 days oral treatment to adult rats with 10 mg/kg TiNPs showed adverse effect on rat reproductive system (Solaiman et al. 2020). Low level of testosterone, sperm count, and Johnson score significantly increased lipid peroxidation and lactate dehydrogenase level, and degenerative alterations in seminiferous tubules of testis were observed in TiNPs-exposed rats.

7.5.3 Effect of TiNPs on Female Reproductive System

TiNPs have also been found to cause adverse effect in female rats (Ali et al. 2019). Immature female Wistar rats were intraperitoneally injected with 50, 100, and 150 mg/kg of TiNPs for 5 days. They found a significant increase in the levels of estrogen and progesterone in 150 mg/kg group. The NPs caused histopathological alterations in the ovary such as loss of Graafian follicles, reduction of granulosa and theca layer thickness and destruction of follicle walls. Higher doses of 100 and 150 mg/kg decreased the number of corpus luteum, growing follicles, and Graafian follicles significantly. Other studies also suggest the adverse effect of TiNPs on reproductive parameters of female rodents (Gao et al. 2012; Tassinari et al. 2014). TiNPs have also been studied for their toxicity to developing embryo. For assessing the toxicity of TiNPs in embryo, the embryos of 8.5 days were isolated from female mice and cultured and incubated with TiO_2 NPs (5–10, 60, and 90 nm) at 0, 50, 100, and 200 μg/ml for 16, 24, and 48 h (Jia et al. 2017). They observed a dose- and time-dependent toxicity of the NPs on the growth and development of embryos. They found that the increasing toxicity like decrease in VXY diameter of embryo, crown-rump length, head length, and number of body sections of embryo and malformation rate was associated with larger particle size. In order to assess the potential toxic effect of TiNPs in pregnant women, Lee et al. (2019) exposed rats orally to 0, 100, 300, and 100 mg/kg from GD (gestation day) 6–19. They found the accumulation of NPs in maternal liver, brain, and placenta. But the NPs did not induce marked toxicities in the maternal rats and did not affect the embryo development. In another experiment on gestational exposure of pregnant mice to TiNPs, the mice were orally exposed to 0, 1, and 10 mg/kg/day TiNPs from GD 1–13 (Zhang et al. 2018). They found no difference in the number of implanted and resorbed embryos and placental weight in the treated groups. However, placental to body weight was reduced in 1 mg/kg dose group at GD13. In the 10 mg/kg dose group, the proportion of spongiotrophoblast was higher than the untreated group, yet the placental labyrinth was significantly lower. The intricate fetal vessel formation was impaired, and the number of uterine natural killer cells was reduced in TiNP-treated rats. The treatment inhibited the proliferation, induced apoptosis in the placenta by nuclear pyknosis with activation of caspase 3, upregulation of Bax, and downregulation of Bcl-2 proteins on GD13. In the TiNP-treated placenta, the expression levels of Exc1, Ascl2, Hand1, Hand2, Eomes, and Fra1 mRNA decreased. Thus, exposure of pregnant mice to TiNPs significantly impaired the growth and development of placenta.

7.6 Cadmium Nanoparticles (Cd NPs)

Nanomaterials containing cadmium have various applications including in electronic, optical, and biological applications (Nirmal et al. 1996; Bowers et al. 2005; Agarwal et al. 2005). Thus, its environmental and human health impacts has become a cause of concern. Toxicity of cadmium selenide NPs (CdSe NPs) is linked to the release of Cd^{2+} which generates ROS and can produce oxidative stress (Kirchner et al. 2005; Haque et al. 2013). Cd NPs have been reported to induce neurotoxicity, hepatotoxicity, and pulmonary and reproductive toxicities (Horvath et al. 2011; Gao et al. 2018; De La Cruz et al. 2019; Blum et al. 2012; Bajaj et al. 2013). Cadmium sulfide (CdS) nanodots are more toxic than nanorods in terms of accumulation in organs, DNA damage, viabil-

ity and abnormality of spermatozoa, oxidative stress, and liver and kidney damage (Liu et al. 2014). CdS nanodots have been reported to be more toxic than microsized CdS (Li et al. 2009).

7.6.1 Effect of Cd NPs on Male Reproductive System

CdS NPs were investigated for its effect in male Wistar rats, where the NPs were intraperitoneally injected at 2.5 mg/rat/day for 30 days (Bajaj et al. 2013). The results showed a reduction in sperm count and motility, fertility index, decrease in testis weight, decrease in testis protein and glycogen, increased cholesterol, and decreased testosterone level. Antioxidants like the enzyme SOD and glutathione significantly decreased and lipid peroxidation significantly increased in the testis. Histopathological observation showed degradation in testicular tissue. Single dose of intraperitoneal administration of dextrin-coated CdS NPs at 100 μg/kg to rats showed quick distribution in the body, with maximum concentration after 72 h in all the analyzed tissues (De La Cruz et al. 2019). Upon continuous administration for 90 days, testis degeneration and chronic lung inflammation were observed.

7.6.2 Effect of Cd NPs on Fertility, Embryo, and Post-natal Development

Blum et al. (2012) performed a study to assess the ability of cadmium oxide (CdO) NPs to reach the placenta and affect the fetus and/or neonate upon exposure of pregnant CD1 mice to CdO NPs. The pregnant mice were exposed every other day to 100 μg (exposure 1) or daily to 230 μg (exposure 2) of CdO NPs/m^3 for 2.5 h from 4.5 to 16.5 days post coitus. The concentration of Cd increased in the uterus, placenta, and other maternal organs but was undetectable in the fetus at 17.5 days post coitus. A decrease in pregnancy incident (i.e., no implantation evidence) by 23%, delay in maternal weight gain, decrease in length of fetus, and delayed neonatal weight gain was observed in exposure 2 group. Inhalation of CdO NPs thus has an adverse effect on reproductive fecundity and fetal and postnatal growth. Yan et al. (2016) investigated the effect of cadmium telluride quantum dots (CdTe-QDs) on gonads of *Bombyx mori*. They injected the organism through dorsal vein with 0.32 nmol of CdTe-QDs. The QDs induced early germ cell death or malformations via mechanisms related to autophagy and apoptosis through lysosomal and mitochondrial pathways. Quantitative analysis of development of germ cells and histological observation of gonads showed that reproductive toxicity was characterized by male sensitivity. The quantity and quality of sperm deteriorated due to QD exposure in early stages of male, which was the main reason that the eggs remained unfertilized. Chan and Shiao (2008) investigated the effect CdSe QD on post-implantation embryonic mice development. They incubated mouse blastocytes in CdSe QDs at 250 and 500 nmol/L for 24 h. They found dose-dependent apoptosis in blastocytes. The QDs induced inhibition of cell proliferation especially in the inner cell mass and inhibition of post-implantation embryo development. Very few blastocysts could reach later stages of development. The QDs also inhibited pre-implantation development of morulas to blastocysts. Furthermore, 500 nmol/L dose group resulted in the resorption of blastocysts and decrease in weight of fetus. Hsieh et al. (2009) investigated the effect of CdSe QDs on mice oocyte maturation, fertilization, and pre- and post-implantation development. The QDS significantly reduced oocyte maturation rate, fertilization, and embryo development (in vitro). 500 nM QDs in vitro treatment resulted in resorption of post-implantation embryo and decreased the fetal and placental weights. Chu et al. (2010) investigated the transfer of CdTe and CdS QDs from pregnant mice to their fetuses. They showed that the QDs could cross the placental barrier and transfer from mice to fetus. Smaller QDs were easily transferred than the larger ones and the number of QDs transferred increased with increase in dosage. Capping the QDs with silica or polyethylene glycol could reduce the transfer of QDs but did not prevent. These results limit

the QDs utility in pregnant women. CdTe QDs may retard hatching of zebrafish and increase the oxidative stress in zebrafish embryos (Tian et al. 2019). Cd NPs can be modified to decrease or delay its toxic effects. For example, carbon-coated Cd NPs reduce toxicity by mitigating the release of Cd^{2+} (Balmuri et al. 2017).

7.7 Conclusion

Nanotechnology is a rapidly expanding field in terms of application and adverse impact on both animals and environment. Its positive impacts are undoubtedly important for the medicinal and industrial sector. However, increasing health hazards due to NM application cannot be overlooked. Therefore, toxicological impacts of NMs need to be explored thoroughly. Safer and sustainable application of nanotechnology cannot be approached without its complete nanotoxicological assessment. Knowledge acquired from the nanotoxicological assessments will allow production of safer and sustainable NMs.

This study discusses that metallic NMs may induce reproductive toxicity. NMs reach the systemic circulation through blood and may get accumulated in reproductive organs (testes) as they are able to cross the blood testes barrier. Bioaccumulation of these metallic NMs lead to the generation of oxidative stress in reproductive organs and cause hazardous effects such as reduced sperm count, sperm mobility and adversely affects hormonal regulation and morphological and ultrastructural changes. Nonetheless, more elaborated studies on reproductive toxicity are needed to demonstrate safer nanotechnological exploitation to benefit mankind. Therefore, by addressing the toxicological concerns of NMs, nanotechnology will be able to be utilized at its most.

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Bisphenol A and Male Infertility: Role of Oxidative Stress

8

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Abstract

Bisphenol A (BPA) is an endocrine-disrupting chemical that is capable of mimicking, antagonizing, and interfering with the normal biological functioning of the endocrine system. BPA is used in diverse industries, hence its vast sources of exposure. Although the half-life of BPA is relatively short (<24 hours), studies have reported its detection in the urine of different populations. It, therefore, became important to investigate its effect on general health, including male reproductive health. The adverse effects of BPA on male fertility have been evaluated and reported from both in vivo and in vitro studies. Up to date, reports from randomized controlled trials remain controversial, as some revealed decreased sperm quality, sperm concentration, and total sperm count, while others reported that no adverse effect was seen after exposure. Findings from animal model studies and in vitro experiments have shown that exposure to BPA led to a reduction in sperm quality and increased sperm DNA fragmentation, and some even revealed altered expression of the gene that encodes gonadotropin-releasing hormone. This shows that BPA not only may adversely affect male fertility by acting as an endocrine disruptor but also can potentially impact male fertility via its possible contribution to oxidative stress. Therefore, this book chapter aims to identify and elucidate the effect of BPA exposure on male fertility, and to as well illustrate the mechanisms through which this occurs, while emphasizing the role of oxidative stress as a potential pathway.

Keywords

Bisphenol A · Oxidative stress · Male infertility · Hormone dysfunction · Endocrine-disrupting chemical

8.1 Introduction

The decline in male fertility has been attributed to diverse etiologies. This includes lifestyle choices (obesity), endocrinological abnormalities (Kallmann syndrome), congenital defects

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(cryptorchidism), idiopathic and genetic anomalies (Y-chromosome microdeletions), and acquired dysfunctions (varicocele) and could as well be caused by endocrine-disrupting chemicals (EDCs) and other environmental toxins (Vander Borght and Wyns 2018; Babakhanzadeh et al. 2020). Numerous types of EDCs have been shown to adversely affect male reproductive health. EDCs are a class of exogenous substances or chemical compounds that interfere with the function of the endocrine system, often exerting estrogen-like and/or anti-androgenic effects, which consequently cause adverse health effects in an intact organism, or its offspring, or subpopulation (Mima et al. 2018; Sharma et al. 2020). These substances, including bisphenol A (BPA), pesticides, and other environmental chemicals, may disrupt normal hormonal stimulatory effect, inhibitory action, or elimination of hormones. Exposure to organophosphates, for instance, a commonly used compound in pesticides, has been associated with abnormal sperm parameters including reductions in sperm counts, motility, viability, increased DNA damage, and abnormal morphology (Krzastek et al. 2021). Several studies have also reported its negative influence on serum reproductive hormones, as a reduction in total testosterone and an increased in luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels were observed following exposure to organophosphates (Mima et al. 2018; Melgarejo et al. 2015). Another compound is cadmium, a heavy metal, which is known to cause toxicity by impacting the hypothalamic-pituitary-gonadal (HPG) axis, testicular function, and spermatogenesis (Rana 2014). Exposure to cadmium can also induce endocrine disruption via interfering with the DNA zinc finger motif and substituting zinc for cadmium subsequently causing a decrease in steroidogenesis (Krzastek et al. 2021; Kumar and Sharma 2019). Another widely used chemical is BPA. BPA is a crystalline chemical compound used as a monomer or plasticizer in the production of epoxy resins and polycarbonate synthesis. It is also used in the production of medical equipment, aluminum cans, thermal papers, toys, food packaging, and jar caps, among others (Fig. 8.1). Due to the vast sources of BPA, more than 90% of people in Western countries have detectable BPA levels in the urine (Castellini et al. 2020). Exposure to BPA has been found to cause reduced sperm count, motility, and normal morphology (Chiang et al. 2017). After exposure to, and absorption of, BPA via inhalation, ingestion or skin contact, and distribution in the body (Fig. 8.1), it directly disrupts the HPG axis by lowering circulating levels of gonadotropins and reduces the expression of the gene that encodes gonadotropin-releasing hormone (GnRH) within cells in the preoptic area. This is caused due to BPA having an affinity to alpha and beta estrogenic receptors (ERα, ERβ), thereby inducing an estrogenic receptor-dependent gene expression which leads to endocrine disruption in the HPG feedback mechanism and, thus, causing hypostimulation and decreased spermatogenesis (Castellini et al. 2020). Additionally, BPA may also impair male fertility by causing imbalance between the generation of reactive oxygen species (ROS) and the antioxidant activities. When this trend persists, oxidative stress ensues. This shows that BPA not only may adversely affect male fertility by acting as an endocrine disruptor but also can potentially impact male fertility via its possible contribution to oxidative stress. Although environmental EDCs generally exist at low concentrations that may cause a negligible impact on general health, daily exposure to these toxins could potentially pose a threat to male reproductive health. Therefore, the purpose of this study is to identify and elucidate the effect of BPA exposure on male fertility and to as well illustrate the mechanisms through which this occurs, emphasizing the role of oxidative stress.

Key Statement

"Several environmental factors are associated with the global decline of male fertility. These compounds are abundant in our modern society, and the daily exposure to these compounds adversely affects male fertility."

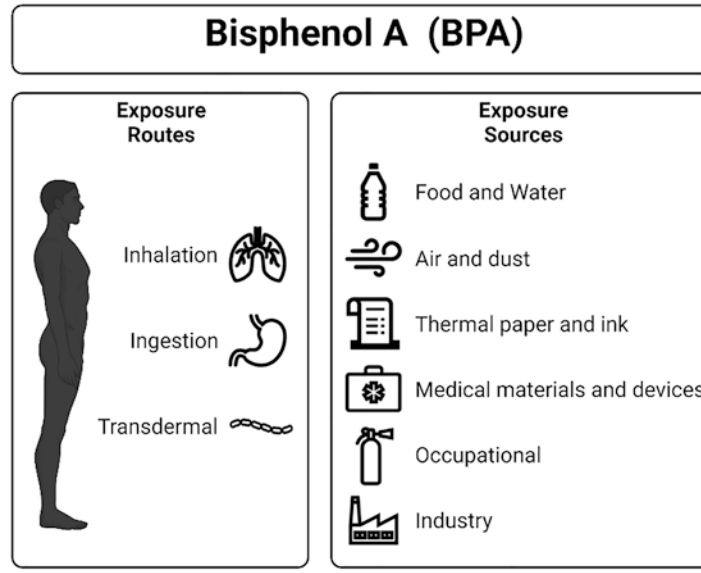

Fig. 8.1 The routes of exposure and sources of bisphenol A (BPA)

8.2 Bisphenol

8.2.1 Overview of Bisphenol

Bisphenols are chemical compounds that acquire two specific hydroxyphenyl capacities. Many of the derivatives of bisphenol are diphenylmethane-based except bisphenols M, P, and S (Liang et al. 2020). Other derivatives of bisphenol are BPA, bisphenol AP, bisphenol AF, bisphenol B, bisphenol BP, bisphenol C, bisphenol C2, bisphenol E, bisphenol F, bisphenol G, bisphenol M, bisphenol S, bisphenol P, bisphenol PH, bisphenol TMC, bisphenol Z, dinitrobisphenol A, and tetrabromobisphenol A. The derivatives are classified based on their reactants. For instance, acetone is the reactant of BPA, the most common derivative of bisphenol (Liang et al. 2020). This chapter will predominantly focus on BPA.

Key Statement
"The classification of the different types of bisphenol compounds depends on their reactant. In comparison to BPA, very limited studies are available on the effect of exposure to other substrates of bisphenol."

8.2.2 Bisphenol A

Approximately nine million tons of BPA are produced worldwide per year (Plasticstoday 2019). BPA is now widely considered as a structural component present in epoxy resin and polycarbonate materials used to manufacture medical devices, water supply pipes, safety equipment, beverage bottles, and food packaging, all of which are items and products that the average individual is exposed to daily (Fig. 8.1) (Castellini et al. 2020). The varied sources containing BPA allow for several different modes of BPA consumption, for instance, oral ingestion from canned foods, inhalation of dust from the air, and transdermal through physical contact (Fig. 8.1) (Loganathan and Kannan 2011). BPA is exceptionally prevalent in consumer merchandise production with approximately 90% of the people in the Western countries having a detectable amount of BPA in the urine, serum, seminal plasma, amniotic fluid, follicular fluid, placental tissue, and umbilical cord blood (Vandenberg et al. 2007). This is partly due to the act of consuming foods that are stored in BPA-containing packaging. The free monomers transfer from the packaging into the food

due to BPA's solubility which is then orally ingested, altering the cellular functionalities and development of the body, which is the leading cause of reproductive damage (Castellini et al. 2020). In recent years, the health risks following exposure to BPA have been examined. The data obtained through standardized toxicological tests displayed evidence that high exposure to BPA affects fertility and fetal development, hormonal levels, neurological and cardiac functionalities, and other physiological aspects of the human body. BPA is a known xenoestrogen, as it mimics estrogen effects due to its characteristic polycyclic phenolic chemical structure, which is similar to estradiol. BPA also affects redox homeostasis by altering the standard equilibrium of oxidative mediators, such as ROS and antioxidant enzymes. This causes the direct induction of cellular dysfunction due to the alteration of cell signaling pathways and activation of apoptosis (Castellini et al. 2020). Although studies have shown that low levels of BPA have no effect on human health, nonetheless, the increased exposure to high levels of BPA through direct and indirect contact causes potential adverse health effects (Gassman 2017).

Key Statement
"BPA has a chemical structure that is similar to estrogen, hence its affinity to bind to the estrogen receptors. Additionally, BPA damages redox homeostasis by altering the standard equilibrium of oxidative mediators, such as ROS and antioxidant enzymes."

8.3 Toxicokinetics of Bisphenol A

Data obtained through toxicological and epidemiological studies presented evidence of high concentrations of BPA in the body causing negative effects on physiological health and developmental capacity. It has been determined that BPA levels from 20 to 400 μg/kg/day and above interfere with normal human physiology (Acconcia et al. 2015). After ingestion, with the aid of uridine diphosphate glucuronosyltransferase, BPA binds to glucuronic acid to form BPA glucuronide (BPA-G) (Fig. 8.2). The same process occurs when BPA binds to sulfonic acid via sulfotransferases, such as phenol sulfotransferase, to form BPA sulfate (BPA-S). The glucuronidation or sulfation of BPA is a rapid process that makes it more soluble in water with a half-life of <24 hours. The toxicokinetic process of BPA can however be influenced by physiological changes (Castellini et al. 2020). The toxic accumulation of BPA is evident as the substance concentration diverts from the regular pharmaceutical range. There is no standard dose that causes detrimental effects in humans, as females and males react differently to specific concentrations of the toxin. Overall, the singular effect of BPA is weak. However, reports indicate that the excessive consumption of the toxin is the leading cause of adverse effects (Acconcia et al. 2015).

Key Statement
"The toxicokinetic process of BPA is rapid. The swiftness of the process makes it more soluble in water, especially when heat is applied. It has a half-life of 5.4–6.4 hours."

8.4 Bisphenol A, Sex Hormones, and Male Fertility

Testosterone is a crucial sex hormone in males as it regulates libido, the production of red blood cells and spermatozoa. Studies have emphasized the importance of preserving a specific level of estrogen in the male body in addition to testosterone to sustain the reproductive capacity. Exposure to BPA decreases the biosynthesis and secretion of testosterone, causing a decline in steroidogenic enzyme expression which affects testosterone production and spermatozoa concentration and quality. Studies have reported the concurrent decrease in testosterone production and an increased levels of FSH and LH following BPA toxicity (Meli et al. 2020).

Estradiol is the predominant hormone derivative of estrogen that plays an important role in maintaining male sexual maturation. During BPA toxicity, the transcription of target genes that are mediated by the estrogen receptor β is affected, as BPA inhibits receptor degradation and ubiquitina-

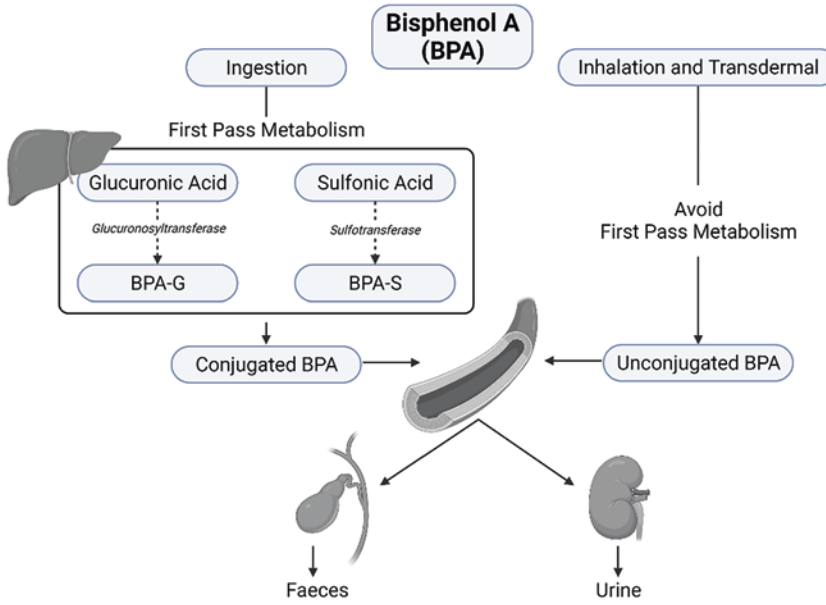

Fig. 8.2 Toxicokinetics of bisphenol A (BPA). Following oral ingestion, BPA undergoes first-pass metabolism in the liver (conjugation phase). Briefly, BPA binds to glucuronic acid with the aid of uridine diphosphate glucuronosyltransferase to form BPA glucuronide (BPA-G). This process is called glucuronidation. The same process occurs when BPA binds to sulfonic acid via sulfotransferases, such as phenol sulfotransferase, to form BPA sulfate (BPA-S), a process termed sulfonation. After glucuronidation or sulfonation, BPA metabolites are excreted either into the bile/GUT and then urine or into the blood and then passed into the kidney to be excreted as urine. The first-pass metabolic phase is avoided when BPA is absorbed through inhalation or transdermal route. This enhances the production of unconjugated BPA in the blood

tion (Masuyama and Hiramatsu 2004). This occurs as a result of BPA affecting the estrogen receptors (α and β) in target cells. The BPA molecule contains specific phenolic structural qualities that allow BPA to bind to the estrogen receptor subtypes α and β by imitating the estrogen receptor features, promoting alterations in cell migration, proliferation, and viability. BPA also stimulates cell growth which has a similar effect to estrogen on the human body, therefore presenting an association between the increased exposure of BPA and the reduced production of sperm and testosterone while prompting male reproductive diseases. Specific levels of the estrogen hormone are required in human males to regulate standard sexual development and fertility. The normal range of estradiol in human males to promote fertility is 10–40 pg/ml (Schulster et al. 2016).

Abnormal levels of the testosterone-to-estrogen ratio provoke damage to important processes such as spermatogenesis and steroidogenesis. The testicular compartments of the two cellular processes are vulnerable to BPA-induced damage due to their functionally similar state. The testosterone hormone is produced during steroidogenesis. BPA-induced oxidative stress alters the synthesis and distribution of the steroid receptors required for the mediation of hormonal activity by binding to the receptors and destroying the steroidogenic enzymes. As a result, the influence of BPA profoundly alters the quality of sperm produced and decreases the likelihood of successful male fertility (Acconcia et al. 2015).

Key Statement

"BPA is a known xenoestrogen as it mimics estrogen effects due to its characteristic polycyclic phenolic chemical structure, similar to estradiol."

8.5 Evidence of Bisphenol A-Induced Male Infertility: The Role of Oxidative Stress

Since the use of BPA in diverse "works of life" keeps increasing, it remains important to continually investigate the effect of its exposure on male reproduction. At present, several studies have pinpointed its adverse effects on male fertility (Li et al. 2021; Liu et al. 2021; Rahman et al. 2021; Mínguez-Alarcón et al. 2021), while some, however, showed no association (Benson et al. 2021). The controversy may be due to differences in methodological approaches, such as in vivo versus in vitro versus ex vivo. To reduce the disparity in findings, some authors have conducted meta-analyses and systematic reviews, establishing a conclusion from a holistic perspective (Castellini et al. 2020; Santiago et al. 2021). This section of the chapter will discuss some studies that have reported the effect of BPA exposure on male fertility (Fig. 8.3).

It has been previously established that BPA has estrogenic and anti-androgenic activity affecting the hypothalamus, which in turn disrupts the hypothalamic-pituitary-gonadal (HPG) axis. The disruption occurs by altering the gonadotropin-releasing hormone (GnRH) pulsatile release, resulting in impairment of adequate secretion of FSH and LH (Santiago et al. 2021). These two hormones play an important role in the male reproductive system, where LH stimulates the Leydig cells and FSH stimulates the Sertoli cells. Leydig cells produce testosterone, and Sertoli cells are responsible for testicular growth and promoting the production of androgen-binding protein. Both of these cells are found in the seminiferous tubules, which are responsible for hosting spermatogenesis and sustaining the maturing spermatozoa (Emedicine.medscape.com 2021). In summation, there is a reduction and/or inhibition of androgen production, as well as a decrease in the number and function of Sertoli cells leading to the degeneration and decline of spermatocytes. In other words, it disrupts the rather complex process of spermatogenesis (Santiago et al. 2021).

Prenatal maternal exposure of BPA when carrying male fetuses has been studied and was found to have an increased risk of urogenital developmental abnormalities. These abnormalities include cryptorchidism, hypospadias, and structural alterations of the testis of male fetuses (Pallotti et al. 2020). Similar to BPA exposure in adults, the main effect of this exposure is related to disruption of the preexisting and well-established hormonal homeostasis. Consequentially, this may hinder the appropriate development of the male genital tract as well as the induction of chronic structural modifications which is partly mediated by oxidative stress through ROS and inflammatory mechanisms (Pallotti et al. 2020). For instance, cryptorchidism may occur due to Leydig cell dysfunction, and it has been suggested that a history of cryptorchidism in boys could potentially increase the risk of fertility issues in the future (Komarowska et al. 2015). It may also be relevant to state that couples with a history of BPA exposure have reported difficulties in conceiving (Komarowska et al. 2015), and this may be attributed to the alteration in the mechanisms involving hormonal homeostasis and histopathological changes in the testicular structure.

Additionally, extensive in vivo and in vitro studies have been conducted to further elucidate the effect of BPA on male fertility. After exposure to BPA, the levels of circulating hormones (testosterone, estrogen) in the animal samples and histopathological changes in the testes were analyzed. It was identified that the circulating hormone levels were altered where testosterone levels decreased and estrogen levels had increased (Jia et al. 2020). Furthermore, the glandular cavity in the BPA group was slightly enlarged, with abnormal morphological changes in the spermatogenic cells and Leydig cells (Jia et al. 2020). It was identified that there is a reduction in the number of spermatozoa, the different phases of spermatogenesis were altered, and there were histopathological changes in the seminiferous tubule as vacuolation and shrinkage of the tubule occurred (Jia et al. 2020). It was also noted that there was a decline in the testicular mitochondrial

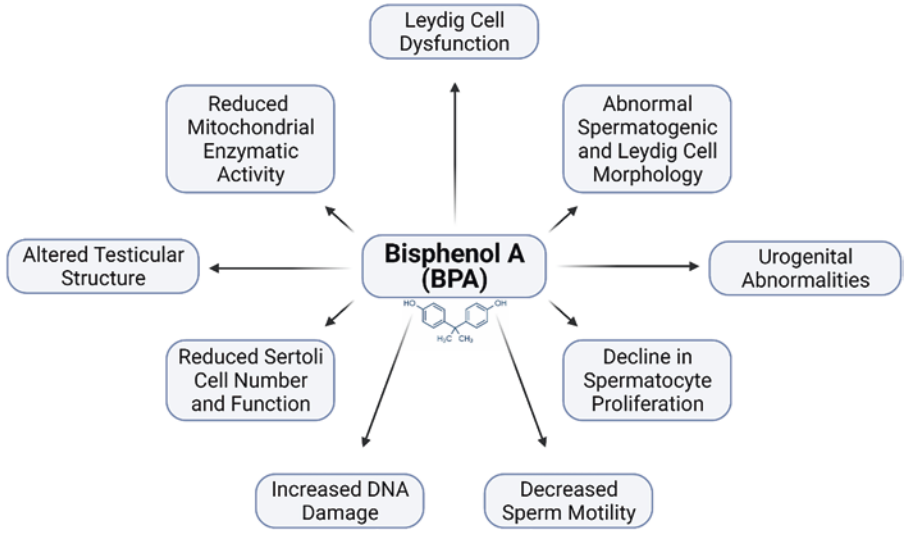

Fig. 8.3 Bisphenol A and male infertility. Exposure to BPA may lead to urogenital developmental abnormalities, decrease in testicular mitochondrial enzymatic activities, altered testicular structure, abnormal morphological changes in the spermatogenic and Leydig cells, increased DNA damage, decreased sperm motility, reduction in the number and function of the Sertoli cells, and decline in spermatocyte proliferation

enzymatic activities such as monoamine oxidase (MOA), NADH dehydrogenase (NDH), malate dehydrogenase (MDH), succinate dehydrogenase (SDH), and isocitrate dehydrogenase (IDH) (Meli et al. 2020; Santiago et al. 2021). Unsparingly, it also reduces the activity of antioxidant enzymes such as superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT), and glutathione peroxidase (GSH-Px) (Meli et al. 2020; Santiago et al. 2021), thus promoting an imbalance that results in oxidative stress (Santiago et al. 2021).

In vitro studies using mice spermatozoa exposed to BPA demonstrated a significant decrease in the percentage of motile spermatozoa, decreased intracellular ATP levels, increased levels of ROS, and impaired epididymal sperm motility and viability potentially attributed to the oxidative stress (Rezaee-Tazangi et al. 2020). Findings from human studies reported mitochondrial dysfunction, reduced sperm motility, and increased oxidative DNA damage (Barbonetti et al. 2016). Furthermore, mitochondrial dysfunction in Sertoli cells may be a resultant of the increased ROS in the testes which propagate DNA damage and cellular apoptosis (Wang et al. 2017).

Exposure to BPA has been demonstrated to be dose-dependent (Rochester 2013), so, in theory, prolonged exposure to BPA and higher concentrations thereof have an increased risk of causing the mechanisms of change described above. Some studies have attempted to evaluate male adults with a known history of BPA exposure in their lifetime with known difficulties in conceiving and without. Nevertheless, there is a variation in these studies according to the dose-dependent exposure that could not be identified (Pallotti et al. 2020). Additionally, the measurements done to identify exposure included urine BPA which has been highlighted to be a suboptimal method of evaluating the exposure to BPA (Konieczna et al. 2015), serum BPA, and seminal BPA levels (Vitku et al. 2015). In comparison to healthy males, infertile males have significantly higher levels of seminal and serum BPA. It was identified that seminal BPA levels were associated with a reduction in the semen parameters (total sperm count and sperm concentration), while this was not true for serum BPA (Vitku et al. 2015). This finding provides an emphasis on the significance of seminal BPA levels which may aid and be a future focus in upcoming research attempting to approach this

issue. Furthermore, BPA exposure was found to be associated with increased serum prolactin levels in males (Liu et al. 2015), and this has a detrimental effect through inhibiting pulsatile GnRH secretion which as a result inhibits the release of FSH and LH (Dabbous and Atkin 2018). This will negatively affect and impact testosterone levels and the process of spermatogenesis. Furthermore, another study analyzed the urine samples of men undergoing IVF to investigate whether there is a link between IVF outcome and BPA concentration (Mínguez-Alarcón et al. 2021). The presence of BPA was identified in the urine samples, and the hazard ratio between cycle failure prior live birth and BPA concentration is greater than 1. This suggests that there is a probability that exposure to BPA may increase IVF failures before live births.

Key Statement
"Several adverse effects of BPA have been reported following its toxicity. This includes altered testicular structure, reduced mitochondrial enzymatic activities, and altered sperm quality, to mention a few. The hallmark mechanisms through which these adverse effects are exerted include (i) initiation of oxidative stress and (ii) disruption of the HPGA signaling."

8.6 Mechanisms Through Which BPA Impairs Male Fertility

Metabolically, BPA exposure will impair the homeostatic balance between the production of ROS and their neutralization. This occurs through an increase in the production of ROS and reduction of the antioxidant enzymes, which will lead to oxidative stress (Santiago et al. 2021). Oxidative stress is one of the main components of inflammatory reactions; these reactions will cause damage and changes to the male reproductive system mainly centered around testicular damage (Pallotti et al. 2020). The oxidative stress occurring in the epididymal and testicular sperm occurs through an increase in the levels of oxidants such as superoxide and hydrogen peroxide (H_2O_2) and a decrease in antioxidants, which consequently results in lipid peroxidation (LPO). Moreover, there are decreased levels and activity of glutathione reductase (GR), glutathione peroxidase (GPx), SOD, CAT, and glutathione (GSH). GSH is a known cofactor for multiple peroxidase enzymes that are involved in the detoxification process of ROS (Santiago et al. 2021). Additionally, BPA toxicity-induced oxidative stress may also cause mitochondrial dysfunction with resultant alteration of diverse cellular signaling and the concurrent initiation of apoptosis.

Hence, the main mechanisms of impairment fall under HPG axis dysfunction with hormonal imbalances and oxidative stress. Be that as it may, the induced oxidative stress and concurrent inflammation have a multitude of pathological and histopathological changes to the testicular structure. This is deemed to be significant and potentially more contributory to dysfunction of the male reproductive system and the risk of male infertility. In addition to altered hormone function and development of oxidative stress, BPA can also impair male fertility by promoting adipogenesis and lipid storage in adipocytes, thereby exhibiting obesity-related metabolic dysfunction. BPA also exerts anti-androgenic activity, as it interferes with androgen receptor signaling. That is, BPA acts as an antagonist of the androgen receptor and consequently results in decreased secretion of androgens. The different pathways through which BPA impairs male fertility are summarized in Fig. 8.4.

Key Statement
"Excessive accumulation of ROS and subsequent development of oxidative stress are key mechanisms through which BPA affects male fertility."

8.7 Summary of the Mechanisms Through Which BPA Impairs Male Infertility

All the available literature confirms that BPA is a potent endocrine disruptor affecting the HPG axis; this may occur during intrauterine and adult life. The two main mechanisms coexist and

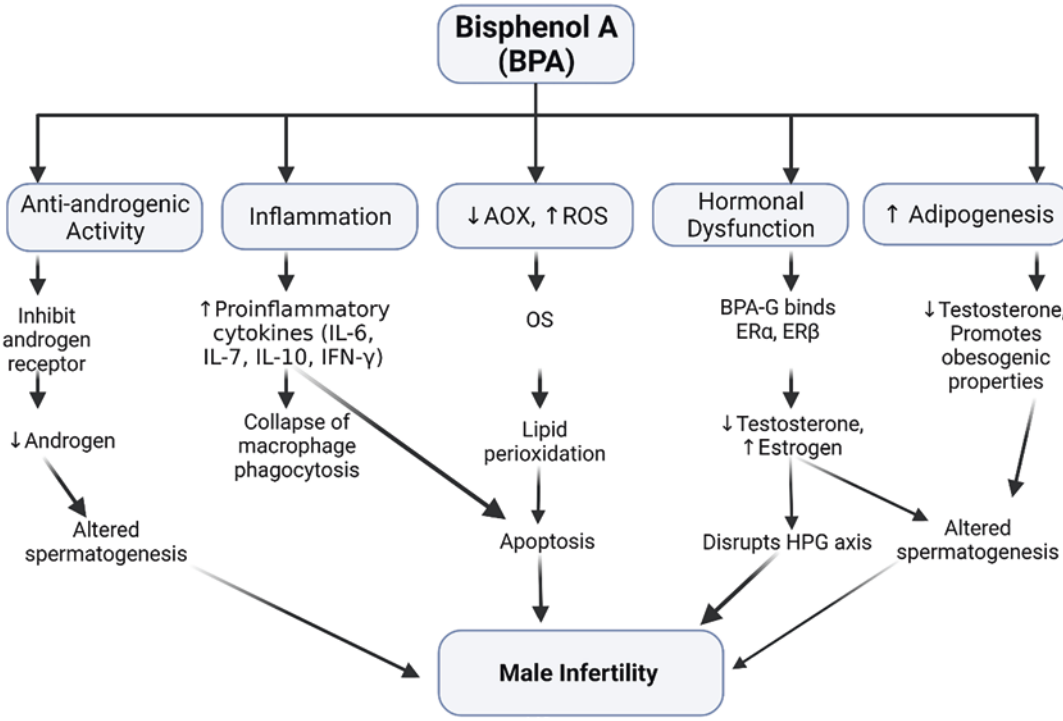

Fig. 8.4 Mechanisms through which bisphenol A (BPA) impairs male fertility. Briefly, BPA may impair male fertility by inducing oxidative stress and inflammation, cause hormonal imbalance via disruption of the HPG axis, and promote adipogenesis and lipid storage in adipocytes, thereby reducing the production of testosterone. It also acts as an antagonist of androgen receptor, which consequently results in decreased expression of androgen. The resultant outcomes of the diverse pathways are male subfertility or infertility

are collectively responsible for causing endocrine dysfunction and an imbalance in the cellular redox system as well as mitochondrial dysfunction, overall resulting in altered development of the testis in terms of structure and function, manifesting with abnormal sperm parameters. These parameters include concentration and motility, both of which are decreased with an increase in genetic abnormalities due to DNA damage. In general, there is a reduction in the semen quality and its parameters in exposed individuals.

While this dilemma has been ongoing for decades, the review and analysis of the existing literature do not provide a definitive answer to whether or not there is direct causation between BPA exposure and male infertility. It influences the HPG axis, estrogenic properties, anti-androgenic properties, oxidative stress, and overall impact on spermatogenesis. Consequently, these factors are known to have an impact on male fertility, yet we cannot conclude that they will ultimately lead to male infertility or that there is a direct causative effect. Hence, the current consensus is that BPA exposure and the sequel of events may increase the risk of male infertility or lead to difficulties in conception.

Key Statement

"Excessive exposure to BPA may not only have an effect on semen parameters such as sperm motility, concentration, or total count but may also cause genetic and epigenetic modifications."

8.8 Reactive Oxygen Species (ROS)

Having provided a background on BPA, the effects of BPA toxicity on male fertility, and the possible mechanisms through which these consequences are exerted, especially, the role of oxidative stress. This section and the succeeding sections of this article will briefly lay emphasis on ROS, the development of oxidative stress, and the management of oxidative stress-induced male infertility.

ROS is a collective term used to describe an array of oxygen-containing reactive species. Variations of ROS contain unpaired electrons and, therefore, are associated with free radicals (unstable atoms that sabotage cell integrity). ROS are predominantly valuable for ensuring appropriate functionality of cell development and proliferation for the maintenance of fundamental physiological processes, namely, immunological defenses to ultimately circumvent cell death. Nonetheless, overproduction of ROS within the human body can be detrimental to crucial cellular and biochemical functions as they are known toxic by-products of aerobic metabolism. The dual biological role of ROS exhibits the importance of a balance between ROS and antioxidants. Low levels of antioxidants in comparison to high levels of ROS obstruct the performance of neutralization activities. Thus, increased concentrations of ROS cause deliberate activation of a physiological cell death pathway and induce oxidative stress (Li and Trush 2016). Please refer to (Du Plessis et al. 2015) for a detailed review on ROS.

8.8.1 Pathophysiology of ROS in Human Semen

ROS play a fundamental role in the pathogenesis of various reproductive processes. An imbalance in ROS to antioxidant ratio due to the overproduction of ROS exhausts antioxidant defenses which directly affect male fertility. Hence, the regulation of ROS is vital. ROS present within human seminal plasma acquires a role in capacitation; ROS facilitates communication using the NADPH oxidase enzyme complex. ROS also modulates sperm chromatin condensation by altering the number of germ cells. Increased levels of ROS in seminal fluid induce apoptosis and proliferation of spermatozoa (Agarwal et al. 2003).

8.9 Oxidative Stress

Oxidative stress is a phenomenon that pertains to the disturbance between levels of production and elimination of ROS in the cells and tissues. The elevated levels of ROS within the human body modify the lipids, proteins, and DNA, thereby inhibiting the body's ability to detoxify the reactive products which activate an oxidative stress response. Cellular processes such as the activation of transcriptional factors, protein phosphorylation, immunity, and differentiation depend on adequate ROS production to commence proper functionality. Deviations made to the desired level of ROS pose harmful effects on crucial cellular structures (Tremellen 2012).

8.9.1 Origin of Oxidative Stress

Oxidative stress is associated with numerous intracellular and extracellular pathologies. ROS are the main cause of oxidative stress as they occur naturally in aerobic cells. There can be multiple sources of ROS, and the origins can include idiopathic and iatrogenic sources, as well as lifestyle and environmental factors such as smoking and pollution.

8.9.2 Idiopathic

The term idiopathic refers to a disease with an unknown cause and unspecified origin. Studies classify the idiopathic origin of oxidative stress-induced male infertility as multifactorial heterogeneous etiologies. Idiopathic male infertility is indicative of sperm abnormalities with no previous familial history of fertility problems,

medical information, or abnormal laboratory test results to corroborate the occurrence. The current consensus on idiopathic male infertility refers to an array of genetic disorders that could potentially affect fertility as a consequence (Alahmar 2019).

8.9.3 Iatrogenic

Iatrogenic causes of oxidative stress are consequences of certain medical treatments or examinations. In terms of oxidative stress-related male infertility, exposure to specific medications or medical equipments containing BPA could harm testicular function, spermatogenesis, and testosterone production due to hypothalamic-pituitary-testicular suppression; a dysfunctional reproductive axis causes irreparable consequences to developmental stages of fertility (Gandhi et al. 2017). Alteration at any stage of spermatogenesis may impair the overall sperm structure and quality, thereby affecting the vital fertilization functions. For instance, varicocele is one of the most common etiologies of male infertility. It is associated with elevated levels of oxidative stress-induced responses such as the impairment of sperm quality due to the overexpression of ROS, causing swollen veins in the scrotum. Moreover, an operative procedure such as varicocelectomy can be performed to remove the swollen varicoceles within the scrotum (Ni et al. 2016).

Several pharmaceutical medications have been approved to impair human spermatogenesis, including fluvoxamine maleate, cortisone acetate, and bosentan, danazol, among others (Ding et al. 2017). The mentioned medications are frequently prescribed to treat an array of psychological and physiological illnesses. Therapeutic drugs affect spermatogenesis function at varying degrees, causing temporary or persistent spermatogenesis impairment depending on their chemical properties and patients' immune response. Moreover, medications that alter the cellular function of either the testis or the epididymis will likely lead to adverse effects on fertility (Twigg et al. 1998).

Diet and lifestyle are among the prime contributory factors increasing the expression of free radicals within the human body. Medical conditions, treatment, and certain medications can also temporarily induce oxidative stress reactions due to mild inflammation. Also of importance is the exposure to BPA via oral ingestion, inhalation, or transdermal route. Oxidative stress occurs when the body is unable to facilitate the appropriate defense mechanisms against oxidative stress. Long-term exposure to oxidative stress leads to the development of chronic medical conditions, such as cardiac and neurological diseases (Pizzino et al. 2017).

8.9.4 Effect of Oxidative Stress on Male Fertility

The imbalance in the concentration of ROS and antioxidants alter DNA integrity, leading to the production of a lower quality of semen and inducing male infertility. ROS is required for sustaining regular cellular function. However, oxidative stress amplifies the production of ROS to a level of toxicity. Ooverproduction of ROS modifies sperm function by breaking DNA strands, altering bases and inducing chromatin cross-linking. The cellular characteristics are vulnerable to the influence of ROS, thereby leading to impaired defense mechanisms against ROS-induced oxidative stress damage (Agarwal et al., 2014).

8.10 Methods of Assessing Oxidative Stress-Related Male Infertility

Assessing the concentration of seminal ROS in infertile men is pivotal in determining therapeutic strategies that would offer the most effective

treatment. Numerous direct and indirect modes of detection have been developed to identify the ROS levels in seminal fluid (Alahmar 2019).

8.10.1 Direct Methods of Identification

Direct methods of identifying oxidative stress-related male infertility include the following:

- Chemiluminescence assay (CLIA) is a diagnostic tool that utilizes a variety of standard enzyme immunoassay methods with immunochemical reactions to detect oxidation or reduction through light generation (Alahmar 2019).
- Flow cytometry is an immunophenotypical mode of identification that is used to measure ROS concentration. A small sample of spermatozoa is required to facilitate the assay. Assessment of ROS concentration occurs by examining the visible light scatter and fluorescence parameters of single cells and particles that migrate past lasers in a buffered salt-based solution. The ability to simultaneously measure markers is a great advantage of the assay. However, it is a costly piece of equipment that is not sustainable for widespread clinical usage (Alahmar 2019).
- Electron spin resonance (ESR) is a spectroscopic method that allows for the detection and quantitative analysis of short-lived free radicals ESR-based methods have become widely used because the process can detect free radicals without interference from the sample properties, including its phase (solid, liquid, or gas). A limitation of ESR is the possibility of neutralization occurring by rapid reactions between a free radical and a molecule rather than a spin-trapping agent (Kohno 2010).
- The MiOXSYS System is used to measure oxidation-reduction potential (ORP). ORP measures the transfer of electrons from a reductant (or antioxidant) to an oxidant. ORP is measured in millivolts (mV). ORP is an overall measure of the oxidative stress to which a biological component is subjected. MiOXSYS System provides two measures of oxidative stress. Static ORP (sORP), measured in millivolts, is the integrated measure of the existing balance between total oxidants and reductants in a biological system. After this initial sORP reading is recorded, the analyzer automatically applies a small current sweep to the sample, resulting in the exhaustion of all antioxidant species, providing a measure of antioxidant capacity reserve (cORP), measured in microcoulombs (μC). Unlike other measures, sORP represents an integrated measure of all oxidants and reductants, making it a more clinically meaningful measure when diagnosing idiopathic cases associated with high levels of oxidative stress (Agarwal et al. 2016).
- Nitroblue tetrazolium assay is used to determine the ability of cells to produce ROS, giving insight into their oxidative metabolism. During this assay, NBT is reduced and precipitated, resulting in dark blue granules (formazan). Phorbol myristate acetate (PMA) in this assay acts as a stimulant, inducing the reduction of NBT to form formazan (Aitken 2018).

8.10.2 Indirect Methods of Identification

Indirect modes of identifying oxidative stress-related male infertility include the following:

- Lipid peroxidation levels are measured through colorimetric and thiobarbituric acid assays. MDA and toxic 4-HNE are detected by identifying the by-products of lipid peroxidation (Alahmar 2019).
- Myeloperoxidase identifies granulocytes in semen. Peroxidase charge (positivity) is measured through staining using benzidine. Myeloperoxidase is suitable for white blood cell differentiation from the immature germs present in semen. However, a disadvantage of the assay is its inability to identify ROS production in spermatozoa (Alahmar 2019).
- Cytochrome c reduction test quantifies oxidation by detecting the decrease in absorbance at 500 nm of ferricytochrome c caused by its oxidation, therefore displaying evidence whether an organism contains cytochrome c, an

enzyme derived from the electron transport chain. The assay measures oxygen released through the respiratory burst of neutrophils or isolated enzymes.

- Increased levels of sperm DNA damage have adverse effects on male reproductivity. Sperm chromatin structure assay (SCSA) is used to measure and identify sperm DNA damage. Sperm with an abnormal chromatin structure is more likely susceptible to acid and heat denaturation. SCSA measures the susceptibility of sperm DNA to acid-induced denaturation in situ.
- Chemokines are generated as a by-product of ROS-induced inflammation. Chemokines are measured using commercial ELISA. The prime disadvantage of using chemokines as a measurement of ROS in semen is that more than 0.5 L of biological material is required in order to facilitate proper ROS identification and measurement.
- Oxygen radical antioxidant capacity (ORAC) is a common assay used to determine antioxidant capacity. The assay measures antioxidant ability to reduce the degradation of fluorescent dye by ROS. Briefly, the assay measures the oxidative degradation of the fluorescent molecule (such as beta-phycoerythrin or fluorescein) after being mixed with free radical generators such as azo-initiator compounds. Azo-initiators are considered to produce peroxyl radical by heating, which damages the fluorescent molecule, resulting in the loss of fluorescence. Antioxidants are considered to protect the fluorescent molecule from the oxidative degeneration (Ou et al. 2001).

8.11 Male Infertility Treatments and Oxidative Stress Management

Oxidative stress-induced male infertility can be managed by combating the underlying cause of the pathogenesis, such as the use of antioxidants to reduce the excessive ROS production accrued due to BPA toxicity, and performing testicular sperm extraction for patients with low sperm count and/or azoospermia.

8.11.1 Antioxidants

Suboptimal fertility in men is associated with oxidative stress due to an increase in the levels of ROS production, which subsequently induces DNA damage resulting in lower rates of pregnancy. Occurrence of oxidative stress due to excessive production of ROS has been reported in BPA toxicity. This means that BPA toxicity can be ameliorated with the use of antioxidants. The role of antioxidants as a method to approach male infertility due to the increase in ROS has been explored. Favorable results such as improvement in sperm quality, mitigation of DNA damage, and combating lipid peroxidation have been elucidated following treatment with antioxidants (Martin-Hidalgo et al. 2019).

The nature and characteristics of antioxidants can be generally divided into enzymatic (SOD, CAT, etc.) and small organic molecules (ascorbate, urate, etc.). Organic molecules can be subsequently classified as lipid-soluble (vitamin E) and water-soluble molecules (glutathione, urate, and ascorbate). The main mechanisms by which antioxidants act are either by inducing a chain break via donating an electron to the free radical present in the system or by removing the ROS via quenching the chain-initiating catalyst. However, antioxidants may still act by other mechanisms such as metal-ion chelation and regulation of gene expression (Lobo et al. 2010; Ali et al. 2020; Stone and Pham 2021).

Antioxidants from a nutritional and synthesis point of view can be classified into endogenous and exogenous antioxidants. Endogenous antioxidants are made of smaller molecules, and it encompasses all enzymatic antioxidants and a few nonenzymatic antioxidants. Endogenous antioxidants depend heavily on the continuous synthesis of the reduced forms of reductants. On the other hand, exogenous antioxidants can only

be obtained via diet and cannot be naturally synthesized in eukaryotic cells due to their synthetic pathways being present only in plant and microbial cells. Common dietary sources for antioxidants include tomatoes, pineapples, watermelons, and all citrus fruits which contain high amounts of vitamin C, as well as vegetable oils, nuts, broccoli and, fish that are mainly abundant with vitamin E (Sharifi-Rad et al. 2020). The primary antioxidants found in the seminal plasma include SOD, CAT, GSH-px, vitamin C, vitamin E, and zinc (Pahune et al. 2013).

Extensive research exploring oral antioxidants and their possible role in terms of treating subfertility or infertility due to an increase in ROS has been performed throughout the years. However, only a few have demonstrated an improvement in terms of fertility rates and live births (Martin-Hidalgo et al. 2019). On the other hand, there is a possibility of causing more harm than good by using oral antioxidants, as demonstrated in the "Selenium and Vitamin E Cancer Prevention Study" (SELECT) where it showed that dietary supplementation with vitamin E significantly increased the risk of prostate cancer among men (Klein et al. 2011). In the interim, the overall evidence that is present in the literature is inconclusive due to a lack of proper methods and outcome reporting on live birth rates and pregnancy. This requires studies to include properly designed randomized placebo-controlled trials that would report the role of antioxidants on pregnancy and live births (Smits et al. 2019). In summary, the use of antioxidants remains essential when treating oxidative stress-induced male sub(in)fertility.

8.11.2 Testicular Sperm Extraction

Testicular sperm extraction (TESE) is the process of sperm retrieval from focal areas of spermatogenesis in the testis. It is usually used in men with nonobstructive azoospermia (NOA) caused by a multifarious array of etiology ranging from genetic disorders to gonadal toxins such as BPA, which impairs the process of spermatogenesis by causing hormonal imbalance. Men with NOA usually have focal areas of spermatogenesis on a background of germinal cell aplasia. There are several methods for sperm extraction, and they include fine-needle aspiration (FNA), percutaneous testicular biopsy, open testicular biopsy, and microdissection TESE. TESE, micro-TESE, and FNA carry the risk of vascular supply injury during the procedure which can lead to an intratesticular hematoma, and FNA could also cause epididymal injury (Janosek-Albright et al. 2015; Schlegel 1999). This method can be used to extract healthy sperm from the focal areas of spermatogenesis in men with BPA toxicity. Additionally, in cases where BPA toxicity resulted in oligospermia, asthenozoospermia, teratozoospermia, or combinations thereof, assisted reproduction such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) can be utilized.

8.11.3 Cryopreservation

Cryopreservation is the process by which biological structures are subjugated to extremely low temperatures. This is done mainly by first introducing the specimen to a cryoprotective agent such as dimethyl sulfoxide or polyvinylpyrrolidone and cooling the samples by using chemicals such as liquid nitrogen and then storing them until they are thawed again for usage (Martin-Hidalgo et al. 2019; Jang 2017). This procedure is useful when the source of oxidative stress cannot be immediately managed. For instance, if it is anticipated that workers would have a higher exposure to BPA, which may eventually lead to BPA toxicity, workers should be advised to cryopreserve their gametes to prevent the repercussions of BPA toxicity.

8.12 Conclusion

BPA is an ideal plasticizer because of its cross-linking characteristics. However, free monomers can be released in food content after polymerization, especially on exposure to high temperatures and with reuse of the containers. This chapter has

identified that BPA exerts both estrogenic and anti-androgenic properties, which interfere and impair testicular homeostasis. Although the half-life of BPA is relatively short, successive exposure to BPA poses a risk to male fertility. These threats are unraveled when the production of ROS begins to increase above the physiological level, thus leading to the induction of oxidative stress. The initiation of oxidative stress results in alteration of diverse signaling pathways which will adversely affect male fertility. Other ways through which BPA toxicity affects male fertility include endocrine dysfunction and the subsequent alteration in spermatogenesis, as well as the ability of BPA to promote adipogenesis and enhance obesogenic phenotype. Hence, caution must be taken, especially by men of reproductive age, to mitigate the exposure to BPA.

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9 Oxidative Stress and Male Infertility: Role of Herbal Drugs

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Abstract

Infertility is a universal health problem affecting 15% of couples, out of which 20–30% cases are due to male infertility. The leading causes of male infertility include hormonal defects, physical reasons, sexual problems, hazardous environment, stressful lifestyle, genetic factors, epigenetic factors, and oxidative stress. Various physiological functions involve reactive oxygen species (ROS) and nitrogen species at appropriate levels for proper smooth functioning. ROS control critical reproductive processes such as capacitation, acrosomal reaction, hyperactivation, egg penetration, and sperm head decondensation. The excessive free radicals or imbalance between ROS and endogenous antioxidant enzymes damages sperm membrane by inducing lipid peroxidation causing mitochondrial dysfunction and DNA damage that eventually lead to male infertility. Numerous synthetic products are available in the market to treat infertility problems, largely ending in side effects and repressing symptoms. Ayurveda contains a particular group of Rasayana herbs, called *vajikarana*, that deals with nourishment and stimulation of sexual tissues, improves male reproductive vitality, and deals with oxidative stress via antioxidant mechanism. The present study aims to describe oxidative stress and the role of herbal drugs in treating male infertility.

Keywords

Oxidative stress · Vajikarana · Antioxidant · Herbal drugs · Male infertility

9.1 Introduction

Infertility (or subfertility) is defined as the failure of couples to establish a clinical pregnancy after 1 year of consistent unprotected sexual intercourse (Zegers-Hochschild et al. 2017). According to the WHO, about 50–80 million people suffer from infertility worldwide. Globally, infertility affects approximately

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13–15% of all couples, and out of all cases of infertility, the male is responsible in 20–30% of cases. Male infertility is widespread not only in developing countries but also in developed ones. The exact figure is unpredictable because majority of cases are not registered often due to the scarcity and high cost of medical resources and treatment, respectively, sociocultural phobia, humiliations, etc. (Leslie et al. 2021). The leading causes of male infertility include hormonal defects, physical reasons like blockage of the ejaculatory pathway, sexual problems like erectile dysfunction or impotence, hazardous environment and stressful lifestyle, genetic factors like chromosomal abnormalities, single-gene mutations, and epigenetic factors (Babakhanzadeh et al. 2020; Iammarrone et al. 2003). Such factors can be broadly categorized (Table 9.1) in primary gonadal and hypothalamic-pituitary disorders which can be both, congenital and acquired, and disorders of sperm transport (post-testicular).

Table 9.1 Causes of men infertility

A. Primary gonadal disorders	
Congenital	Y-chromosome deletions, Klinefelter syndrome, cryptorchidism, congenital anorchia, Noonan syndrome, myotonic muscular dystrophy, sickle cell disease, varicocele, androgen insensitivity syndrome, 5α reductase deficiency
Acquired	Orchitis (mumps pyogenic, traumatic), malignant neoplasm (germ cell, leukemia, lymphoma), trauma, torsion, castration, systemic illness (renal failure, liver cirrhosis or other hepatic disorders, cancer, etc.), retroperitoneal fibrosis, drugs (cytotoxic drugs, alkylating agents, alcohol, marijuana, anti-androgens, histamine receptor antagonists, etc.), environmental toxins (dibromochloropropane, carbon disulfide, cadmium, lead, mercury, etc.), irradiation, hyperthermia
B. Hypothalamic-pituitary disorders	
Congenital	Congenital GnRH deficiency (Kallmann syndrome), hemochromatosis, multiorgan genetic disorders (Prader-Willi syndrome, Laurence-Moon-Biedl syndrome, familiar cerebral ataxia)
Acquired	Pituitary and hypothalamic tumors and cysts, infiltrative disorders (sarcoidosis, histiocytosis, tuberculosis), trauma, postsurgical, post-irradiation, vascular (infarction, aneurysm), hormonal (hyperprolactinemia, androgen, estrogen, and cortisol excess), drugs (opioids and psychotropic drugs, GnRH agonists or antagonists, etc.)
Systemic	Chronic illnesses, obesity, nutritional deficiencies
C. Disorders of sperm transport (post-testicular)	
Epididymal dysfunction (due to drugs, infection, etc.), abnormalities of the vas deferens (congenital absence, Young's syndrome, infection, vasectomy), ejaculatory dysfunction (spinal cord disease, autonomic dysfunction, premature ejaculation)	

GnRH gonadotropin-releasing hormone

9.2 Pathophysiological Factors of Male Infertility

9.2.1 Hormonal Defects

The right concentration of male hormones is required for the proper functioning of the testes and sexual development. They are produced by hypothalamic-pituitary-gonadal axis. Decrease or lack of release of gonadotropic-releasing hormone (GnRH) by the brain produces less testosterone and reduced sperm production, resulting in disorders like Kallmann syndrome (Monaco et al. 2015). Furthermore, the insufficient release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland causes failure to stimulate the testes, decrease in testosterone and sperm production, and defects in spermatogenesis (Wdowiak et al. 2014). Elevated prolactin concentrations also reduce sperm production and impotence (Marrag et al. 2015). The treatment of such disorders includes the use of long-term hormonal therapy involving the use of sex steroids (testosterone injections) or gonadotropin-releasing hormone (Schagen et al. 2016). But such treatment regimens lead to other complications such as diabetes mellitus, heart diseases, etc.

9.2.2 Physical Reasons and Sexual Problems

Various physical or anatomical changes, viz., blockage of the ejaculatory pathway, enlargement of sperm vessels (varicocele), testicular torsions, impaired sperm circulation, genital tract infection, obstruction in semen flow by damaged urinary bladder sphincter (retrograde and antegrade ejaculation), and its movement along the path, also lead to infertility. Erectile dysfunction, early ejaculation, and inability to ejaculate are problems related to intercourse that led to the inability of couples to establish a clinical pregnancy or indirectly cause infertility (Babakhanzadeh et al. 2020; Sun et al. 2018).

9.2.3 Lifestyle and Environment

Changing lifestyle or more of a sedentary lifestyle has been considered as one of the major reasons for infertility. Excessive consumption of alcohol, high-fat diet, smoking, prolonged sitting, poor nutrition, repeated use of drugs, high stress (leading to anxiety, depression, and other psychological disturbances), pollution in the air, etc. causes degradation of sperm, reduce sperm count and motility, and increase the chances of impotency in men. Exposure to harmful radiation, hazardous substances, and high temperature can also lead to infertility (Katib 2015; Mustafa et al. 2019).

9.2.4 Genetic and Epigenetic Factors

Among various genetic factors, chromosomal abnormalities and single-gene mutation are one of the leading causes of infertility. Chromosomal defects like microdeletions and disarrangement in chromosome fragments can cause dysfunction in spermatogenesis. Genetic mutations like a defect in ciliary function, congenital bilateral absence of the vas deferens (CBAVD), and testicular dysgenesis syndrome are also responsible for male infertility. Furthermore, epigenetic modifications, like acetylation, methylation, hypermethylation, etc., of the germ cells cause alteration in the expression of the genes that control spermatogenesis and other characters of sperm, leading to the deficiency in semen parameters (Boissonnas et al. 2013; Iammarrone et al. 2003; Neto et al. 2016; Stouffs et al. 2014).

9.2.5 Oxidative Stress (OS)

Free radicals, of both oxygen and nitrogen, can act as friend and foe for our reproductive system. These free radicals are generated as by-products of normal physiological effects in different cells including sperm cells. When their concentration is at appropriate levels, they act as friends and help in critical processes like capacitation, hyperactivation, egg penetration, and sperm head decondensation (Barati et al. 2020). Under normal conditions, human sperm generates free radicals (ROS and RNS) by numerous pathways, which instigate tyrosine kinases, and cyclic adenosine monophosphate (cAMP) that increases tyrosine phosphorylation levels (Wagner et al. 2018). Free radicals such as hydrogen peroxide, superoxide anion (O_2^-), nitric oxide, nicotinamide adenine dinucleotide (NADH), and nicotinamide adenine dinucleotide phosphate (NADPH) activate phosphorylation of protein tyrosine (p81 and p105). Furthermore, these phospho-tyrosine proteins are overexpressed during sperm capacitation. In the female genital tract, sperm gets hyperactive due to tyrosine phosphorylation in its tail region that further aids in the acrosome reaction which eventually helps the sperm to get attached to the zona pellucida (Fig. 9.1).

It has also been observed that apart from phosphorylation of tyrosine proteins, free radicals also induce sperm capacitation which is a compulsory process for fertilization (Tremellen 2008; Thundathil et al. 2003), while antioxidants like catalases, superoxide dismutase (SOD), and NADPH oxidase inhibit sperm capacitation (Takeshima et al. 2021). Moreover, Ca^{2+} also stimulates cyclic adenosine monophosphate

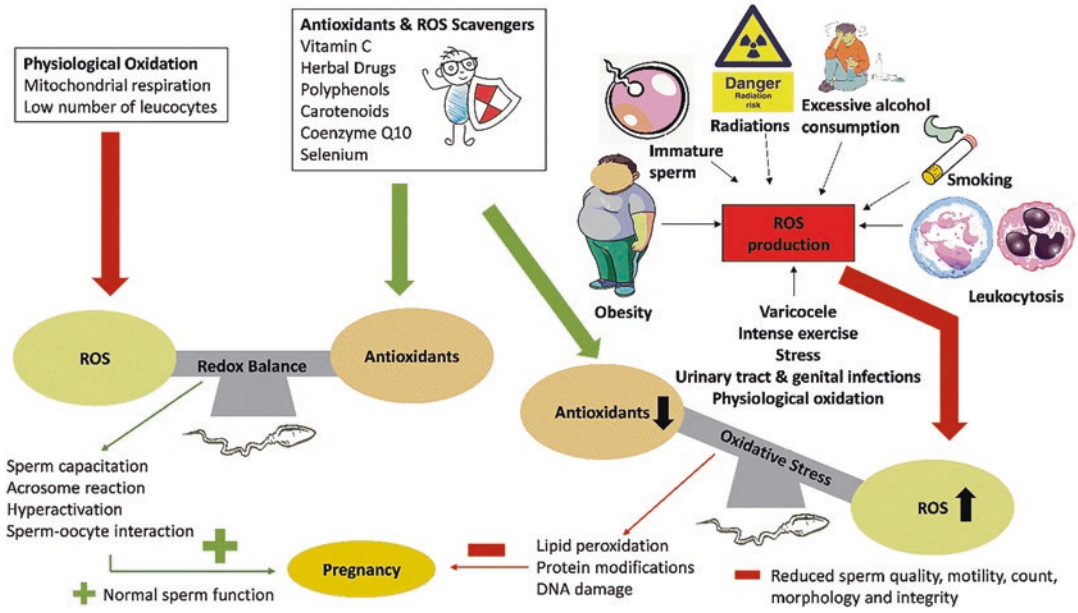


Fig. 9.1 ROS and OS in male infertility

(cAMP) during the capacitation process, which further regulates the superoxide anion generation and leads to rise in phosphorylation of p81 and p105 (Leclerc et al. 1998). Moreover, activities of adenylyl cyclase (AC) and phosphodiesterases (PDEs) also regulate the production and degradation of cAMP, thereby regulating its levels (Lefièvre et al. 2002). The above literature showed that many intricate pathways are involved during sperm capacitation and acrosomal reaction, which require ROS for functioning.

Human semen contains antioxidants, which can be categorized into enzymatic and nonenzymatic antioxidants, in the seminal plasma to protect sperm from OS. The former group includes include glutathione peroxidase (GPX), SOD, glutathione S-transferase, catalase, etc., whereas the latter one includes ascorbic acid, alpha-tocopherol, coenzyme Q10, myoinositol, astaxanthin, taurine, transferrin, L-carnitine, urate, melatonin, and lactoferrin. Under normal conditions, these antioxidants scavenge free radicals and preserves redox homeostasis in the sperm so that the sperm delivers the healthy and intact DNA to the oocyte during fertilization. Disproportion amid production and scavenging or neutralization of these free radicals heads to oxidative stress and acts as foe for our system (Fig. 9.1).

Various conditions that lead to such an oxidative stress include both endogenous [immature sperm (Sabeti et al. 2016), leukocytospermia (Fariello et al. 2009), metabolic syndromes, etc.] and exogenous [smoking (Aboulmaouahib et al. 2018), alcohol (Akang et al. 2017), radiations, environmental causes, etc.] factors. Free radicals during OS also have an indirect effect on the production of male reproductive hormones which leads to increased production of immature sperm (Barati et al. 2020). Immature sperm are the ones that have excess cytoplasmic residues, and because of this, they develop mitochondrial dysfunction which eventually leads to depletion of energy required for sperm motility and also produce excess of ROS (Sabeti et al. 2016). Leukocytes, though few, are found in normal semen, but when their number is more than 10,00,000/ml, the condition is known as leukocytospermia. Infection or inflammation of the reproductive tract is the main reason for leukocytospermia which also increase the levels of ROS in the semen leading to OS (Fariello et al. 2009). OS leads to various pathological and biochemical changes in sperm such as damage to axoneme,

decrease in ATP levels, and generation of 4-hydroxynonenal and malondialdehyde (MDA) which further initiates lipid peroxidation and DNA and mitochondrial damage (Fang and Zhong 2019).

During OS, ROS affects the double bonds present in the structure of the membrane lipids, thereby initiating lipid peroxidation of sperm membrane that changes its structure, dynamics, and fluidity (Gaschler and Stockwell 2017). ROS also cause oxidation of sulfhydryl groups, along with the change in membrane structure, which also decrease the sperm motility (Saleh and Agarwal 2002). Moreover, lipid peroxidation of membranes also initiates a series of reduction-oxidation reactions of mutagenic and genotoxic electrophiles causing sperm damage (Bui et al. 2018). Furthermore, hydrogen peroxide (H_2O_2), a non-radical ROS, easily passes through the sperm membrane and enters into its cytoplasm. It inhibits glucose-6-phosphate dehydrogenase (G6PD) enzyme, thereby reducing the levels of NADPH, a molecule required for the activity of glutathione peroxidase, an antioxidant enzyme (Said et al. 2004). Due to the reduction of NADPH, the activity of glutathione peroxidase also reduces, thus weakening the antioxidant defense against OS (Walczak-Jedrzejowska et al. 2013). All these events eventually lead to sperm damage or sperm with poor motility. High levels of ROS also cause damage to mitochondrial membranes resulting in the activation of caspases which finally initiate apoptosis in sperm (Wagner et al. 2018).

ROS also attacks the guanine base of the DNA, thereby converting it to 8-hydroxyguanine, and under stress conditions, these further get oxidized to 8-hydroxy-2-deoxyguanosine (8-OHdG) (Noblanc et al. 2013). Glycosidase enzyme present in sperm acts on 8-OHdG and releases another base compound, thereby causing further damage to DNA which can further lead to other changes like changes in single- or double-strand fragment, DNA fragmentation, base pair configuration, etc. OS is considered as on the major reasons for sperm DNA fragmentation (Muratori et al. 2015). Such changes eventually lead to genomic instability that effect the production or genesis of sperm (Barati et al. 2020).

Numerous synthetic products are available globally to treat infertility problems, largely ending in side effects and repressing only symptoms. Thus, once again, the focus has been shifted on herbal and Ayurvedic treatments for curing male reproductive problems (Dutta and Sengupta 2018). Ayurveda contains a particular group of Rasayana herbs, called *vajikarana*, that deals with nourishment and stimulation of sexual tissues and improves male reproductive vitality. In Sanskrit, *vaji* means "horse," and *karana* means "power," giving the idea of horse's strength. *Vajikarana* herbs revitalize the seven dhatus (body elements) and restore equilibrium in the body. They act on the hypothalamus and limbic system and modulate the neuroendocrine immune system. Besides, another category of herbs known as *shukrala* increases spermatogenesis and can improve sperm count, quality, and motility (Dalal et al. 2013). The herbs can also be classified on the basis of their beneficial effects on the male reproductive health, such as herbs that (i) enhance or stimulate semen production (*Mucuna pruriens*, *Asparagus racemosus*, etc.), (ii) improve semen quality (*Vetiveria zizanioides*, *Sesamum indicum*, etc.), (iii) revitalize ejaculatory functions (*Strychnos nux-vomica*, *Cannabis sativa*, etc.), (iv) improve nourishment and ejaculatory performance (*Cinnamomum tamala*, *Asparagus racemosus*, etc.), and (v) increase libido (*Asparagus racemosus*, *Withania somnifera*, etc.) (Chauhan et al. 2014).

9.3 Some Common Plants Used to Treat Male Infertility

9.3.1 *Nigella sativa* (Family: Ranunculaceae)

Nigella sativa, also known as *black cumin*, *black seed*, *Habbatus sauda*, and *kalonji*, is a medicinal plant widely used traditionally in Ayurveda, Unani Tibb, and Siddha systems of medicine. In Islamic literature, it is considered as *Tibb-e-Nabawi* (Prophetic medicine) and is believed to be the most extraordinary form of healing medicine (Shuid et al. 2012; Yimer et al. 2019). It has

been extensively used as anti-inflammatory, spasmolytic, bronchodilator, immunomodulator, antioxidant, antidiabetic, antihypertensive, liver tonics, diuretics, digestive, and analgesics and in skin disorders (Yimer et al. 2019). The main active compounds isolated from the seeds are thymoquinone (30–48%), thymohydroquinone, dithymoquinone, p-cymene (7–15%), carvacrol (6–12%), 4-terpineol (2–7%), anethole (1–4%), longifolene (1–8%), α-pinene and thymol, and alkaloids nigellimine and nigellimine-N-oxide, nigellidine, and nigellicine (Fig. 9.2). Furthermore, the seeds also contain a water-soluble pentacyclic triterpene alpha-hederin, used in treating cancer; unsaturated fatty acids, mainly dihomolinoleic (10%), linoleic (50–60%), eicosadienoic (3%), and oleic acid (20%); saturated fatty acids mainly palmitic and stearic acid; and α-sitosterol (44–54%) followed by stigmasterol (6–20%). Most of the beneficial properties of *N. sativa* are attributed due to the presence of thymoquinone (Fig. 9.2), the major bioactive component of the essential oil (Ahmad et al. 2013).

Alcoholic extract of *N. sativa* increases the production of sperm cells, increases sperm motility, and improves epididymal sperm reservation, gonadotropin content, testosterone levels, and fertility indexes in male rats. Black cumin seeds induce a rise in spermatogenesis, levels of hormones like testosterone and luteinizing hormone (LH), and an increase in the weight of reproductive organs (Parandin et al. 2012). The treatment with seeds also increased the sperm count, fertility index, and overall characters of sperm. A randomized, double-blind, placebo-controlled clinical study conducted on 68 infertile Iranian men revealed that 2.5 mL of black seed oil twice daily for 2 months significantly improved the sperm count and motility (Kolahdooz et al. 2014). Thymoquinone (TQ) at 50 mg/kg p.o. protected the testicular tissue by alleviating inflammation and apoptosis and by restoring the average balance of sex hormones (Alyoussef and Al-Gayyar 2016). Moreover, TQ treatments significantly increase testosterone level in serum, testicular GSH, and

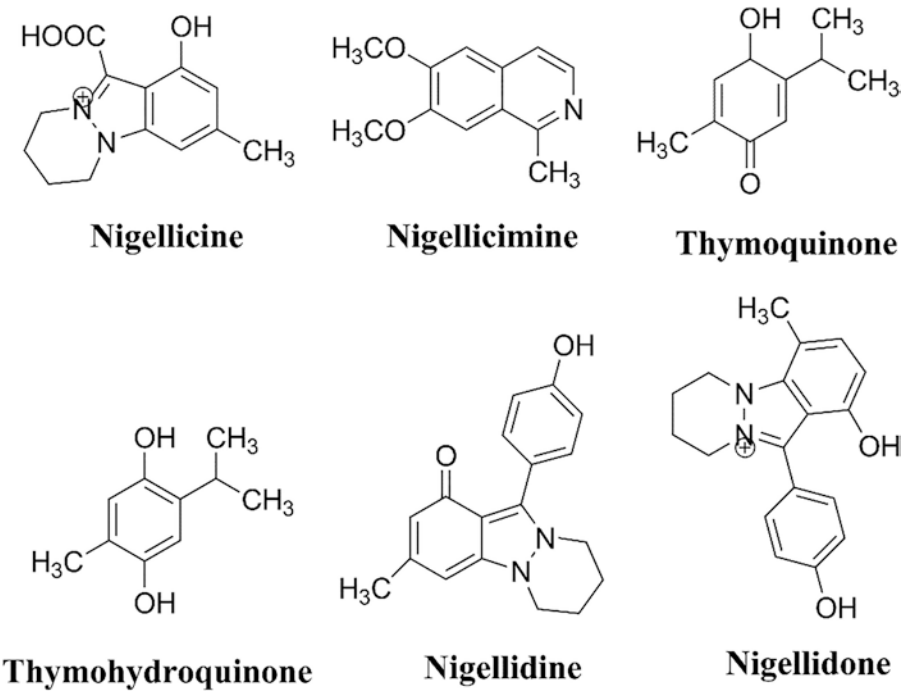


Fig. 9.2 Chemical constituents of *Nigella sativa*

SOD activity and lower MDA and nitric oxide activity when compared with the control group (Fouad et al. 2014). It also increases the mean volumes of testis and seminiferous tubules, count of spermatogenic cells, and Leydig cells (Darand et al. 2019; Yimer et al. 2019). These studies indicate that *N. sativa*/TQ can be used as an alternate source for developing natural aphrodisiac agents.

9.3.2 *Mucuna pruriens* (Family: Fabaceae)

It is also known as *velvet bean* or *Kapikacchu* or *Konch* and is an underutilized wild legume that is spread throughout the tropical and subtropical regions across the globe. It is largely utilized as fodder, forage, and green manure crop. It is mentioned in Rigveda as a *Balavardhaka aushadhi* (strength-promoting medicine) (Pandey and Lalitha 2018). The plant is used to treat impotence and diabetes mellitus, whereas the seeds are used to manage Parkinson's, diabetes, arthritis, atherosclerosis, and analgesic, antipyretic, and antioxidant activities. It is a common ingredient in itching powder due to the presence of 5-hydroxytryptamine (serotonin) in the seed pods which causes severe itching (Yadav et al. 2017).

The seeds of velvet beans contain beta-sitosterol, 3-(3,4- dihydroxyphenyl)-l-alanine (L-DOPA), gallic acid, and glutathione. It also contains alkaloids like mucunadine, mucunine, pruriendine, and prurienine, 3-methoxy-1,1-dimethyl-6,7-dihydroxy-1,2,3.4-tetrahydroquinoline, and 3-methoxy-1,1-dimethyl-7,8-dihydroxy-1,2,3.4-tetrahydroquinoline (Fig. 9.3). A β-carboline alkaloid, 6-methoxyharman, has also been isolated from leaves. Seeds also contain oil rich in palmitic, stearic, oleic, and linoleic acids (Sathiyanarayanan and Arulmozhi 2007; Yadav et al. 2017).

Alkaloids present in seeds of *M. pruriens* are considered as main bioactive compounds as they trigger spermatogenesis and increase the weight of the testes in male rats (Saksena and Dixit 1987). The plant escalates mounting frequency, duration to ejaculate, and intromission frequency in male rats (Amin et al. 1996; Suresh et al. 2010). It also significantly recovers the spermatogenic loss induced by ethinyl estradiol administration in male rats. The plant showed beneficial effects by reducing ROS, regulating apoptosis, and increasing germ cell number. Aphrodisiac activity of *M. pruriens* can also be due to L-DOPA, the principal constituent of the plant, which accounted for pro-spermatogenic properties (Singh et al. 2013). The L-DOPA metabolite, dopamine, may further stimulate the hypothalamus and anterior pituitary to secrete GnRH, FSH and LH, which improves testosterone synthesis in the Leydig cells of the testicles (Sriraman et al. 2003). The ethanolic extract of seeds exhibited noticeable improvement in sexual potency, libido, sperm parameters, and endocrine levels (Suresh and Prakash 2012). *M. pruriens* improves semen quality by attenuating OS-induced lipid peroxidation in the seminal vesicles and reinstating GSH, ascorbic acid, catalase, and SOD levels (Ahmad et al. 2008; Shukla et al. 2010). The plant also raises LH, adrenaline, testosterone, noradrenaline, and dopamine levels and lowers FSH and prolactin levels in infertile men. It also improves steroidogenesis and semen quality in infertile males (Ahmad et al. 2008). Many drugs like "Tentex forte," "Speman," and "Confido" by Himalaya Drug Company contain *Mucuna* for sexual well-beings (Bhagwati and Singh 2017).

9.3.3 *Asparagus racemosus* (Family: Asparagaceae)

Asparagus racemosus, commonly known as *Shatavari or Shatavar* or *Shatmul*, is an ingredient of *Vajikarana Rasayana* in Ayurveda for its aphrodisiac role. It acts as a vitalizer and regulates hormone imbalance. The primary chemical constituents present in the plant are steroidal saponins, shatavarin I to IV, sarsasapogenin, adscendin (A, B, C), and asparanin (A, B, C); alkaloid, asparagine; isoflavones, 8-methoxy-5,6,4′-trihydroxyisoflavone-7-O-β-d--glucopyranoside; flavonoids, quercetin and rutin (Fig. 9.4); and sterols, sitosterol, 4,6-dihydroxy-2-O-(2′-hydroxyisobutyl). Unani traditional sys-

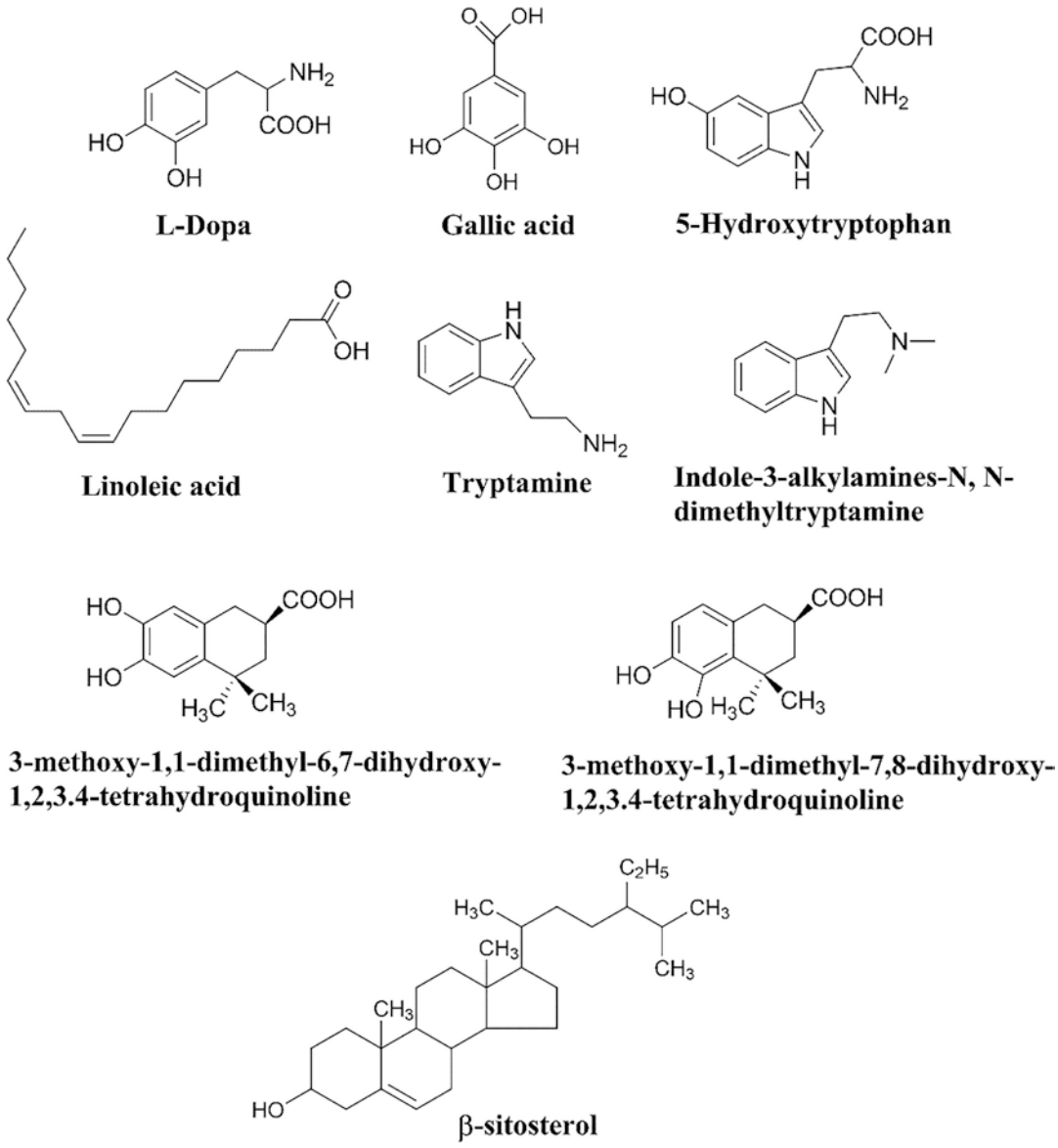
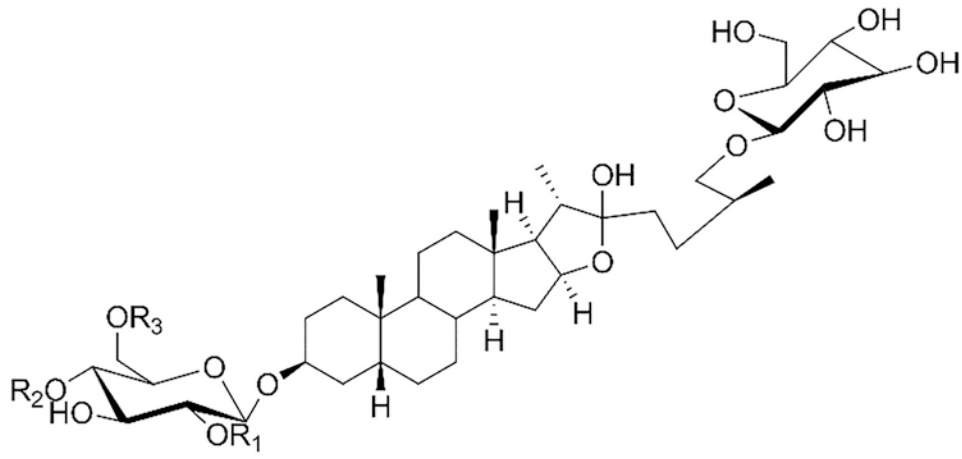
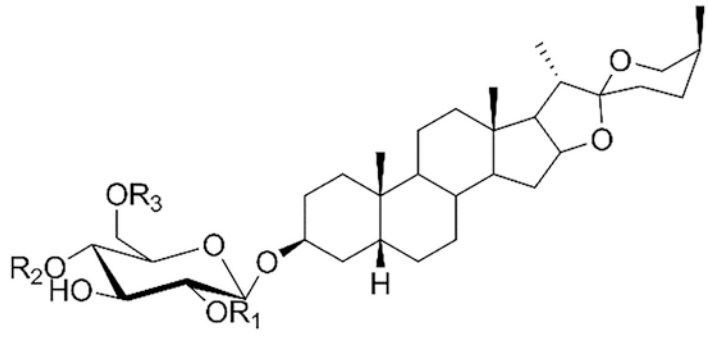


Fig. 9.3 Chemical constituents of *Mucuna pruriens*

tem of medicine used shatavari as *Mwallide mani* (ovulation-inducing), *Mwallide labn* (galactogogue), *Mugallize mani* and *Muqawwie bah* (aphrodisiac), *Dafe jiryan* (prevent spermaturia), *Dafe sailanur rehm* (prevent leucorrhoea), *Muqawwie qalb* (cardiac tonic), and *Muhallile warm* (anti-inflammatory) (Shameem and Majeedi 2020). The cooling property of the herb controls *pitta* in the small intestine and balances the heating effect of herbs like garlic, onion, and ashwagandha, which improve sperm count. The effect of Shatavari on pitta also helps in preventing the sperm damage, thereby improving/maintaining the sperm count. It can also be given in combination with Brahmi (*Centella asiatica* also known as gotu kola) to boost libido and benefit in overcoming emotions like anger and irritability (Dutta and Sengupta 2018).



Shatavarin I (or Asparoside B): R_1= β-D-Glc, R_2 = α-L-Rha, R_3 = H



Shatavarin IV (or Asparinin B): R_1= β-D-Glc, R_2 = α-L-Rha, R_3 = H
Shatavarin V : R_1= α–L-Rha, R_2 = β-D-Glc, R_3 = H
Shatavarin VIII : R_1= β-D-Glc, R_2 = α-L-Ara, R_3 = β-D-Glc
Shatavarin IX : R_1= β-D-Glc, R_2 = β-D-Glc, R_3 = H
Shatavarin X : R_1= β-D-Glc, R_2 = β-D-Glc, R_3 = H

Fig. 9.4 Chemical constituents of *Asparagus racemosus*

Shatavari root extract significantly increased the number of mounts and mounting frequency as well as mating performance in adult male albino rats (Mishra et al. 2010; Wani et al. 2011). The plant has shown to enhance sexual activity and treat numerous sexual disorders like lack of sexual desire, erectile failure, and premature ejaculation in males. Moreover, the aqueous extract of *A. racemosus* rich in steroidal saponins and fructo-oligosaccharides restores the sexual functions deteriorated by alloxan or streptozotocin treatment and can be used to treat sexual dysfunctions related to diabetes due to hyperglycemia (Thakur et al. 2009). Shatavari (at 100 mg/kg dose) along with three other herbs increases sperm count and nitric oxide release and improves penile erection in male albino rats (Thakur et al. 2011). A herbo-mineral preparation including Shatavari and Gokshura (50 g each) along with 5 g of Anhraka bhasma has shown to increase the production and

quality of sperm, thereby improving the fertility of a 28-year-old male (Kumar and Venkatesh 2020).

9.3.4 *Withania somnifera* (Family: Solanaceae)

Withania somnifera, also known as *Ashwagandha*, *Indian ginseng*, and *winter cherry*, is a well-known Ayurvedic *Rasayana* drug widely used for increasing energy and longevity (Winters 2006). The plant has been reported for its effects against inflammation, as an antioxidant, immune-modulating, antistress, memory enhancer, and anticonvulsant activities. Phytochemical studies on Ashwagandha revealed the presence of various steroidal lactones (collectively known as withanolides) and alkaloids (withanine, somniferine, somnine, somniferinine). The prime withanolides isolated from the plant include withaferin A, withanone, withanolide A–Q, sitoindoside VII–X, and withanamides A–I (Fig. 9.5) (Mirjalili et al. 2009; Singh et al. 2010).

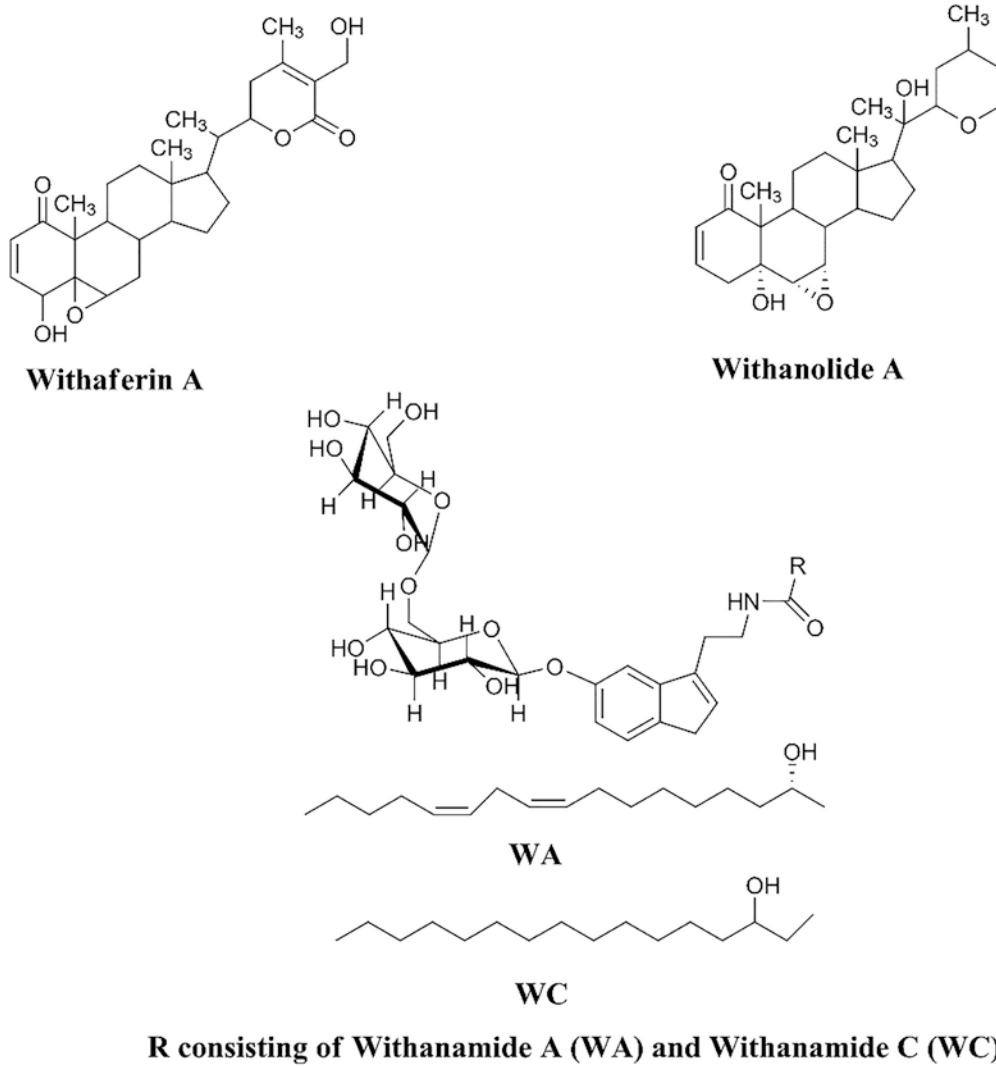


Fig. 9.5 Chemical constituents of *Withania somnifera*

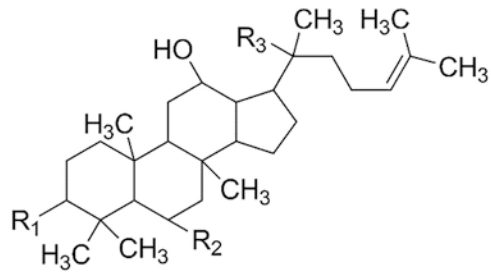
Studies exhibited that ashwagandha roots increase sperm motility, semen volume, and sperm count, stabilize testosterone production in oligospermic male, and, therefore, it can be used as a valuable treatment for infertility (Pathak et al. 2020). The plant acts via oxidative and non-oxidative process to exert its effect on male fertility. In oxidative mechanism, *W. somnifera* maintains antioxidant enzymes and the cofactors responsible for antioxidant activity. Non-oxidative mechanism includes regulation of the hypothalamus-pituitary-gonadal axis and hypothalamus-pituitary-adrenal axis for the proper functioning of reproductive organs. It regulates endocrine homeostasis, reduces the stress response, and normalizes the cortisol levels to improve male fertility (Sengupta et al. 2018). Moreover, the roots of the plants hold considerable amounts of lactate and lactate dehydrogenase (LDH) which stimulate Krebs cycle and increase ATP and cAMP levels, improving sperm concentration, quality, and motility (Gupta et al. 2013; Teixeira and de Araujo 2019).

W. somnifera exhibited enhancement in spermatozoa factors in males who have idiopathic infertility (Azgomi et al. 2018). *W. somnifera*, along with *Cynomorium coccineum*, showed testosterone-like effect and influence spermatogenesis in the seminiferous tubules of immature rats (Abdel-Magied et al. 2001). Treatment with the plant in infertile males has also improved semen quality by inhibiting lipid peroxidation, restoring antioxidant enzymes, increasing testosterone and LH levels, and reducing FSH and prolactin levels (Ahmad et al. 2010). Moreover, withanone exhibited GABA-mimetic action which regulates gonadotropin-releasing hormone (GnRH) at cellular levels which support the claims of Ashwagandha extracts in improving sexual function and testosterone production in male rats (Kataria et al. 2015). Some of the market formulations for male infertility problems, containing *W. somnifera*, by Himalaya Drug Company, are "Speman," "Himplasia," "Confido," and "Tentex Forte."

9.3.5 *Panax ginseng* (Family: Araliaceae)

Ginseng is one of the ancient herbs used in traditional Chinese medicine. The most common varieties of ginseng are Asian ginseng (*Panax ginseng*), American ginseng (*Panax quinquefolius*), and Japanese ginseng (*Panax japonicus*) (Leung and Wong 2013). Based on their structural differences, the plant consists of three tetracyclic dammarane triterpenoid saponin glycosides (called as ginsenosides): panaxadiols (e.g., Rb1-Rb3, Rc, Rd., Rg3, Rh2, and Rs1), panaxatriols (e.g., Re, Rf, Rg1–2, and Rh1), and oleanolic acid derivatives (e.g., Ro) (Fig. 9.6). Ginseng has been used mainly as a tonic to rejuvenate fragile bodies and restore proper metabolism in the body. It possesses antioxidant, anti-apoptotic, anti-inflammatory, immune-stimulatory activities and showed beneficial effects on aging and neurodegenerative diseases. The plant and its various chemical constituents reduce lipid peroxidation, maintain cellular ATP levels, and inhibit excitotoxicity and Ca^{2+} over-influx into neurons (Choudhary et al. 2013).

Ample studies have exhibited the importance of ginseng in boosting of sex hormone levels, sperm numbers, and testicular antioxidants, restoring Leydig cells, and improving spermatogenesis and sperm motility (Kopalli et al. 2015; Ku et al. 2020). Many ginsenosides especially Rg1 (10 mg/kg) help in treating erectile dysfunction by inducing synthesis of nitric oxide (NO) in endothelial cells by glucocorticoid receptor-dependent, non-genomic mechanisms. NO release causes smooth muscle relaxation which allows more blood to enter into the corpus cavernosum, causing an erection (Leung and Wong 2013). Experimental models revealed that the intake of *Panax ginseng* (5%), ginsenoside Rg1 (10 mg/kg), and ginsenoside Rb1 (10 μg/kg) increases serum testosterone levels, improves copulatory behavior, and increases LH secretion (Fahim et al. 1982; Tsai et al. 2003; Wang et al.



Ginsenoside Rb_1: R_1 = OGlc(2-1)Glc, R_2 = H, R_3 = OGlc(6-1)Glc
Ginsenoside Rb_3: R_1 = OGlc(2-1)Glc, R_2 = H, R_3 = OGlc(6-1)Xyl
Ginsenoside Rc: R_1 = OGlc(2-1)Glc, R_2 = H, R_3 = OGlc(6-1)Araf
Ginsenoside Rd: R_1 = Glc(2-1)Glc, R_2 = H, R_3 = Glc
Ginsenoside Re: R_1 = OH, R_2 = OGlc(2-1)-Rha, R_3 = OGlc
Ginsenoside Rg_1: R_1 = OH, R_2 = OGlc, R_3 = OGlc

Fig. 9.6 Chemical constituents of *Panax ginseng*

2010). Apart from this, saponins from the stem and leaves of *P. ginseng* also reduce the oxidative stress linked with hyperthermia and heat stress. They also inhibit the activation of mitogen-activated protein kinase (MAPK) signaling pathways and the expression of apoptotic proteins (Liu et al. 2021). Rg3-enriched extract of Korean red ginseng attenuates heat stress-induced testicular damage and change in expression of sex hormone receptors that affect spermatogenesis (Kopalli et al. 2019). Long-term administration of its aqueous extract significantly delays the aging-induced testicular dysfunction by modulating the expression of enzymes that regulate oxidation, acetylation, and growth-related activities linked with spermatogenesis (Kopalli et al. 2017; Kopalli et al. 2015). It also prevents or treats psychological stress-induced male infertility by increasing antioxidant enzyme expression, sex hormone receptor expression, and functioning of spermatogenesis-related proteins (Lee et al. 2019). The plant also prevents oxidative stress and apoptosis linked with monobutyl phthalate in human Sertoli cells by increasing the expression of nuclear factor erythroid 2-related factor 2 (NRF-2), sirtuin (SIRT-1), and antioxidant enzymes (De Freitas et al. 2019).

9.3.6 *Trigonella foenum-graecum* (Family: Fabaceae)

Trigonella foenum-graecum Linn, also known as *fenugreek*, is an aromatic annual plant widely grown in Egypt, India, China, France, Spain, and Turkey. The plant comprises of active constituents, such as alkaloids (choline, trigonelline, carpaine), saponins (yamogenin, fenugrin, gitogenin, yuccagenin, tigonenin, diosgenin, neotigogenin, sarsasapogenin), flavonoids (luteolin, kaempferol, naringenin, quercetin, tricin 7-O-D glucopyranoside, iso-vitexin, vitexin), coumarins (methyl coumarin, trigocoumarin, trimethyl coumarin), steroids, and phenolics like gallic acid, catechin, chlorogenic acid, vanillic acid, and syringic acid (Fig. 9.7) (Dini 2018). The seeds contain diosgenin which is an essential precursor for synthesizing several sex hormones including testosterone and estrogen. Traditionally, fenugreek seeds were given to lactating females as a stimulant for milk production (El-Hak and Elrayess 2018).

Fenugreek seed aqueous extract improved the sperm damage caused by bisphenol by reducing MDA levels, decreasing the expression of apop-

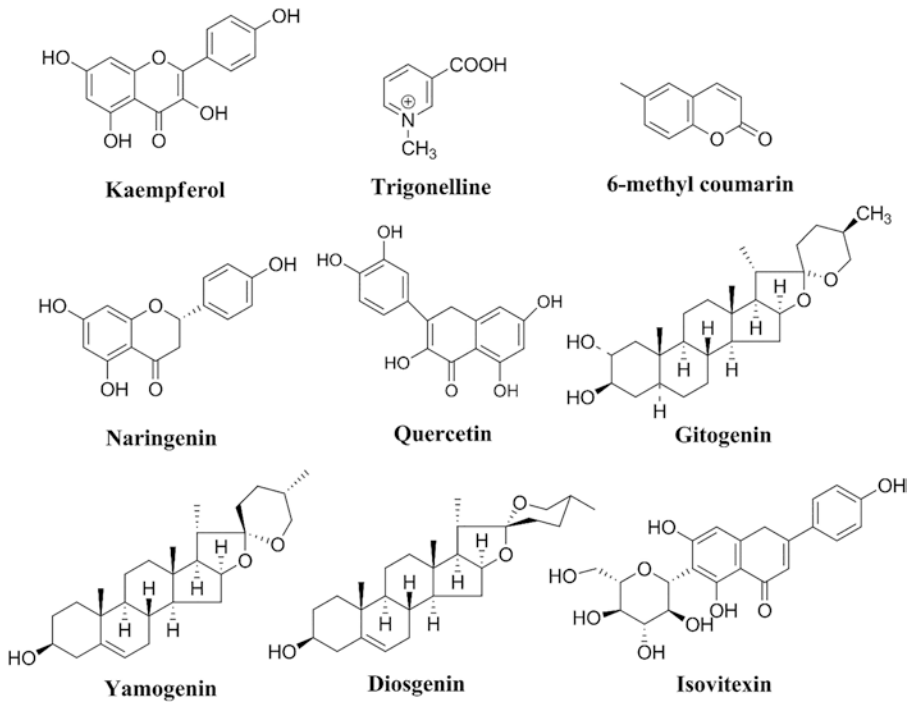


Fig. 9.7 Chemical constituents of *Trigonella foenum-graecum*

totic markers, increasing levels of antioxidant enzymes and thereby improving sperm parameters (Kaur and Sadwal 2020). Furosap™, a patented 20% protodioscin-enriched seed extract of fenugreek, increases free testosterone levels, sperm count, and sperm motility and causes significant alleviation in mood, reflex erection, and overall performance in male subjects (Maheshwari et al. 2017; Swaroop et al. 2017). Testofen®, a patented formulation by Gencor Pacific Lifestage Solutions, containing standardized extract of *Trigonella foenum-graecum* boosts male libido and maintains prolactin, testosterone, and prostate-specific antigen levels (Rao et al. 2016). A 12-week, single-site, double-blind, randomized clinical trial, on 120 male subjects, aged 43–70, showed that Testofen® effectively improved sexual health by remarkably increasing sexual desire, arousal, and testosterone levels (Rao et al. 2016). The mixed extract of fenugreek seeds and *Lespedeza cuneata* exhibited significant improvement in testosterone deficiency syndrome (Park et al. 2018). Moreover, consumption of aqueous extract of fenugreek seeds improves fertility and reproductive function in male rats (Hind et al. 2017).

9.3.7 *Allium sativum* (Family: Liliaceae)

Allium sativum, also known as *garlic*, is an intensely aromatic bulb crop that is cultivated across the globe. Traditionally, garlic is used as an aphrodisiac, to relieve cough problems, prevent graying of hair, lower cholesterol, and treat eczema, rheumatism, and high blood pressure. The *Kashyapa Samhita* contains a special chapter called "Lasunkalpa Addhyaya," which deals with the uses and pharmaceutical preparations containing garlic for treating infertility in males and females (Vaijnath and Manikrao 2018). Fresh garlic bulb contains water (65%), carbohydrates (28%), organosulfur compounds (2.3%), proteins

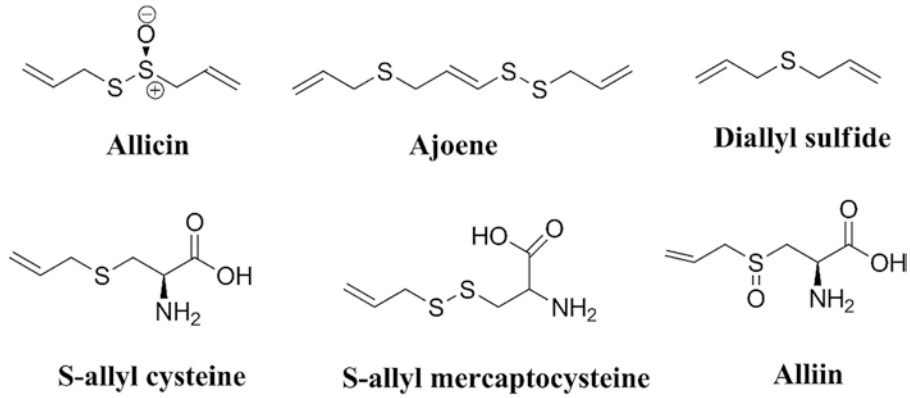


Fig. 9.8 Chemical constituents of *Allium sativum*

(2%), fiber (1.5%), amino acids (1.2%), saponins, and phenolics (Nouroz et al. 2015). Many sulfur-containing compounds include alliin, diallyl polysulfides, S-allylcysteine, diallyl sulfide, diallyl disulfide, diallyl trisulfide, S-allyl mercaptocysteine, and vinyldithiins (formed in the breakdown of allicin) (Fig. 9.8). Saponins reported from *Allium sativum* extract include proto-eruboside B; eruboside B; voghierosides A1, A2, B1, B2, C1, C2, D1, D2, E1, and E2; gitogenin 3-O-tetrasaccharide; and agigenin 3-O-trisaccharide. The phenolics isolated from garlic extract contain caffeic, ferulic, vanillic, *p*-hydroxybenzoic, and *p*-coumaric acid (Fig. 9.8) (Kuete 2017).

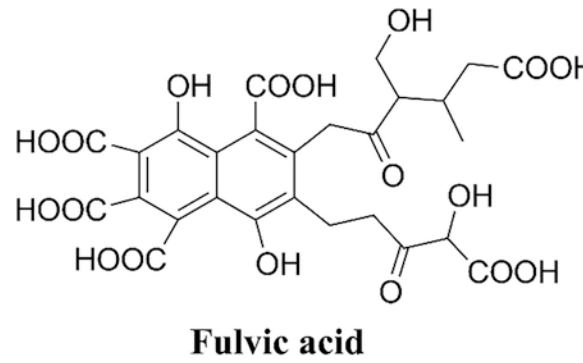
Many studies revealed the importance of garlic in the treatment of male infertility due to sulfur compounds that directly affect the uptake of CYP450 and glutathione S-transferase and protect spermatogenesis. It inhibits caspase-3 and CYP450 enzymes, which had a deleterious effect on the testicles (El-Akabawy and El-Sherif 2016). Moreover, garlic has antioxidant properties which reduce lipid peroxidation and increase fertility in men (Hammami and El May 2013). It also acts as a precursor to testosterone production, which further stimulates sexual cells and testosterone secretion from the testicles, boosts LH from the pituitary gland, and improves spermatogenesis. Garlic also inhibits reactive oxygen species and increases sperm motility and survival (Musavi et al. 2018). It also increases the flow of blood in the testis. Fresh garlic juice (120 mg/kg) protects semen oxidation by decreasing MDA levels and increasing antioxidant activity in rat testes (Ghalehkandi 2014). The aged garlic extract (250 mg/kg, 14 days) causes an increase in sperm count, motility, testis weight, recuperation of seminiferous tubules, and decreased sperm abnormality and death via antioxidant mechanism (Nasr 2017).

Similarly, treatment with single bulb garlic (250 mg/kg) improves sperm count, sperm motility, and sperm normality in male mice with hyperlipidemia (Qadariah et al. 2020). Also, aqueous garlic extract enhances spermatogenesis and ameliorates testicular and hematological alterations induced by cadmium poisoning in male rats (Mbegbu et al. 2021). The primary protective mechanism of garlic for testicular damage is to combat oxidative stress by decreasing free radicals and increasing antioxidant parameters in the semen (Adeyemi et al. 2021; Alsenosy and El-Aziz 2019; Eric et al. 2020; Ifeoma et al. 2018; Nasr et al. 2017).

9.3.8 Shilajit (Asphaltum, Mineral Pitch)

Shilajit, also known as *salajit*, *shilajatu*, *mimie*, *or mummiyo*, is a drug of mineral origin. It occurs

Fig. 9.9 Fulvic acid



as a blackish-brown powder or an exudate from high mountain rocks between India and Nepal. It is known as a rejuvenator and as an antiaging compound in Ayurveda. Shilajit is composed of humic substances, mainly fulvic acid (60–80%) (Fig. 9.9) plus some oligo-elements, including selenium. The composition of the phytocomplex varies from region to region (Carrasco-Gallardo et al. 2012).

The pure extract of shilajit and processed shilajit capsules improve spermatogenesis and male fertility and have beneficial effects on oligozoospermia (Biswas et al. 2010; Chouhan et al. 2018). It decreases oxidative stress in sperm and improves sperm quality parameters, such as motility, plasma membrane integrity, etc., in the semen of buffalo (Sultan et al. 2021). Furthermore, when those sperm were stored under cryogenic conditions, shilajit preserves their viability, livability, and DNA integrity, indicating its beneficial effects on sperm quality. In another study, shilajit increases the serum LH and testosterone levels and the number of seminiferous tubular cell layers in the testes and improves spermatogenesis in the treated rats (Park et al. 2006). Moreover, treatment with shilajit also increases the weight of reproductive organs and sperm production, enhances activities of testicular enzymes, and reverts the adverse effects of cadmium on motility and concentration of spermatozoa (Mishra et al. 2018). Inhibition of phosphodiesterase 5A (PDE5A) has become the first-line therapy for treatments of erectile dysfunction. Fulvic acid, the main constituent of shilajit, binds to the catalytic site of PDE5A and blocks the degradative action of cGMP for penile erection (Bhavsar et al. 2016). Shilajit displayed a peripheral parasympathomimetic effect for endothelium-dependent relaxation of corpus cavernosum smooth muscles, which supports the traditional claims of shilajit on libido and fertility (Kaur et al. 2013).

Apart from these extensive studied plants to treat male infertility, there are numerous other plants that have exhibited beneficial effects against male infertility. Some of the plants/phytoconstituents responsible for enhancing male fertility and fertilization process are mentioned in Table 9.2.

9.4 Conclusion

OS is considered as one of the major causes of male infertility. It leads to increase in lipid peroxidation and DNA damage which disturbs sperm functions making early diagnosis of infertility essential to avoid permanent impairment in the long run. Proper diet, exercise, reduced smoking, moderate consumption of alcohol, and nonexposure to radiation have substantial effects on lowering OS levels, thus improving male fertility. Besides lifestyle changes, antioxidant therapy is also used to prevent OS in the body, but additional studies are required for modulating their doses and duration.

Table 9.2 Miscellaneous plants responsible for improving male fertility

S. no.	Plant name (family)	Part used	Extract/phytoconstituent category	Mechanism of action	Effects	References
1	*Ajuga iva* (L.) *Schreb.* (Lamiaceae)	Leaves	Methanol extract	Enhancing the testosterone and 17β-estradiol levels	Protecting from oxidative stress and cellular toxicity and maintaining the number and motility of spermatozoids	Hamden et al. (2008)
2	*Apium graveolens L.* (Apiaceae)	Leaves and seeds	Phyto-estrogens	Stimulating effects on testosterone levels	Enhance spermatogenesis	Abarikwu et al. (2020)
3	*Camellia sinensis* L. (Theaceae)	Leaves	Powder	Upregulated the gene expression of SOD1, SOD3, and XRCC1 and improved the levels of SOD and GSH	Reduce the rate of sperm malformation by affecting the change of androgen levels and oxidative stress	Han et al. (2020)
4	*Cardiospermum halicacabum* L. (Sapindaceae)	Aerial parts	Saponins and flavonoids	Boosted serum testosterone level	Increase in caput and epididymal sperm count and sperm motility	Adewoyin et al. (2017)
5	*Chlorophytum borivilianum* Santapau & R.R. Fern. (Asparagaceae)	Roots	Aqueous extract/phenolic compounds	Increase SOD, CAT, and GP_X, increase epididymal sperm density	Increase sperm count, motility, and viability	Giribabu et al. (2014)
6	*Cinnamomum zeylanicum* L. (Lauraceae)	Bark	Phenolic compounds and essential oil	–	Improvement of male infertility	Yüce et al. (2013)
7	*Cistanche tubulosa* (Schrenk) Wight (Orobanchaceae)	–	Echinacoside	Enhancing star, CYP11A1, 3β-HSD, 17β-HSD, and CYP17A1 levels	Increased sperm motility	Santiago et al. (2021)
8	*Crocus sativus* Linn. (Iridaceae)	Flowers/ stigmas	Safranal	–	Improved normal morphology and total and progressive sperm motility	Heydari et al. (2008)
9	*Curcuma longa* (Zingiberaceae)	Rhizomes	Curcumin	Reduced MDA and inflammatory markers	Increased sperm count, concentration, total motility, and vitality	Alizadeh et al. (2018)
10	*Eruca sativa* Mill. (Brassicaceae)	Aerial parts	Aqueous extract/polyphenols and flavonoids	Restores the activity of antioxidant enzymes such as SOD, CAT, and GPX in rat testis	Increase sperm progressive motility and viability, mitochondrial function, and decreased immotile sperm	Grami et al. (2020), Grami et al. (2018)
11	*Lepidium meyenii* Walp. (Brassicaceae)	Roots	Ethanolic extract/macamides, macaenes, and glucosinolates	Improvement of the systemic antioxidant capacity of stallions	Increase testis weight, sperm count, motility, ejaculate volume, improved erection, mounting activity, and sperm quality	Clément et al. (2010) Del Prete et al. (2018)

12	*Ionidium suffruticosum* (L.) Ging (Violaceae)	Leaves	Alkaloids, steroidal lactones, and flavonoid	Increasing testicular oxidative biomarkers, SOD, and CAT	Increased spermatogenesis, increased sperm counts, lessened sperm agglutination	Chenniappan and Murugan (2017)
13	*Moringa oleifera Lam.* (Moringaceae)	Leaves	Hydroethanolic extract/ naringin	–	Increase testicular weight and volume	Santiago et al. (2021)
14	*Petasites japonicas* (Asteraceae)	Leaves	Butanol fraction of methanolic extract/ eremophilane-type sesquiterpenoids	Stimulate spermatogonial stem cell proliferation and through its antioxidant effects	Increase spermatogenesis	Kang et al. (2015)
15	*Punica granatum* (Lythraceae)	Fruit rind	Tannins, phenols (ellagic acid), and flavonoids	Reduced oxidative damage by suppressing extra-creation of free radical	Improved semen and biochemical parameters by scavenging ROS and preventing oxidative DNA damage	Al-Olayan et al. (2014), Türk et al. (2010)
16	*Sesamum indicum* (Pedaliaceae)	Seed, seed powder/ oil	Unsaturated fatty acids and lignans such as sesamin and sesamolin, sesamol, sesamin, butylated hydroxytoluene	Increasing body antioxidant activities	Increasing seminal parameters, testosterone level	Abbasi et al. (2013), Ashamu et al. (2010), Khani et al. (2013)
17	*Solanum lycopersicum* L. (Solanaceae)	Fruit	Lycopene	Normalizing the activity of antioxidant enzymes	Protect rat testis from germ cell loss, preventing testis and epididymis loss of weight and restoring the impairment of sperm motility. Improve sperm parameters in oligozoospermia	Ateşşahin et al. (2006), Santiago et al. (2021), Nouri et al. (2019)
18	*Tinospora cordifolia* (Menispermaceae)	Whole plant	–	–	Increases semen cholesterol and antioxidant parameters	Jayaganthan et al. (2013)
19	*Tribulus terrestris* (Zygophyllaceae)	Fruits	Methanol extract/4,5-di-p-coumaroylquinic acid	Increased release of testosterone, FSH, and LH and enhanced tissue antioxidant capacity	Pretreatment with methanolic extract has protective and antioxidant effects in sodium valproate intoxicated rats	Hammoda et al. (2013), Shalaby and Hammouda (2014)
20	*Vitis vinifera* (Vitaceae)	Seeds	Anthocyanin oligomers (flavonoid)	Increases intracellular vitamin C levels and scavenges ROS and free radicals	Protected sperm cell against DNA damage, reduced activities of nitric oxide synthase, and attenuated apoptosis of germ cells induced by torsion/detorsion of testicles	Bayatli et al. (2013), Hala et al. (2010), Shi et al. (2003)
21	*Zingiber officinale* (Zingiberaceae)	Roots/ rhizomes	Aqueous, ethanolic extract/ zingerone, gingerdiol, zingiberene, gingerols, and shogaols	Increase total antioxidant capacity and androgenic activity	Increases sperm parameters (viability, concentration and motility), decrease sperm cell abnormality	Abo-Ghanema et al. (2012), Bordbar et al. (2013), Khaki et al. (2009), Khaki et al. (2008), Zahedi et al. (2010)

SOD superoxide dismutase, *GSH* glutathione, *GSH-PX* glutathione peroxidase, *MDA* malondialdehyde, *ROS* reactive oxygen species, *T* testosterone, *LH* luteinizing hormone, *FSH* follicle-stimulating hormone

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10

Natural Products as the Modulators of Oxidative Stress: An Herbal Approach in the Management of Prostate Cancer

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Abstract

Prostate cancer is the most commonly diagnosed and frequently occurred cancer in the males globally. The current treatment strategies available to treat prostate cancer are not much effective and express various adverse effects. Hence, there is an urgent need to identify novel treatment that can improve patient outcome. From times immemorial, natural products are highly recognized for novel drug development for various diseases including cancer. Cancer cells generally maintain higher basal levels of reactive oxygen species (ROS) when compared to normal cells due to its high metabolic rate. However, initiation of excess intracellular ROS production can not be tolerated by the cancer cells and induce several cell death signals which are in contrast to normal cells. Therefore,

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small molecules of natural origin that induce ROS can potentially kill cancer cells in specific and provide a better opportunity to develop a novel drug therapy. In this review, we elaborated various classes of medicinal compounds and their mechanism of killing prostate cancer cells through direct or indirect ROS generation. This can generate a novel thought to develop promising drug candidate to treat prostate cancer patients.

Keywords

Oxidative stress · Prostate cancer · ROS

10.1 Introduction

Cancer is the major health concern in the entire world due to the increase in prevalence, mortality rate, as well as cost in the treatment. Cancer not only causes remarkable damage to health but also affects the economy of the country, and it is also the second most leading cause of death worldwide (Casey et al. 2015; Salehi et al. 2019a). Cancer growth and progression always depend on the association of the cancer cells and the microenvironment, and it is the most important reason for tumorigenesis. Tumor generally originates as an uncontrolled cell proliferation in different tissues and migrates to its surrounding tissues (Casey et al. 2015; Shokoohinia et al. 2018). In spite of tremendous development in the treatment of cancer, cancer remains as one of the leading causes of mortality. Moreover, the cases of cancer are increasing sharply because of many risk factors such as the use of tobacco, consumption of alcohol, consuming diet with low fruits and vegetables, physical inactivity, overweight, and obesity (Institute of Medicine Committee on Cancer Control in and Middle-Income 2007). Moreover, patients who survive from cancer had to face persistent complications related to physical, cognitive, and psychosocial struggles and other side effects (Shokoohinia et al. 2018).

Among several cancers, prostate cancer (PC) is the most frequently occurring and most frequently diagnosed cancer in the men after the lung cancer (Rawla 2019). The prostate gland is a part of human male reproductive system, which is of walnut size located at the bottom of the urinary bladder. Androgen receptor (AR) signaling via dihydrotestosterone (DHT) is the primary motivating force behind prostate development (Berman et al. 2004). The prostate gland consists of three kinds of cells such as gland cells that secrete the liquid part of the semen, the muscle cells that control urine flow and ejaculation, and the fibrous cells that support the gland. Additionally, the prostate gland plays a prominent role in keeping the semen in alkaline conditions and maintaining the life span of the sperm (Kim and Kim 2013).

Prostate cancer is initiated with a mutation in normal prostate cells, usually beginning within luminal cells since prostate cancer predominately consists of luminal cells and lacks basal cell antigens CK5/14 and p53 expression (Xin 2013). The malignant cells develop and begin to multiply, invading the surrounding prostate tissue to form a tumor nodule. These nodules may remain localized within the prostate for years. PC may metastasize to the nearby tissues including bones and lymph nodes (Alukal and Lepor 2016). The available data from animal and human genetic studies reveals strong evidence for the role of genetics in development of PC. Various candidates of genes, with multiple signaling pathways, and those participate in androgen action (ETS family of genes), DNA damage restoration, carcinogenesis, and sex steroid hormone metabolism and inflammation play important roles in PC (Caruso et al. 2009).

Prostate cancer is a major health concern globally during the last few decades, approximately 1.6 million new cases were diagnosed in the year 2015, and also 366,000 deaths were documented (Pernar et al. 2018). In recent years, attention has increased on PC due to the increasing mortality and morbidity rates in the world (Attard et al. 2016). According to the American Cancer Society,

the chances of cancer development in men in their entire life are 1 in 9 persons, and moreover, 1 man out of 41 will die of PC (American Cancer Society. *Key Statistics for PC*; American Cancer Society: Atlanta, GA, USA, 2018).

In India, the prevalence of PC is lower as compared to western countries. However, due to the rise in migration of rural population to the urban areas, industrial development and occupational hazards are leading to an increase in the number of cases (Hariharan and Padmanabha 2016; Jain et al. 2014).

Personage biology and lifestyle changes are influential risk factors for developing PC. The considerable risk factors include age, height and weight of the individual, familial history (Giovannucci et al. 1997; Graff et al. 2018), diabetes, body mass index, and vasectomy (Hariharan and Padmanabha 2016). Additionally, the modifiable risk factors include smoking, physical activity, and food habits (Pernar et al. 2018).

Serum prosthetic acid phosphatase estimation was used as the standard test to diagnose PC in patients until the arrival of prostate-specific antigen (PSA) (Sarwar et al. 2017). General and traditional methods to diagnose PC are traditional rectal examination, PSA level, ultrasound-guided biopsy (USGB), sum of Gleason patterns, and clinical stage. Novel biomarkers are available for effective diagnosis of PC even in nanolevels, for example, PC gene-3 mRNA overexpression in urine (Wei et al. 2014) and Ga68 prostate-specific membrane antigen (PSMA) represent a new emerging image-based technique used for lymph node staging of PC (Descotes 2019).

Current treatment protocols for PC include surgery, androgen deprivation therapy, chemotherapy, and radiation. Androgen deprivation therapy is the common and oldest strategy from past decades, but soon it develops resistance and progresses into castration-resistant PC (CRPC). Moreover, the conventional chemotherapy usually soon develops severe side effects as well as drug resistance in PC patients. Therefore, new therapeutic agents or strategies are highly encouraged to improve PC patient conditions (Chen et al. 2018).

Due to huge chemical diversity, natural products and derived compounds act as a better source in identifying suitable therapeutic agents for various diseases including cancer (Dutta et al. 2022; Huang et al. 2018; Mandal et al. 2013; Nelson et al. 2016; Nelson et al. 2020). From the ancient times, human beings are well associated with natural products especially medicinal plants for treating multiple diseases (Dutta et al. 2021). Several evidences suggest that secondary metabolites derived from the natural sources were used for treating different kinds of cancer and moreover approximately 50% of drugs that are clinically used today were obtained from natural origin (Gach et al. 2015). For example, plant-derived compounds such as taxol analogs, vinca alkaloids like vincristine and vinblastine, and podophyllotoxin analogs were used against different kinds of cancers through modulating the pathways related to growth and progression of cancer (Choudhari et al. 2019).

In addition, there are several other phytochemicals that are reported to induce cytotoxicity in numerous cancer cells. However, the induction of cell death is not specific to the cancer cell alone. Hence, there is a huge requirement of therapeutic strategies that can only trigger cell death in cancer cells but not in normal cells (Xu et al. 2013).

Several documented reports suggest that due to higher metabolic rate, cancer cells like PC cells generally maintain higher oxidative stress level for its growth and progression, which is in contrast to the normal cells (Gach et al. 2015). Reactive oxygen species (ROS) are generally small molecules and are short-lived. These are the by-products of normal aerobic cellular metabolism and are highly reactive in nature (Reczek and Chandel 2017). The presence of a single unpaired electron in their outermost shell makes them highly reactive in nature (Liou and Storz 2010). Generation of ROS within accepted limits is necessary for the regulation of cellular redox homeostasis (Ivanova et al. 2016), including cellular response against infections, and signal transduction and to induce mitogenic response (Sarsour et al. 2008; Valko et al. 2007).

Nevertheless, upregulation of reactive ROS higher than the threshold level or downregulation of the antioxidant system in cancer cells will make the cancer more sensitive toward ROS-induced cell death, while the normal cells via adaptive mechanism preserve its redox homeostasis. Several anticancer drugs were reported to upregulate the ROS specifically in cancer cells, which lead to trigger cell death via activating apoptosis or necrosis or other cell death signals. Hence, treatment of cancer through increasing the level of intracellular ROS can be considered as a successful approach to treat PC (Chen et al. 2017; Xu et al. 2013). Any exogenous biological or plant-derived ROS-generating agents that induce redox imbalance in cancer cells lead to more vulnerability as compared to normal cells and cause cell death (Raza et al. 2017). Accordingly, many research scientists attempted to prove this and succeeded (Kim et al. 2019).

In this review, we summarize various naturally originated compounds and their mechanisms underlying the effects of anticancer utilized in oxidative stress-inducing chemotherapy for direct or indirect ROS generation. This can develop a better idea to generate promising therapeutic tool to treat PC.

10.2 ROS and Its Signaling in Prostate Cancer Cell Death

In the cellular metabolism, several reactive oxygen molecules will be generated, which are so reactive with very short life span. ROS at optimum concentration in cancer cells is safe and helps in maintaining many physiological functions such as cell cycle progression, proliferation, and migration. Interestingly, cancer cells like PC require high level of ROS (optimum concentration is high) to maintain its physiological functions, and this makes the cancer cells highly vulnerable to ROS-mediated cell death. Hence, the slightest increase of ROS via small molecules leads to oxidative stress, and cancer cells are pushed toward the cell death via various signaling pathways including apoptosis, necrosis, and autophagy-associated cell death, and it is in contrast to normal cells. ROS-induced cell death in PC takes place via several signaling pathways, as explained below.

10.2.1 Apoptosis

Apoptosis, also called programed cell death, is a highly regulated physiological process of cell death; in this, the cell will undergo self-destruction upon stimulation with appropriate trigger. In this process, the cells that are no longer needed, damaged, mutated or aged, and unrepairable are removed (Ismail et al. 2019).

ROS is vital part in both cancer and apoptosis though the two are opposed phenomena. Many researchers agreed that there is increased amount of ROS during the apoptosis, but it is always debatable since even under anaerobic conditions the cell will undergo for apoptosis (Matés and Sánchez-Jiménez 2000). On other hand, some anticancer agents like cisplatin, vincristine, and etoposide require ROS to induce cell death (Inoue et al. 2000).

The generation of ROS has been suggested to occur at increased rates during apoptosis (Rollet-Labelle et al. 1998). ROS does not induce apoptosis directly; rather, it stimulates some factors which induce the direct apoptosis process. On the other hand, mitochondrial-derived ROS accountable for full activation of the caspase cascade which play important events of the apoptosis (Schulze-Osthoff et al. 1992). The mitochondrial-derived ROS may activate sphingomyelinase-generating ceramide, which is a type of intracellular mediator of apoptosis (Liu et al. 1998).

The characteristic features of apoptosis process are contraction, and fragmentation of nuclei, along with enlarged endoplasmic reticulum, cell and cytoplasm shrinkage, and loss of grip with other cells (Hotchkiss et al. 2009). Apoptosis could be induced by either mitochondrial membrane pathway or tumor necrosis factor (TNF) cell death receptor pathway. Mitochondrial membrane pathway is activated by various intracellular stimuli or signals like DNA damage,

deprivation of growth factor, and oxidative stress or by various toxic agents (Su et al. 2015). The positive stimulus activates the mitochondrial outer membrane permeabilization, which is under the control of antiapoptotic (Bcl-2, Bcl-xL MCL-1, and BCL-W) and pro-apoptotic (Bax, Bak, Bad, Bid, Bim, Bik, Hrk, Bcl-XS, Bcl-G,) genes, a group of bcl2 family proteins. The anti-apoptotic genes guard the membrane structure and evade the discharge of cytochrome c, but this can be opposed by the pro-apoptotic genes (Arumugam and Abdull Razis 2018). Imbalance between these genes causes the release of cytochrome c in to the cytosol, and it binds to monomer of adapter protein apoptotic protease-activating factor-1 (APAF1) at WD domain of APAF-1 monomers and results in a conformational change in APAF-1 exposing a nucleotide-binding and oligomerization domain that is able to bind deoxy-ATP (dATP) (D'Arcy 2019). Few numbers of this oligomerized complexes form together as a heptameric structure called the apoptosome. The apoptosome binds to pro-caspase-9 and activates to caspase-9, which activates the executioner caspases-3 from its pro-form (Lopez and Tait 2015) which ultimately ends the cell death. The executioner pathway follows endonuclease activation, and chromosome degradation causes chromatin and cytoskeleton condensation and apoptotic body formation.

TNF associated cell death pathway or extrinsic pathway of apoptosis is initiated by the binding of death ligands like TNF-α or Fas ligand (FasL) APO-2 L and CD95L which are produced from natural killer (NK) cells or macrophages to the TNF superfamily receptors also called as death receptors plays a central role in the process of apoptosis. Upon binding with TNF-α receptor (TNF-R1, also called complex I) leads to activation of TNF-R1-associated death domain (TRADD) on death receptor and allows to recruit receptor-interacting protein (RIP) which interacts directly with its death domain leading to activation of the NF-kB pathway, as well as mitogen-activated protein kinase (MAPK) pathways (Singh et al. 2015). Binding of TNF-α to TNFR1 causes the formation of two successive complexes: complex I and complex II. Complex I elicits a non-apoptotic signaling pathway, whereas complex II, internalized, triggers cell death (Fouqué et al. 2014). The apoptotic pathways are activated by a second complex, known as complex II which includes RIP, Fas-associated death domain (FADD) protein, and TRADD (Micheau and Tschopp 2003). Binding of adaptor protein FADD is necessary for the activation of pro-caspase-8 and pro-caspase-10 to caspase-8 and caspase-10 activating caspase-3 and caspase-7 (Schultz and Harrington Jr. 2003). The executioner pathway follows endonuclease activation, and chromosome degradation causes chromatin and cytoskeleton condensation and apoptotic body formation.

10.2.2 Autophagy

Autophagy is a catabolic mechanism stimulated by different conditions like nutrient deprivation, growth factor depletion, cellular stress infection, and hypoxia. In the autophagy, lysosome releases various hydrolytic enzymes which destruct the cell and its organelles. Cell homeostasis is regulated by autophagy by removing cancer causing molecules and damaged organelles; by this, it protects the cell (White 2012). Failure of autophagy may potentially permit the development of cancer and accumulation of protein aggregates in the neurons, and the development of neurodegenerative conditions including Alzheimer's disease (D'Arcy 2019).

The character of cancer cell i.e., rapid metabolic rate even at normal conditions helps to produce more ROS. This condition makes cancer cells more susceptible to ROS-mediated insults and cell death (Ling et al. 2011). Mitochondrial-mediated ROS control various signaling molecules involved in signal transduction processes (Li et al. 2015) (Scherz-Shouval and Elazar 2007).

Free radicals and autophagy are important factors in the regulation of signaling pathways (Underwood et al. 2010). In response to stressful markers like cellular stress, ischemia reperfusion, and nutritional deprivation, significant levels of ROS generated subsequently induce autophagy

(Essick and Sam 2010). Another possible mechanism proposed by Dadakhujaev et al. is that autophagy cannot remove excess free radicals when its levels exceed the autophagy capacity which results to autophagic cell death (Dadakhujaev et al. 2009).

Autophagy holds anticancer mechanisms by removing aged and damaged organelles and activating the apoptosis which would help tumorigenesis (Lin et al. 2017). Autophagy will occur as macro-autophagy, micro-autophagy, and chaperone-mediated autophagy; all these mechanisms promote proteolytic degradation of cytosolic components at the lysosome.

Autophagy is a programmed process controlled by various genes (Mizushima et al. 2011). The process includes initiation of autophagosome, nucleation of the autophagosome, expansion and elongation of the autophagosome membrane, closure and attachment with the lysosome, and finally degradation of intravesicular products (Mulcahy Levy and Thorburn 2020).

In the cytosol, the process of autophagy is initiated in response to various stress markers such as starvation, hypoxia, oxidative stress, protein aggregation, and endoplasmic reticulum stress. Generally, these stress markers target the complex of proteins called ULK1 (Unc-51-like kinase 1). This complex comprised of ULK1, autophagy-related protein 13 (ATG13), RB1-inducible coiled-coil protein 1 (FIP200), and ATG101 and activates the nucleation process of phagophore by phosphorylating components of the class III PI3K (PI3KC3) complex I including class III PI3K, vacuolar protein sorting 34 (VPS34), Beclin 1, ATG14, activating molecule in Beclin 1-regulated autophagy protein 1 (AMBRA1) and general vesicular transport factor (p115). This complex will activate the phosphatidylinositol-3-phosphate (PI3P) in the cytosol. PI3P recruits its effector proteins WIPI-2 (WD repeat domain phosphoinositide-interacting protein-2) and zinc finger FYVE domain-containing protein 1 (DFCP1) to the omegasome by binding with their PI3P-binding domains.

WIPI-2 binds ATG16L1 protein, which directly increases the ATG-3-mediated conjugation of ATG-8 family proteins including microtubule-associated protein light-chain 3 (LC-3) proteins and γ-amino butyric acid receptor-associated proteins (GABARAPs), to membrane-resident phosphatidylethanolamine (PE). During this conjugation, LC-3-I is converted into LC-3-II. LC-3 protein plays a key role in the attraction of the autophagic components which contain LC-3-interacting region and involves in elongation and closure of the phagophore membrane. LC-3 is seriously involved in the sequestration of cytoplasmic cargos into autophagosomes via LC-3-interacting region containing cargo receptors. Organelles like plasma membrane, mitochondria, and Golgi complex contribute to the elongation of the autophagosomal membrane by donating their membrane material. Sealing of the autophagosomal membrane gives rise to a double-layered vesicle called the autophagosome; later on, it fuses with the lysosome. The lysosomes hydrolyzes the autophagosome, and rescued nutrients are released back to the cytoplasm for reuse (Dikic and Elazar 2018; Li et al. 2019; Mulcahy Levy and Thorburn 2020).

10.2.3 Necrosis

ROS has a significant role in tumor-targeted therapy. Increasing ROS levels is one of the novel therapeutic regimens for treating cancer patients with drug resistance (D'Arcy 2019). ROS are highly reactive molecules that function as second messengers in various signal transduction pathways including the regulation of cell death. Necrotic cell death induced by TNFα requires the production of ROS (Morgan and Liu 2010).

Necrosis is an irreversible inflammatory form of cell death in which cytoplasmic granulation along with organelle and/or cellular swelling leads to disruption of plasma membrane and spillage of cellular contents to cause cell death (Conrad et al. 2016). It has been difficult to characterize the essential regulators of necrotic cell death in the absence of apoptosis (Amaravadi and Thompson 2007). Hence, the regulated form of necrosis happens along with apoptosis molecular

machinery and the so-called necroptosis (Fulda 2016) (hereafter called necroptosis). Originally, necrosis happens under pathological conditions when the cell or tissue is directly exposed to various stimuli like radiation, trauma, or bacterial and viral infection. This can be activated by death receptors, predominantly TNFR1, Toll-like receptor 3 (TLR3), and TLR4 (Oliveira et al. 2018).

The molecular mechanism of cell death in necroptosis is initiated by the phosphorylation of receptor-interacting protein kinases 1 and 2 (RIPK1, RIPK3) and mixed lineage kinase domain-like (MLKL) pseudokinase. RIPK1 plays a pivotal role in death receptor signaling pathways by activation of NF-κB and MAP (mitogen-activated protein) kinases and the induction of apoptosis and necroptosis (Green 2019).

Binding of TNF to its receptor activates receptor-associated complex I. Complex I is composed of TNFR1, TNFR1-associated death domain (TRADD), RIPK1, TNFR-associated factor 2 (TRAF2), cellular inhibitor of apoptosis protein 1 (cIAP1), cIAP2, andlinear ubiquitin chain assembly complex (LUBAC). Complex I will undergo ubiquitylation and deubiquitylation reactions and activate NF-κB signaling, cell survival signals, and cell death-inducing mechanisms. If complex I is stabilized by cIAP1 and cIAP2, the cell will undergo survival mode (Dondelinger et al. 2015). On the other hand, complex I may switch to cell death signaling, causing destabilization of the receptor-associated complex I, and transforming complex I to a cytosolic complex IIa, which may lead to caspase dependent apoptosis (Wang et al. 2008).

In another way, complex IIb is composed of RIPK1, RIPK3, FADD, and caspase 8 and favours RIPK1 kinase activity-dependent apoptosis, which is pharmacologically inhibited by necrostatin-1 (Takahashi et al. 2012). However, complex I transforms to complex IIc, also called as necrosome, which consists of RIPK1, RIPK3, and mixed lineage kinase domain-like (MLKL) pseudokinase, when caspase-8 activity is inhibited or inactive (Yu et al. 2020).

RIPK3 recruits MLKL by the kinase domain, phosphorylates at Thr357 and Ser358, causes destabilization of the closed structure, and allows oligomerization of MLKL at the plasma membrane (Hildebrand et al. 2014). The oligomers of MLKL like cardiolipid, a negatively charged phospholipid, will bind to the plasma membrane of the cell. MLKL oligomers with plasma membrane act directly as a pore-forming complex and cause plasma membrane destabilization (Dondelinger et al. 2015; Wang et al. 2008), or failure of the Ca^{2+} or Na^{+} ion channels will allow an increase in intracellular osmotic pressure and contribute to cell oncosis (swelling of cell) (Conrad et al. 2016). Oncosis results in leakage of cellular debris into surrounding tissues and causes damage to surrounding cells (inflammation) (Pasparakis and Vandenabeele 2015).

10.3 ROS-Mediated Cell Death in Prostate Cancer Through Phytocompounds

Prostate cancer is a heterogeneous and more frequently diagnosed disease in male. However, the current treatment approaches were are not able to meet the needs of the patients and also exhibit severe adverse effects as the drugs do not act specifically on the cancer cells alone. Due to the lack of sensitive treatment, the cases of PC were increasing in the entire world very rapidly. Hence, there is a great need of identifying novel drug treatment with minimal toxicity (nontoxic to normal cell).

Due to the availability of never matched chemical library and also due to less toxicity, all the time, medicinal plants and derived chemical moieties act as a better source for drug discovery (Nelson et al. 2020; Pullaiah et al. 2018; Pullaiah et al. 2017; Pullaiah et al. 2021; Singh et al. 2018). Several documented evidences revealed that cancer cells like PC maintain high level of ROS for growth and progression, unlike normal cells. Therefore, slightest increase of ROS through small molecules in the cancer cells can not be tolerated and promotes cell death. This provides an opportunity to develop a promising drug treatment that can target cancer cells alone.

In this study, we describe a mechanism of killing PC cells by several important phytoconstituents through ROS associated with various molecular pathways.

10.3.1 Apigenin

Apigenin, a plant-derived phytochemical, belongs to the class of flavonoid. It is widely available in vegetables (parsley, celery, and onions), fruits (orange), and herbs (chamomile, thyme, oregano, basil) (Salehi et al. 2019b). Apigenin is majorly found in the family Apiaceae also known as Umbelliferae (Shankar et al. 2017). Apigenin exhibits a wide range of pharmacological activities and was used to treat different kinds of diseases such as depression, insomnia, cancer, amnesia, diabetes, and Alzheimer's disease (Salehi et al. 2019b). Apigenin shows anticancer effect against various kinds of cancer cells (Salehi et al. 2019b). It was reported that it induces cell death in PC cells via generating ROS (Morrissey et al. 2005; Shukla and Gupta 2008). It induces cell death in PC cell 22Rv1 cells via ROS mediated apoptosis, which is connected with p53 upregulation and downregulation of MDM2 protein and NF-kB/p65 (Shukla and Gupta 2008). Another study reavels apigenin cell death in PWR-1E, LNCap, PC-3, and DU145 cells by means of ROS-mediated caspase-dependent apoptosis. It also revealed apigenin decrease Bcl-2 expression and mitochondrial permeability (Morrissey et al. 2005).

10.3.2 Artesunate

Artesunate is a sesquiterpenoid and a water-soluble derivative of artemisinin isolated from Chinese herb *Artemisia annua* L. associated belonging to the family Asteraceae. Artesunate is specifically used to treat malaria disease (Cen et al. 2018; Hamacher-Brady et al. 2011). It shows many biological activities such as anti-inflammatory, antiseptic, antiangiogenic, and anti-fibrosis (Cen et al. 2018). It also exhibits antitumor effect against various cancer cells like BGC-823, HGC-27, and MGC-803 (gastric cancer cells), MDA-MB-231 (breast cancer cells), and IMR-32 (neuroblastoma cells) (Michaelis et al. 2010; Zhang et al. 2015a). It was reported that artesunate induces cell death in breast cancer cell lines through mitochondrial apoptosis via ROS generation (Hamacher-Brady et al. 2011). Artesunate also shows antitumor effect on human pancreatic ductal epithelial (HPDE) cells through activation of programed cell death. It was reported that artesunate induces maximum cell death in pancreatic ductal adenocarcinoma (PDAC) cell lines with constitutively active KRas via ROS-mediated iron-dependent apoptosis (Eling et al. 2015).

10.3.3 Andrographolide

Andrographolide, a diterpenoid lactone type of medicinal compound extracted from traditional medicinal herb *Andrographis paniculata*, belongs to the family Acanthaceae (Liu et al. 2017; Wei et al. 2018). It possesses a wide range of pharmacological activities such as anti-inflammatory, antiviral, antibacterial, antihypertensive, antimalarial, anti-HIV, hepatoprotective, and neuroprotective. Andrographolide also exhibits antitumor effect against many cancer cells such as HCT-116, MCF-7, B16, LNCaP, and PC-3 (Banerjee et al. 2016; Islam et al. 2018; Malik et al. 2021; Rajagopal et al. 2003). Numerous reports suggest that andrographolide causes cell cycle arrest through upregulation of p27 and downregulation of cyclin-independent kinase 4. It is also known to trigger caspase-8-dependent apoptosis via ROS induction (Chun et al. 2010). Moreover, in PC cells, andrographolide promotes TRAIL-induced apoptosis through upregulation of DR4 and p53-mediated ROS generation (Wei et al. 2018).

10.3.4 Carvacrol

Carvacrol is a phenolic monoterpenoid class of secondary metabolite isolated from the essential oils of bergamot (*Citrus aurantium*), oregano

(*Origanum vulgare*), thyme (*Thymus vulgaris*), and pepperwort (*Lepidium flavum*). It was known from the earlier studies that carvacrol shares a variety of biological functions such as antimicrobial, antioxidant, anti-inflammatory, antimutagenic, antiparasitic, hepatoprotective, and angiogenic properties (Baser 2008; Sharifi-Rad et al. 2018). From the earlier reports, it was revealed that carvacrol shows anticancer effect against several cancer cell lines through ROS-induced apoptosis signals (Fan et al. 2015; Khan et al. 2018). In addition carvacrol promotes antiproliferative effect on human prostate cancer cells (PC-3) linked with downregulation of pSTAT3, pAKT, and pERK1/2 levels (Heidarian and Keloushadi 2019). In a study on DU-145 cells, carvacrol retards the proliferation of the cells in a time- and concentration-dependent way. Moreover, the anticancer potential on DU145 is associated with elevation of ROS and disruption of mitochondrial membrane potential which in turn leads to caspase-3-dependent apoptosis (Khan et al. 2017). Another study conducted by the same group of scientists revealed the anticancer effect of carvacrol on PC-3 cells. It discloses that carvacrol triggers the ROS-mediated apoptosis through modulation of Bax, Bcl-2, and caspase expression. The study also reveals carvacrol promotes the cell cycle arrest at G0/G1 phase in connection with downregulation of cyclin D1, cyclin-dependent kinase 4 (CDK4), and Notch signals (Khan et al. 2019).

10.3.5 Curcumin

Curcumin, a polyphenol and structurally diarylheptanoid compound, belongs to the class of curcuminoids. Curcumin was isolated from rhizome of *Curcuma longa* species that belongs to the family *Zingiberaceae*. Generally, curcumin is used in wide variety of food materials as a spice, condiment, and pigment. It also shows various pharmacological functions such as anti-inflammatory, antioxidant, neuroprotective, radioprotective, and antitumor effects (Amalraj et al. 2017). The anticancer effect of curcumin was well studied, and it is effective against variety of cancer cells. Clinical studies have proven that curcumin's anticancer effect on PC cells via targeting AR signaling, Bcl-2, NF-κB, AP-1 protein, PI3k/Akt/mTORWnt/β-catenin/TGF-β/MYC pathways, and other signaling pathways (Abd Wahab et al. 2020; Katta et al. 2019). From the reports, it was known that curcumin exerts its antitumor effect on the PC cells like LnCap and PC3 cells through modulating several pathways including downregulation of EGF-R and EGF-R tyrosine activity (Dorai et al. 2000; Suvranil et al. 2021). Curcumin blocks the cancer-associated fibroblast (CAFs) formation through inhibiting tumorigenesis, metastasis invasion, and EMT in PC cells. Moreover, the inhibition of CAFs is associated with upregulation of ROS, IL-6, and CXC chemokine receptor-4 (CXCR-4) receptor expression and downregulation of MAO-A/mTOR/HIF-1α signaling (Du et al. 2015). The study conducted by Rivera et al. revealed that curcumin induces apoptosis in PC cells which mediated through increased ROS and endoplasmic stress response. It also discloses that increased ER stress is associated with increase of pro-apoptotic markers such as caspases (3,9, 12) and poly-(ADP-ribose) polymerase (Rivera et al. 2017). Curcumin analogue WZ35 showed more potent anticancer effect on PC cells than curcumin, and similar to curcumin, it also triggers ER stress-mediated cell cycle arrest and apoptosis in human PC cells. Moreover, the induction of endoplasmic reticulum stress is associated with upregulation of ROS and decrease of CHOP (Zhang et al. 2015b).

10.3.6 Guggulsterone

Guggulsterone, a phytosteroid compound extracted from the gum obtained from Ayurvedic medicinal plant *Commiphora mukul*, belonging to the family Burseraceae. It contains many secondary metabolites such as flavonoid, terpenes, and phytosterols that show various pharmacological activities such as anti-inflammatory, antimicrobial, anticancer, and various other biological functions. Guggulsterone, a steroid secondary

metabolite, also exhibits huge biological functions and helps in treating bone fracture, inflammation, arthritis, and cardiovascular and lipid disorders (Gujral et al. 1960; Sharma and Sharma 1977; Urizar and Moore 2003). Guggulsterone is active against various cancer cells by triggering apoptosis, which is linked with inhibition of various antiapoptotic signals such as IAP1, xIAP, Bfl-1/A1, Bcl-2, cFLIP, survivin, and activation of caspases (Shishodia et al. 2016). Singh et al. revealed that guggulsterone-induced apoptotic cell death in PC cells like LNCaP and PC-3 is connected with upregulation of reactive oxygen intermediate (ROI) and activation of c-Jun NH(2)-terminal kinase (JNK) signaling (Singh et al. 2007).

10.3.7 Isoalantolactone

Isoalantolactone, a medicinal plant-derived small molecule separated from the plant extract of *Inula helenium* L., which belongs to the family Asteraceae (Compositae). Isoalantolactone is a secondary metabolite placed under the class of sesquiterpene lactone. It shares wide range of biological functions such as antibacterial, anti-helminthic, antioxidant, and neuroprotective (Huang et al. 2021; Seo et al. 2014). Besides this, isoalantolactone also exhibits anticancer property against various cancer lines such as leukemia, prostate, lung, colon, ovary, and melanoma cells (Rasul et al. 2013b). Isoalantolactone triggers cell death in PC like PC-3 and DU145 cells through activation of apoptosis signal in connection with endoplasmic reticulum stress through the production of ROS, and it eventually involves downregulation of protein levels of p-STAT3 and STAT3 (Chen et al. 2018) . In another study, it was found that isoalantolactone retards the growth of both androgen-dependent (LNCaP) and androgen-independent (PC-3 and DU145) PC cells through induction of apoptosis that is linked with ROS generation and dissolution of mitochondrial membrane potential. Moreover, it also alters the apoptosis signals including activation of Bax and caspase-3 and deactivation of Bcl-2 and survivin signals (Rasul et al. 2013a). In a study, it was revealed that isoalantolactone in combination with cisplatin enhances the sensitivity of PC cells via production of ROS that enables endoplasmic reticulum stress and JNK signaling in response to cisplatin treatment (Huang et al. 2021).

10.3.8 Parthenolide

Parthenolide is a sesquiterpene lactone type of naturally occurring small molecule isolated from medicinal herb *Tanacetum parthenium* which belongs to the family Asteraceae (Pareek et al. 2011). The plant holds potential medicinal values and is traditionally used to treat migraine headaches, infertility, rheumatoid arthritis, insect bites, menstrual cycle problems, stomachache, and during labor problems (Pareek et al. 2011). *Tanacetum parthenium* contains many pharmacologically active principles such as flavonoid glycosides and pinenes including sesquiterpene lactones. Parthenolide is a major active principle present in *Tanacetum parthenium*; it shares multiple pharmacological properties such as antibacterial, anti-inflammatory, and anticancer, and in addition, it is now approved for headache and migraine based on the clinical trial results (Snezana and David 2018). It shows cytotoxicity to various cancer cells; however, the anticancer potential is more specific to the malignant cells but not to the normal cells (Yang et al. 2016). From earlier studies, it was revealed that parthenolide exaggerates the production of ROS in the cancer alone and promotes apoptotic cell death. In a study on PC cells, it was disclosed that parthenolide radiosensitizes specifically PC-3 cells but there is no effect on normal prostate epithelial cells. In addition, it also promotes oxidative stress specifically in PC cells leading to NADPH oxidase activation followed by downregulation of reduced thioredoxin, FOXO3a, and upregulation of PI3K/Akt. Besides this, parthenolide also targets and decreases the level of antioxidant enzymes like manganese superoxide dis-

mutase and catalase (Sun et al. 2010). Moreover, parthenolide treatment increases the cellular ROS in mouse xenograft model developed by injecting PC-3 cells subcutaneously. It reduces the tumor volume by promoting the oxidation of thioredoxin, which leads to KEAP1 linked PGAM5 and Bcl-xL degradation. In addition, parthenolide induces cell death in LNCaP, DU145, and PC3 cells by enhancing the sensitivity toward the radiation through inhibition of NF-kB and activation of phosphatidylinositol-3-kinase/Akt signaling in the presence of PTEN (Sun et al. 2007).

10.3.9 Plumbagin

Plumbagin, a plant-derived secondary metabolite placed under the class of naphthoquinone, separated from the root extract of traditional medicinal plant *Plumbago zeylanica*, commonly called as chitrak. The plant belongs to the family Plumbaginaceae. It shows various biological functions such as anticancer, antifertility, antiulcer, antimicrobial, wound healing, and hepatoprotective property (Shukla et al. 2021). In addition, the plant also is used traditionally in many diseases likes diabetes, cardiovascular disorders, obesity, and cancer. Plumbagin is the major active principle in the *Plumbago zeylanica*, which possess wide varieties of biological functions including antitumor effect. Plumbagin exhibits an anticancer effect in several cancer cells via inhibition of NF-kB and elevating the intracellular ROS in the cancer cells, directing the cells to apoptosis (Huang et al. 2018; Powolny and Singh 2008; Shukla et al. 2021; Srinivas et al. 2004). Moreover, plumbagin induces PC cell death via ROS-induced endoplasmic reticulum stress. It also helps significantly to retard the tumor growth of PC xenograft via apoptosis through upregulation of ROS and endoplasmic reticulum stress (Huang et al. 2018). In another study, it was reported that plumbagin triggers cell death in prostate cells like PC-3, LNCaP, and C4–2 which are associated with DNA fragmentation, altering cell cycle, and moreover the cytotoxic activity of plumbagin toward PC-3, LNCaP, and C4–2 is actually independent of p53 and accompanied by upregulation of ROS and downregulating antioxidant enzyme superoxide dismutase 2 (Powolny and Singh 2008).

10.3.10 Sparstolonin B

Sparstolonin B (SsnB), a secondary metabolite, comes under the class of polyphenol and has structural similarities with isocoumarins. Isocoumarins in general are known for anticoagulant, anti-inflammatory, and antitumor properties. It was isolated from a traditional Chinese herb *Sparganium stoloniferum*, which was used mostly to treat different kinds of inflammatory diseases. The tubers of the plant are also used for antispasmodic and antitumor purposes (Liu et al. 2021). The anticancer effect of SsnB on PC is not well explored. However, a study conducted by Liu et al. revealed that SsnB suppresses the growth of prostate cells via arresting the cell cycle at G2/M phase. The study further revealed that the cell cycle arrest caused by SsnB in the PC cell is due to ROS-induced apoptosis. The results obtained from the experiment conducted to study the effect of SsnB on the PC-3-induced mouse xenograft disclosed that reduction of tumor growth is due to apoptosis linked with ROS-mediated downregulation of PI3K/AKT signaling (Liu et al. 2021).

10.4 Structures of the Phytocompounds

The phytocompounds inducing ROS-mediated cell death in PC cells have been presented in Table 10.1.

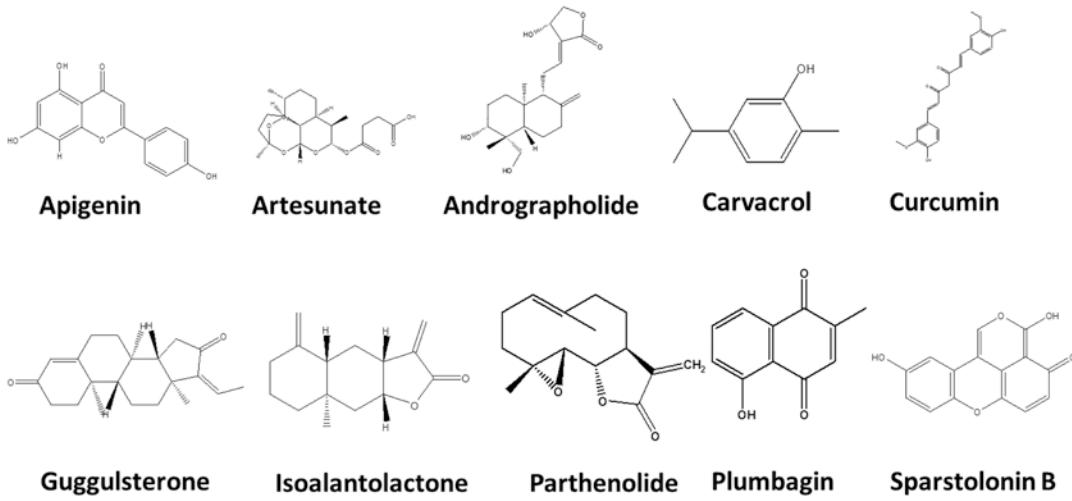
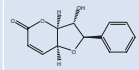
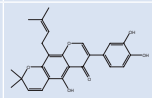
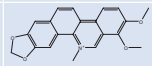
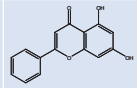
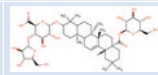
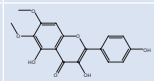
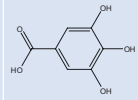
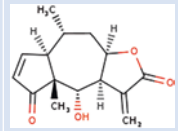
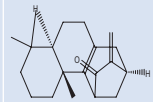
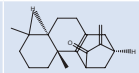
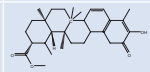
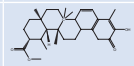
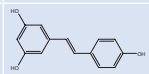
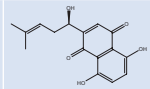


Table 10.1 Phytocompounds inducing reactive oxygen species (ROS)-mediated cell death in prostate cancer (PC) cells

Phytoconstituent	Structure	Biological source and nature	Mechanism of action	Reference
Altholactone		Styryl lactone derivative isolated from *Goniothalamus* spp. Family: Annonaceae	Apoptosis is induced via ROS generation linked with downregulation of NF-kB and upregulation of STAT3 in **DU-145** cells	Jiang et al. (2017)
Auriculasin		Prenylated isoflavone extracted from *Flemingia philippinensis* Family: Fabaceae	Triggers apoptosis in **LnCaP** cells via ROS-mediated decrease in phosphorylation of AKT/mTOR/p70s6	Cho et al. (2018)
Chelerythrine		Phenanthridine alkaloid isolated from *Chelidonium majus* Family: Papaveraceae	Induces apoptosis via ROS-mediated endoplasmic reticulum stress in **PC-3** cells	Wu et al. (2018)
Chrysin		Chrysin a natural flavone mainly found in numerous plant extracts, honey, and propolis	Promotes apoptosis in **DU-145** and **PC-3** cells through ROS-mediated endoplasmic reticulum stress that results in loss of mitochondrial membrane potential (MMP)	Ryu et al. (2017)
Chikusetsu		Saponin derivative isolated from *Aralia taibaiensis* Family: Araliaceae	Promotes apoptosis in **PC-3** and **LNCaP** cells via generation of ROS that leads to activation of caspase, apoptosis-inducing factors (AIF), and endonuclease G (EndoG)	Zhu et al. (2017)
Eupalitin		O-methylated flavonol obtained from *Ipomopsis aggregate* Family: Polemoniaceae	Induces ROS-mediated caspase-3-dependent apoptosis in **PC-3** cells	Kaleem et al. (2016)

(continued)

Table 10.1 (continued)

Phytoconstituent	Structure	Biological source and nature	Mechanism of action	Reference
Gallic acid		Polyphenolic compound extracted from red fruits, onions, and black radish	Causes apoptotic cell death of **LnCaP** cells through ROS-mediated and caspase-dependent mitochondrial potential loss, cytochrome c release	Russell Jr. et al. (2012)
Helenalin		Pseudoguaianolide sesquiterpene lactone isolated from various plant species of Asteraceae family	Triggers ROS-associated apoptosis in **DU-145** and **PC-3** cells via downregulation of thioredoxin reductase-1 (TrxR1) expression	Yang et al. (2021)
Isobavachalcone		Chalcone derivative isolated from *Psoralea corylifolia* Family: Leguminosae	Promotes ROS-induced apoptosis in **PC-3** cells via reduced expression of thioredoxin reductase-1 (TrxR1)	Li et al. (2018)
Jungermannenone A and B		Diterpenoid isolated from liverwort *Jungermannia fauriana* Family: Jungermanniaceae	Triggers ROS-mediated caspase-dependent apoptosis in **PC-3** cells via targeting JNK pathway	Guo et al. (2016)
Piperlongumine		Amide alkaloid isolated from *Piper longum* L. Family: Piperaceae	Induces cell death in **DU-145** cells through ROS production and STAT3 inhibition	Kim et al. (2018)
Pristimerin		Quinone methide triterpenoid isolated from *Maytenus ilicifolia* Family: Celastraceae and Hippocrateaceae	Promotes apoptosis in **LNCaP and PC-3** cells through ROS-mediated caspase-dependent and ubiquitin-proteasomal degradation	Liu et al. (2013)
Resveratrol		Polyphenol isolated from *Polygonum cuspidatum* Family: Polygonaceae	Triggers ROS-dependent apoptosis in **TRAMP** cells through upregulation of HIF-1α and p53 expression	Wang et al. (2018)
Shikonin		Naphthoquinone derivative obtained from *Lithospermum erythrorhizon* Family: Boraginaceae	Induces cell death in **PC-3 and DU145** cells through ROS-mediated reduction of MMP-2 and MMP-9 expressions via targeting AKT and mTOR	Chen et al. (2014)

10.5 Conclusion

In summary, in spite of the huge advancement in the treatment for PC, cases are increasing rapidly in the entire world. Hence there is a huge demand for promising therapeutic tools to treat PC patients for better outcome. Therefore, small molecules of natural origin that induce ROS can kill PC cells in specific and exhibit negligible toxicity to the normal cells. In this study, most important phytochemicals that induce ROS and specifically induce death in PC cells have been well elaborated. Moreover, this approach can develop a highly promising future drug candidate to treat PC patients.

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11 Heat Shock Factors in Protein Quality Control and Spermatogenesis

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Abstract

Proper regulation of cellular protein quality control is crucial for cellular health. It appears that the protein quality control machinery is subjected to distinct regulation in different cellular contexts such as in somatic cells and in germ cells. Heat shock factors (HSFs) play critical role in the control of quality of cellular proteins through controlling expression of many genes encoding different proteins including those for inducible protein chaperones. Mammalian cells exert distinct mechanism of cellular functions through maintenance of tissue-specific HSFs. Here, we have discussed different HSFs and their functions including those during spermatogenesis. We have also discussed the different heat shock proteins induced by the HSFs and their activities in those contexts. We have also identified several small molecule activators and inhibitors of HSFs from different sources reported so far.

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11.1 Introduction

Maintenance of cellular protein homeostasis is crucial for normal cellular health. To be in native functional states, proteins must be in their proper three-dimensional conformations. Cells carry dedicated protein quality control machinery for this purpose. In fact, cells are under constant challenge by different intracellular and environmental stressors such as altered oxidative conditions/pH and elevated temperature which destabilize their proteomes. Of course, proteins could misfold through incorporation of mutation in their transcripts due to transcriptional mistakes or in the encoding genes. Accumulation of misfolded proteins is a burden or toxic for a cell, and thus it requires that these are immediately cleared off by degradation if not possible to refold back to their native conformations (Pellegrino et al. 2013). Misfolding of a protein can result in its loss or gain of function. Accumulation of misfolded proteins in cells leads to development of various pathologies such as cancer, neurodegenerative diseases, and infertility. Cells carry dedicated protein quality control machinery in cytoplasm, mitochondria, and endoplasmic reticulum (ER) to maintain protein quality of each of these cellular compartments to their full capacity (Lindholm et al. 2017; Saito and Imaizumi 2018). In addition to maintaining active surveillance on protein quality through clearing off misfolded mature/functional proteins, these compartments accommodate new polypeptide synthesis (Santiago-Lopez et al. 2021). The protein quality control mechanism called unfolded protein response (UPR) or heat shock response (HSR) at play in the cytosol is called heat shock response (HSR). The UPR is also active in the ER and in the mitochondria (Santiago-Lopez et al. 2021). Ubiquitin proteasome system plays a crucial role in the process through degradation of the misfolded proteins or protein aggregates. As expected, protein quality control mechanism must be in its full capacity for optimal gametogenesis and thus fertility which is a global public health issue (Jaradat and Zaid 2019) in both males and females in the developed as well as in developing countries. Globally, about 10% of couples suffer with issues of infertility, and 60–80 million people experience infertility issues every year, about 25% of which belong to India (Katole and Saoji 2019). According to the World Health Organization, one of every four couples experience issues with infertility. In general, lifestyle factors, such as smoking, excessive alcohol consumption, uncontrolled use of contraceptives, abortion, and rising maternal age, are believed to add to infertility cases. Genetic problems, health issues such as problems with the endocrine system, and psychological disorders deter couples from their parenthood (Alahmadi 2020; Jaradat and Zaid 2019). Other factors such as delaying childbirth, inappropriate age for marriage, and economic problems promote infertility (Katole and Saoji 2019). Unfortunately, till now, there is no guaranteed treatment for infertility. In fact, it is one of the major challenges for the scientific community (Hrometz and Gates 2009). Taken together, there is an urgent need to advance our understanding on the molecular basis of infertility at the cellular level to better design strategies for therapeutic interventions.

Like many other cell types, the spermatozoa formed in the testes require high rate of protein synthesis. It is obvious that the testes to function at its highest capacity would require to have its HSR at the highest efficacy. It is however reported that in the spermatozoa (i.e., the male gamete), high level of HSPs or activation of HSR results in apoptosis. Notably, spermatozoon is a highly specialized cell that lacks most of the usual organelles and even cytoplasm and transcription and translational activities which may explain its contentious relationship with HSPs/HSR (Santiago-Lopez et al. 2021). It is reasonable to assume that precursors such as spermatogonium cells need to carry out optimal proteostasis through well-regulated protein quality control. It is noteworthy to mention that spermatogenesis occurs at a temperature below (4–5 °C) the nor-

mal physiological temperature. In fact, the so-called HSR guided by the heat shock activated at elevated temperature induces distinct set of genes which drive apoptosis instead of cytoprotection. It is believed that by this mechanism spermatogenesis eliminates defective or damaged cells. Thus, HSR is activated at heat shock temperature that acts as a quality control mechanism during spermatogenesis. It is understood that heat shock factors (HSFs) function through upregulating distinct sets of genes at permissible temperature for spermatogenesis. Here, we describe the role of HSF(s) and protein chaperones with demonstrated functions in human fertility. This study also discusses the phytochemical(s) and small molecules that modulate HSF1 activity.

11.2 Heat Shock Factors (HSFs)

The mammalian genome as such produces six different HSFs – HSF1, HSF2, HSF3, HSF4, HSF5, HSFY, and HSFX. All are expressed in humans except HSF3 which among the mammals is expressed in mice (Table 11.1). Several of these factors occur in isomeric forms and may interact with each other to exhibit their functions through binding to their recognition sequences – heat shock elements (HSEs) composed of multiple 5′-nGAAn-3′ units which are usually from three to eight functional HSEs.

HSF1 and HSF2 bind to their distinct targets. Their targets also overlap. Moreover, these two factors also function as heterotrimers (Vihervaara et al. 2013). Nonoverlapping functions of these HSFs play important roles in many cellular processes as determined by their recognition of specific/unique HSE(s) (Widlak and Vydra 2017; Yamamoto 2009). Their expression during mammalian spermatocytes and round spermatid formation may imply their involvement during spermatogenesis. Cooperation between HSF1 and HSF2 is especially well known as double knockout of these two factors caused infertility along with arrest in meiosis and apoptosis of spermatocytes (Abane and Mezger 2010; Widlak and Vydra 2017). Sensitivity of spermatogenesis to an elevated temperature has been correlated with sensitivity of HSF1/HSF2 heterodimers which were reported to be sensitive to an elevated temperature. HSF1 and HSF2 have also been reportedly involved in packaging of chromatin structure during spermatid differentiation (Widlak and Vydra 2017).

A major regulator of general HSR is HSF1, a transcription activator (Neef et al. 2010; Shi et al. 1998). HSF1, a relatively well-studied factor, carries the major task of cellular protein homeostasis. HSF1 is present in an inactive monomeric state in the absence of stress in a repressive complex stabilized by HSP70, HSP90, HSP40, and T-complex protein ring complex/chaperonin containing TCP-1 (TRiC/CCT) (Nelson et al. 2016; Singh et al. 2018). As per chaperone titration model (Fig. 11.1), misfolded proteins produced in a cell under stress attract molecular chaperones HSP70 and HSP90 engaged in the repressive complex. HSF1 monomer in the repressive complex with substoichiometric amount of HSP70 and HSP90 being unstable forms homotrimer and accumulates in the nucleus to bind to its recognition element HSE and its target gene promoters which include those encoding the HSP genes to upregulate their expression (Clos

Table 11.1 Expression of heat shock factors (HSFs) in various stages of mammalian spermatogenesis

HSFs	Spermatogonium	Primary spermatocyte	Secondary spermatocyte	Round spermatids	Elongating spermatids	Spermatozoa
HSF1	_	+	+	+	_	+
HSF2	+	+	+	+	_	+
HSF3	_	_	_	_	_	_
HSF4	_	_	_	_	_	_
HSF5	_	+	+	+	_	_
HSFX	_	_	_	_	_	_
HSFY	_	+	+	+	+	_

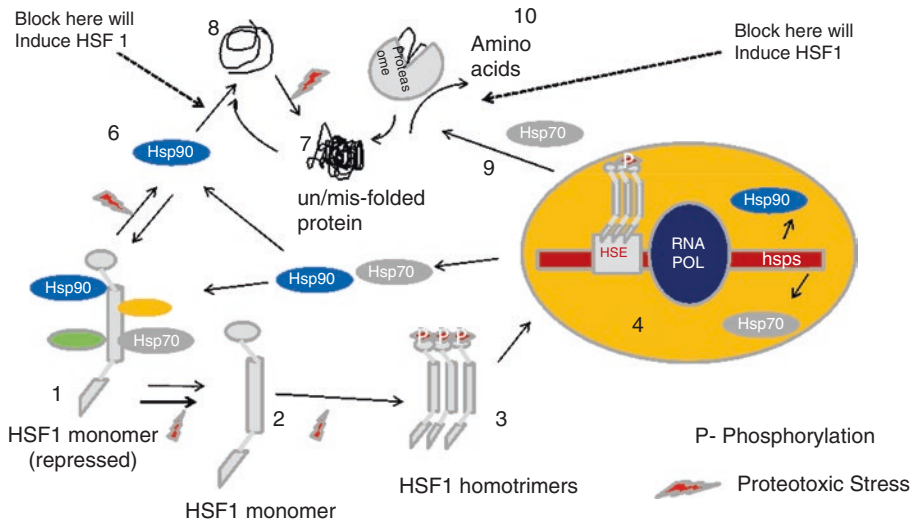


Fig. 11.1 Chaperone titration model of HSF1 activation – (1) HSF1 in an inactive monomer sequestered in a repressive complex in association with HSP90, HSP70, and cochaperones; (2, 3) exposure to proteotoxic stress results in dissociation to monomer which forms homotrimer along with undergoing posttranslational modification such as phosphorylation (P) in the activation pathway; (4) HSF1 homotrimer engages on its recognition sequences (HSE) on their target promoter driving expression of HSP70 and HSP90; (5, 6) The HSPs bind to the misfolded proteins (7) to refold them to their native conformation (8); (5) HSP70 and HSP90 produced can repress HSF1 by feedback mechanism. Inhibition of HSP90 would result in the accumulation of misfolded proteins leading to HSF1 activation; (9) proteins that were not refolded are degraded by proteasome to their constituent amino acids; (10) inhibition of proteasome would result in the accumulation of misfolded proteins leading to HSF1 activation

et al. 1990; Rabindran et al. 1993). In support of this chaperone titration model, downregulation of HSPs has been proposed to be in the repressive complex such as HSP70, HSP90, and TRiC/CCT results in HSF1 activity (Abravaya et al. 1992; Lee et al. 2013; Neef et al. 2014; Powers et al. 2008; Powers and Workman 2007; Whitesell et al. 2003; Zou et al. 1998).

The other activation model proposed HSF1 as an intrinsic thermosensor. As per this model, HSF1 stays as a monomer by intramolecular folding stabilized by leucine zipper formation by interaction between HRA/B and HR-C regions. This concept of this model was strengthened by constitutive oligomer formation by HSF1 mutants deleted of HR-C region (Rabindran et al. 1993). That HSF1 in different organisms and organs is activated at distinct temperatures has made this model not solely sufficient to explain its activation mechanism (Baler et al. 1993; Clos et al. 1990; Widlak and Vydra 2017). However, temperature does play an important regulatory role; temperature-dependent homotrimer formation concomitant with unfolding in the regulatory region of mammalian HSF1 was demonstrated by deuterium exchange mass spectrometry. The study also revealed the transition to DNA-binding competence of HSF1 to occur through a highly cooperative process (Hentze et al. 2016). In addition, different posttranslational modifications such as phosphorylation, acetylation, and simulation regulate HSF1 activity either positively or negatively. HSF1 occurs to be phosphorylated in multiple residues, and multiple kinases have been implicated in HSF1 phosphorylation. In fact, multiple kinases such as Gsk3b, casein kinase II, MEK1 and ERK, and AMPK were implicated in the phosphorylation of ser303 and ser307. Phosphorylation of these residues facilitates HSF1 degradation as observed in metabolic diseases, cancer, and Huntington's disease (Dai and Sampson 2016; Dai et al. 2015; Gomez-Pastor et al. 2017; Gomez-Pastor et al. 2018; Jin et al. 2011; Kourtis et al. 2015). E3 ubiquitin ligase FBXW7 degrades HSF1 phosphorylated at specific residue (Kourtis et al. 2015). It was revealed

that HSF1 activity under normal condition is not much influenced by its phosphorylation status. Different disease conditions, however, were influenced by the phosphorylation status of HSF1 (Budzyński et al. 2015; Gomez-Pastor et al. 2018). Acetylation at Lys208 and Lys298 by P300 was shown to regulate the steady-state level of HSF1 by interfering with its proteasomal degradation. Acetylation at Lys80 was shown to influence the DNA-binding efficacy of HSF1 by preventing its interaction with HSE – a process counteracted by NADP-dependent deacetylase SirT1 (Anckar and Sistonen 2011; Westerheide et al. 2009). Like phosphorylation, HSF1 can be subjected to SUMOylation at multiple Lys residues (K126, K157, K208, K224, and K298) influencing its transcription efficacy (Hendriks et al. 2017). HSF1 is SUMOylated by single SUMO at Lys298 which is influenced by phosphorylation at ser303 and ser307 which was thought to influence HSF1 transcription activity (Kmiecik et al. 2021). HSF1 protein SUMOylated at Lys82 in a chimera carrying HSF2 DNA-binding domain influenced its DNA binding in vivo (Jaeger et al. 2016). HSF2 was shown to be SUMOylated at Lys82 – a modification that was shown to increase its DNA-binding efficacy (Fan et al. 2008; Hilgarth et al. 2004). HSF1 and HSF4 are not SUMOylated at similar residue suggesting that HSFs can be differentially regulated though this type of posttranslational regulation. Posttranslational modification-induced degradation of HSFs plays important roles in regulating their activity. For example, HSF1 level is upregulated in cancer and downregulated in neurodegenerative diseases and is degraded during mitosis (Goetzl et al. 2015; Jiang et al. 2013; Lee et al. 2008). HSF2 level also went down during mitosis (Elsing et al. 2014; Gomez-Pastor et al. 2018).

HSF1 is activated in response to heat shock as well as other various physiological stresses/stimuli. HSF1 can function in a stress-specific manner through modulating expression of a subset of its target genes (Ali et al. 2019; Gomez-Pastor et al. 2018; Hazra et al. 2017). Eukaryotic HSF1 carries distinct functional domains, namely, amino-terminal winged helix-turn-helix DNA-binding domain and leucine zipper domain consisting of two short hydrophobic repeat regions (HR-A/B) which mediate multimerization of HSF1 monomers, following the leucine zipper domain which is regulatory domain and another heptad repeat HR-C. The C-terminus portion harbors the activation domain (Clos et al. 1990; Harrison et al. 1994; Hentze et al. 2016; Rabindran et al. 1993; Voellmy 2004; Vuister et al. 1994).

HSF2 was predominately activated in the testes and was also seen in the brain cells, but the HSF4 is expressed majorly in the eye lenses (Akerfelt et al. 2010; Gomez-Pastor et al. 2018). Studies in mouse and rat revealed that HSF1 and HSF2 are expressed in the spermatocytes and round spermatids although the quantity of both HSF1 and HSF2 decreased after elongation of spermatids (Akerfelt et al. 2010; Korfanty et al. 2014). HSF1 and HSF2 were also located in the heads of epididymal spermatozoa; in general, the testes of mouse and rat exhibit majorly the larger α-isoform of HSF1 and HSF2 (Neueder et al. 2014). Moreover, due to the influence of testosterone hormone, HSF1 expression was downregulated by androgen receptor in Sertoli cells (Yang et al. 2014). HSF5 (which is as important as HSF1 and HSF2) expression is confined completely to spermatocytes and round spermatids (Chalmel et al. 2012).

HSFY is primarily expressed during spermatogenesis, in the elongated spermatids. It was observed to be expressed rarely in type A spermatogenesis and Sertoli cells (Sato et al. 2006; Shinka et al. 2004). Involvement of HSF3, HSF4, and HSFX protein in the spermatogenesis is not known yet. Table 11.1 lists differential expression of various HSFs in the process of spermatogenesis (Widlak and Vydra 2017).

The importance of HSF1 in fertility was implied by the observation that HSF1 knockout mice exhibited a rise in prenatal lethality and retardation of postnatal growth. Female mice with HSF1 knockout were infertile due to failure of oogenesis and preimplantation development, although males were fertile with 20% decreased sperm count. HSF1 knockout males exhibited abnormal head morphology of epididymal sper-

matozoa (Abane and Mezger 2010; Salmand et al. 2008; Xiao et al. 1999). HSF2 knockout male mice showed defects in spermatogenesis leading to embryonic lethality and nervous system impairment, with females exhibiting hypofertility and defective ovary (Abane and Mezger 2010). These conditions also led to apoptotic death in spermatocytes and type A spermatogonia. The testes with HSF2 null male mice were found with decreasing size with lower number of epididymal sperm (Kallio et al. 2002). Male mice with HSF2 knockout showed decreased fertility, whereas HSF2 null men showed serious deficiency in spermatogenesis and were infertile (Kallio et al. 2002; Wang et al. 2003). Mice with double knockout of HSF1 and HSF2 showed serious deficiency in spermatogenesis with decreased spermatogonia due to apoptosis and were infertile (Wang et al. 2003). Lack of knockout models of other HSFs such as HSF3, HSF5, HSFY, and HSFX could not verify the roles of these factors in fertility yet (Widlak and Vydra 2017). However, HSFY, located in Y chromosome, is expressed in the testes, and deletion of this gene caused degeneration of spermatogenesis, azoospermia, and infertility (Fujimoto and Nakai 2010). HSFX is located in X chromosome, but significance of this factor in fertility is not known so far (Tessari et al. 2004). The HSF functions are listed in Table 11.2.

11.3 Heat Shock Proteins (HSPs)

Also called molecular chaperones, HSPs are upregulated upon various environmental stresses or developmental signaling. HSPs guide folding of newly synthesized polypeptides, refolding of misfold proteins, and/or degradation of denatured/misfolded proteins (Miller and Fort 2018;

Table 11.2 Heat shock factors (HSFs) and their functions

Heat shock factors (HSFs)	Functions	References
HSF1	Major regulator of proteotoxic stress process, also regulates genes involved in other cellular functions such as cell survival	Gomez-Pastor et al. (2018), Mendillo et al. (2012), Santagata et al. (2013)
HSF2	Mainly controls heat shock protein (HSP) genes in spermatogenic cells. HSF2 α-isoform (71 kDa) expressed mainly in the testes, while the HSF2 β-isoform (69 kDa) mostly activated in the heart and brain cells; roles in early development, including as testes development process; involvement in upregulation of nonclassical HSP	Fujimoto and Nakai (2010), Gomez-Pastor et al. (2018), Goodson et al. (1995)
HSF3	Occurs in mouse (not in humans) involvement in protection of cells from stress/heat shock without inducing classical heat shock genes	Fujimoto and Nakai (2010), Gomez-Pastor et al. (2018)
HSF4	Essential for development and differentiation of eye lens, mutation in this gene leads to cataract formation, also localized in the heart, brain, skeletal muscle, and pancreas	Fujimoto et al. (2004), Fujimoto et al. (2008), Gomez-Pastor et al. (2018) Nakai et al. (1997)
HSF5	Plays a prominent role in spermatogenesis in zebra fish; mutation in HSF5 leads to infertility in males, with decreased sperm count and increased sperm head size	Gomez-Pastor et al. (2018), Saju et al. (2018)
HSFX	Located on the X chromosome (function not well known)	Fujimoto and Nakai (2010), Gomez-Pastor et al. (2018)
HSFY	Located on the Y chromosome, plays essential role in spermatogenesis in human and other animals; removal of HSFY gene results in infertility in males	Gomez-Pastor et al. (2018), Tessari et al. (2004)

Ponomarenko et al. 2013) (Table 11.2). They are commonly grouped into distinct families based on molecular weight, scheme of activation or cell localization, and some other features. According to the recent guidelines for nomenclature, HSPs are grouped as HSPH (HSP110), HSPC (HSP90), HSPA (HSP70), DNAJ (HSP40), HSPB (small HSP), HSPD/E (HSP60/HSP10), and CCT (TRiC) (Kampinga et al. 2009). HSPs can also be classified broadly into two families depending on their size/molecular weight – those with molecular weights ranging from 8 kDa to 28 kDa, such as ubiquitin/α-crystallins which function independent of ATP requirement as HSPB1 (also called as HSP25 in mice or HSP27 in rats and humans). The second group includes large HSPs with molecular weights in the range from 40 kDa to 105 kDa such as HSP70 and HSP90 which function through an ATP-requiring process (Jee 2016; Kampinga et al. 2009). HSPs are thought to be important in fertility as adult male mice knocked out of HSP70 and HSP72 lack sperm cells and are thus sterile (Allen et al. 1996; Mori et al. 1997).

11.3.1 HSP27

It is involved in modulation of various physiological processes including cell survival and immunity. HSP27 physically interacts with actin and controls actin polymerization. It activates manganese superoxide dismutase activity associated with neuroprotective and cardioprotective functions via blocking apoptosis (Graceffa 2011). Higher expression has been implicated in testicular tumorigenesis (Spisek and Dhodapkar 2007; Trieb et al. 2000). HSP27 also seems to play a prominent role in reproduction since it has been exhibited in various estrogen-dependent organs such as the uterus, breast, oviduct, and vagina and is also seen in hair follicles of the human skin (Adly et al. 2008; Bany and Schultz 2001). It is expressed in Sertoli cells of rat testis and during spermatogenesis (Liu et al. 2000). Studies on the human testes revealed varying levels of HSP27 expression with the states of spermatogenesis (Adly et al. 2008). HSP27 reportedly regulates oocyte maturation (Kronja et al. 2014). *Ceratitis capitata* HSP27 (CcHSP27) was shown to be involved during oogenesis and spermatogenesis via expression under thermal shock as well as normal condition in a stage-specific manner (Economou et al. 2017). CcHSP27 was colocalized with actin cone suggesting the involvement of this protein in the stabilization of the processes of spermatid development individualization (Economou et al. 2017).

11.3.2 HSP60

It is widely distributed in eukaryotic and prokaryotic cells specifically in the cytoplasm and mitochondria and is mainly associated with proper protein folding with the help of other HSPs. HSP60 activity was implicated in several functions such as apoptosis, immunity, and oncogenesis. HSP60 was shown to be involved in hepatitis B infection (Wyżewski et al. 2018). Involvement of HSP60 in fertility was implicated based on its localization in the rat testis specifically in Sertoli cells, Leydig cells, germ cells, and initial primary spermatocytes. Expression of HSP60 in the initial spermatocytes indicated its requirement in the beginning stage of spermatogenesis (Meinhardt et al. 1995). In an experiment conducted by Meinhardt and Seitz, it was concluded that HSP60 exhibits distinctly in different stages of spermatogenesis and during spermatogonia type A mitosis (Paniagua et al. 1987; Paranko et al. 1996). High expression of HSP60 was noted in dividing spermatogonia which is important in promoting mitochondria into the daughter cells; cells deficient in HSP60 led to the testes with spermatogenic defect (Meinhardt et al. 1995; Werner et al. 1997). Another study revealed the presence of HSP60 in porcine testes and in developmental changes (Huang et al. 2005).

11.3.3 HSP70

HSP70 family of proteins is one of the major chaperones involved in refolding, maturation, degradation, and transport of cellular proteins. Some of these proteins are constitutively expressed, while others are activated in response to stressors (Mayer and Bukau 2005; Miller and

Fort 2018). HSP70s comprise HSP70–1 (also called HspA1A) and HSP70–2 (also called HSP A1B), collectively termed HSP70 or HSP70–1, and are major stress-inducible proteins. These proteins differ from each other by only two amino acids. HSPA1A/B basal expression levels vary in most tissues which exceed levels of expression of other HSP70 isoforms in humans (Daugaard et al. 2007). Constitutively expressed HSP70–1 t (also called HSPA1L) carries about 90% identities with HSP70.2 (also named as HSPA2). These proteins are abundantly expressed in the testis (Radons 2016). By clearing protein aggregation, these proteins provide protection from neurotoxicity, inflammation, and apoptosis. They are implicated in immune response and autoimmune diseases (Turturici et al. 2011). HSP70 is also involved in spermatogenesis and was seen both in interstitial and in spermatogenic cells of mature testis (Huang et al. 2005). In mice, HSP70 proteins were highly expressed during spermatogenesis; HSP70–2 is expressed in pachytene spermatocytes at meiotic phase (Dix et al. 1996). HSP70–2 that carries ~85% homology with HSP70–1 is highly expressed in different cell types including the testis and implicated in spermatogenesis and meiosis (Zhu et al. 1997). The miR-15a and HSPA1B (HSP70–2) levels were shown to be altered in the spermatozoa of varicocele patients implicating miR-15a-mediated stress regulation of HSP70–2 in sperm (Ji et al. 2014; Radons 2016). HSP70–2 was involved in synaptonemal complex function at the meiosis phase in male germ cells (Dix et al. 1996). In *D. melanogaster*, HSP70 proteins were upregulated in the oogenesis and early development stages only. Cobreros et al. showed the importance of DNAJ/HSP40 and HSP70 in border cell migration, which is an important process in the fly to build egg chamber to accommodate maternal factors for normal embryogenesis, and border cells without HSP70 genes which resulted in malfunctioning of actin cytoskeleton and failure in cell migration (Cobreros et al. 2008). Another study suggested an importance of HSP70–4 (CG4264) in the production of healthy eggs. By RNAi screen, Jia et al. found the importance of HSP70–4 in Notch-mediated follicle cell differentiation where it is implicated in Notch trafficking (Jia et al. 2015). In experiments to identify the genes that participate in regulating transposon silencing in oocytes, HSP70–5 (also called GRP78) gene of HSP70 family was identified as essential for female fertility (Gong and Golic 2006; Radons 2016).

11.3.4 HSP90

HSP90 plays a crucial role in cell cycle control, cell survival, and hormonal balancing (Jarosz 2016). HSP90 client proteins are also involved in DNA repair, immune response, growth, and proliferation of cancer cells (Schopf et al. 2017). The role of HSP90 in murine embryo development was suggested by its presence in the murine preimplantation embryos (Neuer et al. 2000). Studies conducted on *Caenorhabditis elegans* observed that mutation in daf-21, the HSP90 version of the worm, leads to the formation of dauer larva and decreased brood size and reproduction capability (Christians 2017; Vowels and Thomas 1994). This study also revealed a strong correlation between fecundity/reproduction/germ cells and decline in HSR (Labbadia and Morimoto 2015). Besides this, two mammalian cytosolic isoforms of HSP90, i.e., HSP84 and HSP86, were shown to be involved in reproduction; both HSP84 and HSP86 were upregulated in the midgestation period of the mouse embryo (Lee 1990). Although HSP84 and HSP86 were located in each and every tissue, the maximum level was found in adrenal gland and ovary. These genes are found to be distinctly expressed in an adult mouse tissue. HSP86 was shown to be expressed in the germ cells of the testis, too (Lee 1990).

11.3.5 GRP78

Glucose-regulated protein 78 (GRP78), commonly known as immunoglobulin heavy-chain-binding protein (Bip), belongs to the family of ER chaperone proteins and is a member of the HSP70 family. It is a central regulator of ER UPR engaging in protein folding and degradation of abnormal proteins via proteasomal pathway

Table 11.3 Heat shock proteins (HSPs) associated with spermatogenesis

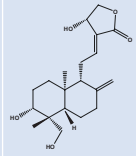
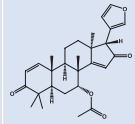
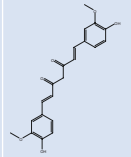
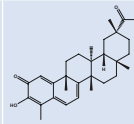
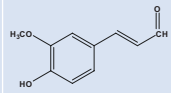
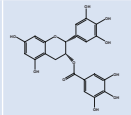
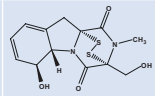
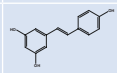
Heat shock protein	Site of expression	Function(s) in reproduction	References
HSP27	Sertoli cells, spermatogonia, Leydig cells, spermatocytes, and spermatids	Required for normal spermatogenesis, deregulation of HSP27 led to development of unusual sperm and male infertility	Adly et al. (2008), Purandhar et al. (2014)
HSP40	Testis, trachea, lung, and oviduct	HSP40 cochaperone function involved in spermiogenesis and motility of mature spermatozoa	Li and Liu (2014), Meccariello et al. (2014), Purandhar et al. (2014)
HSP60	Intramitochondrial chaperone, expressed in spermatogonia, primary spermatocytes, and Sertoli cells	Involved in the maintenance of the spermatogenic function, decreased expression led to downregulation of spermatogenic potency	Purandhar et al. (2014), Werner et al. (1997)
HSP70	Sperm surface, specific spermatogenic cell, and seminal plasma	HSP70 required for male fertility. Lack of HSP70 arrested spermatogenesis. Along with HSP70–2, HSP70 involved in synaptonemal complex function and male fertility	Dix et al. (1996), Erata et al. (2008), Huang et al. (2005), Hunt and Morimoto (1985), Purandhar et al. (2014)
HSP90	Testis and epididymis	Important in ovarian biology, involved in regulating the ability of mammalian spermatozoa to fertilize the oocyte	Ecroyd et al. (2003), Pires (2017), Purandhar et al. (2014)
GRP78	Uterine luminal and glandular epithelia	Required for implantation of the embryo	Lin et al. (2012), Marín-Briggiler et al. (2010), Zhang (2017)

(Hebert-Schuster et al. 2018; Marín-Briggiler et al. 2010; Yang et al. 2016). It plays a crucial role in cell survival and apoptosis via regulating the functions of client proteins PKR-like ER-associated kinase (PERK), inositol-requiring kinase 1 (IRE1), and activating transcription factor 6 (ATF6) (Zhang 2017). Report suggested involvement of ER UPR in female reproductive processes such as embryo implantation, decidualization, preimplantation embryonic development, follicle atresia, and placenta development (Yang et al. 2016). Several reports revealed the involvement of GRP78 in the fertilization process; GRP78 is upregulated and secreted by oviduct epithelial cells (OEC). GRP78 was reported to be present in native oviductal fluid (OF) with higher protein concentration in the periovulatory period (Marín-Briggiler et al. 2010). Another report revealed the presence of GRP78 along with other chaperone proteins in the mammalian male germ line suggesting its role in the beginning stages of spermatogenesis (Marín-Briggiler et al. 2008). GRP78 was reported to be secreted by oviduct epithelial cells. The protein was thought to bind to the gametes for modulation of their interaction in a calcium-dependent manner (Marín-Briggiler et al. 2010). The study also explained the GRP78 produced in the oviductal cells in feedback to spermatozoa connected with sperm surface and helps in the fertilization process (Marín-Briggiler et al. 2008). GRP78 plays an important role in steroid synthesis, and its interaction with steroids was shown to be important in reproduction as well as tumorigenesis (Hebert-Schuster et al. 2018). Important functions of HSPs are summarized in Table 11.3.

11.4 Phytochemicals as Upregulators of Cellular Protein Quality Control Mechanism

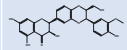
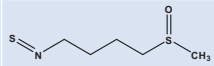
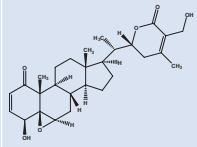
Proteins are crucial mediators of cellular functions. Therefore, they must be in proper functional state and free of any defective or nonfunctional forms. Cells maintain their proteins in healthy states by maintaining its protein quality control mechanism at optimal level. Many disease states result due to the deregulation of protein quality control mechanism. For example, HSF1 is constitutively active in cancer, while it is

Table 11.4 Small molecule HSF1 inducers of natural origin

Compound	Structure	Source and nature	Mechanism(s)	Reference(s)
Andrographolide		Labdane diterpenoid extracted from *Andrographis paniculata* leaf. Family: Acanthaceae	Indirectly induced HSF1 via ROS generation. Inhibited mTORC1 and activated Nrf2	Dutta et al. (2021)
Azadiradione		Seed of *Azadirachta indica*, triterpenoid. Family: Meliaceae	Physically interacted with HSF1 and induced HSF1 activity through enhancing its binding to HSE. Did not interfere with HSP90 or proteasome	Nelson et al. (2016), Singh et al. (2018)
Curcumin		Rhizome of *Curcuma longa*, polyphenolic compound	Indirectly activated HSF1 via inhibiting proteasome and HSP90. Induced ROS level	Hamaguchi et al. (2010), Jana et al. (2004), Valentine et al. (2019)
Celastrol		Triterpene form roots of *Tripterygium wilfordii*	Indirect HSF1 activator. Inhibited HSP90 and proteasome activity. Induced ROS level	Allison et al. (2001), Cleren et al. (2005), Wang et al. (2015), Westerheide and Morimoto (2005)
Coniferyl aldehyde		Phenolic compound extracted from *Eucommia ulmoides*. Family: Eucommiaceae	Enhanced the HSF1 protein stability via phosphorylation at Ser326 accompanied by EKR1/2 upregulation	Kim et al. (2015b)
Epigallocatechin -3-gallate		Polyphenolic compound extracted from leaves of *Camellia sinensis*. Family: Theaceae	Indirect HSF1 activator. Inhibited proteasome and induced ER stress	Modernelli et al. (2015), Neef et al. (2010)
Gliotoxi		Fungal epipolythiodioxopiperazine	Indirect HSF1 activator. Inhibited proteasome through targeting catalytic subunits of proteasome system	Kroll et al. (1999)
Resveratrol		Stilbene polyphenol compound extracted from roots of *Veratrum grandiflorum*. Family: Melanthiaceae	Activated HSF1 function through deacetylation of HSF1 which facilitated its DNA-binding activity	Dayalan Naidu and Dinkova-Kostova (2017), Westerheide et al. (2009), Zeng et al. (2017)

(continued)

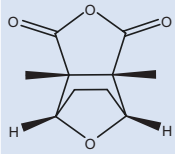
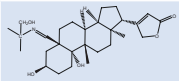
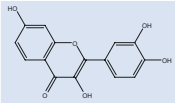
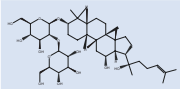
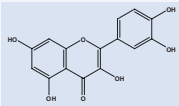
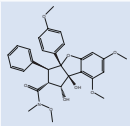
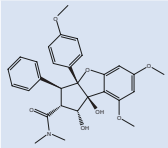
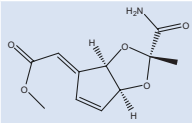
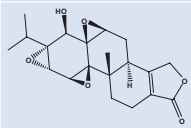
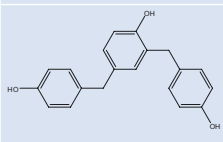
Table 11.4 (continued)

Compound	Structure	Source and nature	Mechanism(s)	Reference(s)
Silibinin		Polyphenolic flavanolignin isolated form of *Silybum marianum*. Family: Asteraceae	Indirect HSF1 activator. Inhibited HSP90 protein function	Amolins and Blagg (2009), Cuyàs et al. (2019)
Sulforaphane		Isothiocyanate class of organosulfur compound obtained from Cruciferous vegetables (*Brassica oleracea*) like broccoli, Brussels sprouts, and cabbages. Family: Brassicaceae	Indirect activator. Initiated proteasome activity	Dayalan Naidu and Dinkova-Kostova (2017), Dutta et al. (2020), Gan et al. (2010)
Withaferin A		A steroid lactone isolated from Indian traditional medicinal plant Ashwagandha *Withania somnifera.* Family: Solanaceae	Indirect HSF1 activator. Inhibited proteasome function and induced endoplasmic reticulum stress. Induced ROS level	Dayalan Naidu and Dinkova-Kostova (2017), Khan et al. (2012)

less active or inactive in different neurodegenerative conditions such as Parkinson's or Huntington's disease (Nelson et al. 2016; Singh et al. 2018). In spermatogenesis, protein quality control involving HSF1 could mediate the removal of defective/damaged cells, apart from its function in temperature at normal gametogenesis process. Different small molecule activators of HSF1 have been reported by several independent laboratories which have been shown to upregulate HSF1 function with different mechanisms. Some were shown to activate HSF1 by indirect mechanism, while small molecules were reported to activate HSF1 by a unique mechanism which includes direct interaction with HSF1 (Table 11.4). In particular, azadiradione has been unique due to the fact that it activates HSF1 without interfering with HSP90 or proteasome functions (Nelson et al. 2016). Several small molecules remain to be tested with regard to their effect on HSF2 and other HSFs.

Several small molecule inhibitors of HSF1 of natural origin were isolated. Quercetin, a flavonoid, carries a wide variety of pharmacological activities (Table 11.5). It was shown to inhibit the binding of HSF to HSE in vitro and HSF1 activity in vivo (Hosokawa et al. 1992; Sharma and Seo 2018). Triptolide belonging to the class of diterpene triepoxide, isolated from medicinal plant *Tripterygium wilfordii*, was also reported to inhibit HSF1. In an experiment conducted on pancreatic cells, PANC-1 and MIA PaCa-2 cells concluded that triptolide inhibited transcription of HSP70 by blocking the HSF1 Hsp70 promoter region (Phillips et al. 2007; Sharma and Seo 2018). Cell-based screening experiments identified Fisetin and Cantharidin as inhibitors of HSF1. Fisetin, a flavonoid isolated from *Elaeagnus indica*, was reported to inhibit binding of HSF1 on HSP70 promoter, and a terpenoid cantharidin secreted from male blister beetle blocked the function of HSF1 through inhibiting its transcription activity (Kim et al. 2013; Kim et al. 2015a). Rocaglamide, a flavagline, again was shown to inhibit HSF1 activity by inhibiting its recruitment on its target HSP70 promoter (Santagata et al. 2013). 2,4-Bis(4-hydroxybenzyl)phenol inhibited HSF1 function through enhancement of dephosphorylation of HSF1 at S326 leading to HSF1 protein instability (Yoon et al. 2014). CL-43, a cyclopentanoperhydrophenanthrene derivative of cardenolide inhibited HSF1 function in colon cancer cells (Yamane et al. 2010). Ginsenoside Rg3, a triterpene saponin isolated from *Panax ginseng*, was reported to inhibit HSF1 activation via blocking its transcription activity (Aziz et al. 2016).

Table 11.5 Natural compounds that inhibit HSF1

Compound	Structure	Biological source and nature	Mechanism(s)	Reference(s)
Cantharidin		Terpenoid class of secondary metabolite secreted from male blister beetle. Family: Meloidae	Inhibited the binding ability of HSF1 to its targeted Hsp70 promoter region	Kim et al. (2013)
CL-43		Cyclopentanoperhydrophenanthrene derivative of cardenolide isolated from *Acokanthera ouabain*. Family: Apocynaceae	Inhibited HSF1 and its targeted chaperones' function	Dutta et al. (2020), Hisakazu et al. (2010), Yamane et al. (2010)
Fisetin		A flavonoid extracted from *Elaeagnus indica*. Family: Elaeagnaceae	Enhanced inhibitory function of HSF1 to its promoter region	Khan et al. (2013), Srinivasan et al. (2020)
Ginsenoside Rg3		Steroid glycosides or triterpene saponin type of secondary metabolites isolated from *Panax ginseng*. Family: Araliaceae	HSF1 transcription regulation	Aziz et al. (2016), Kim and Kim (2021), Nakhjavani et al. (2019)
Quercetin		Plant pigment, present in various fruits and vegetables, belonging to the class flavonoid (flavanol)	Blocked the HSF binding to its DNA-binding domain HSE	Anand David et al. (2016), Sharma and Seo (2018)
Rohinitib		Structural analogue of the compound rocaglamide A	Inhibited DNA-binding activity of HSF1 to its promoter region	Agarwal et al. (2015), Dong et al. (2019), Santagata et al. (2013)
Rocaglamide A		Pyrrolidine amide derivative belonging to the class of flavaglines, isolated from species like *Aglaia oligophylla.* Family: Meliaceae	Blocked binding of HSF1 to its promoter region of the targeted gene via inhibition of translation triggering factors like e1F4A	Dutta et al. (2020), Santagata et al. (2013), Sharma and Seo (2018)
Stresgenin B		Extracted from the cultured broth of *Streptomyces* species	Blocked binding of HSF1 to its promoter region	Akagawa et al. (1999), Dutta et al. (2020)
Triptolide		Diterpenoid triepoxide, isolated from the roots of *Tripterygium wilfordii.* Family: Celastraceae	Prevented binding of HSF1 to Hsp70 promoter leading to inhibition its transcriptional activity	Westerheide et al. (2006), Yuan et al. (2019)
2,4-Bis(4-hydroxybenzyl) phenol		Phenolic derivative isolated from rhizomes of *Gastrodia elata*. Family: Orchidaceae	Triggered the dephosphorylation of HSF1 at S326 leading to HSF1protein instability	Pyo et al. (2004), Yoon et al. (2014)

11.5 Conclusion and Future Perspective

Heat shock proteins play critical roles in the process of spermatogenesis as evidenced by correlation between infertility and mutation/defect in the HSF(s) and/or HSP(s). Studies of spermatogenesis have revealed that the conventional HSR that is at play in a somatic cell is different in spermatozoa, as upregulation of temperature, i.e., heat shock, results in apoptotic death of these cells, because the process of spermatogenesis occurs at a temperature below the physiological temperature. Important goals of current and future research are to understand the molecular basis of this distinction and design therapy to modulate the process as appropriate for the benefit of mankind. Identification of natural products as modulators of HSF1 has shown their potential for their inclusion in the future therapy regimen which should encourage future investigation in this direction as well.

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12 Pathological Role of Reactive Oxygen Species on Female Reproduction

Lisa Goutami, Soumya Ranjan Jena, Amrita Swain, and Luna Samanta

Abstract

Oxidative stress (OS), a clinical predicament characterized by a shift in homeostatic imbalance among prooxidant molecules embracing reactive oxygen species (ROS) and reactive nitrogen species (RNS), along with antioxidant defenses, has been established to play an indispensable part in the pathophysiology of subfertility in both human males and females. ROS are highly reactive oxidizing by-products generated during critical oxygen-consuming processes or aerobic metabolism. A healthy body system has its own course of action to maintain the equilibrium between prooxidants and antioxidants with an efficient defense system to fight against ROS. But when ROS production crosses its threshold, the disturbance in homeostatic balance results in OS. Besides their noxious effects, literature studies have depicted that controlled and adequate ROS concentrations exert physiologic functions, especially that gynecologic OS is an important mediator of conception in females. Yet the impact of ROS on oocytes and reproductive functions still needs a strong attestation for further analysis because the disruption in prooxidant and antioxidant balance leads to abrupt ROS generation initiating multiple reproductive diseases such as polycystic ovary syndrome (PCOS), endometriosis, and unexplained infertility in addition to other impediments in pregnancy such as recurrent pregnancy loss, spontaneous abortion, and preeclampsia. The current article elucidates the skeptical state of affairs created by ROS that influences female fertility.

Keywords

ROS · RNS · Polycystic ovary syndrome (PCOS) · Endometriosis

12.1 Introduction

Elevation in reactive oxygen species (ROS) level is an emerging health concern during aging and also in several other diseases in both humans and animals. High ROS concentration can also be the reason for increasing oxidative stress (OS) or decreasing efficiency of antioxidant system. It acts like a double-edged sword for its involve-

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ment in physiological processes as a major signaling molecule and also plays a role in pathological processes like fertility and reproduction, maturation and fertilization of oocyte, development of the embryo, and maintaining pregnancy. Several studies have reported that age-related decline in fertility is due to the modulation of OS. It is also reported to play a role in normal parturition and initiation of preterm labor. It is found that antioxidants can prevent from damage to ovulation-induced OS and also disruption of DNA of the ovarian epithelium. Growing evidences support that OS has effect on pathophysiology of female reproduction like free radical-induced birth impairment, pre-eclampsia, hydatidiform mole, and other situations such as abortions (Agarwal et al. 2008, 2012). Studies reveal that OS also has a pathophysiological role in infertility and assisted fertility. Moderate concentration of ROS is also involved in growth and apoptotic protection signal transduction. Increased ROS levels alter macromolecules like proteins, lipids, and nucleic acids that significantly damage the cellular structure and further lead to OS. Cells have the capability to escape the damage caused due to ROS by the presence of its nonenzymatic antioxidants like glutathione, vitamin C, and vitamin E and enzymatic antioxidants like superoxide dismutase (Mn-SOD and Cu/Zn SOD) that helps in conversion of superoxide to hydrogen peroxide, glutathione peroxidase, and catalase which neutralize the hydrogen peroxide. Complex interaction among prooxidants and antioxidants ensures the maintenance of intracellular homeostasis of ROS in female reproduction (Fujimura et al. 2000). The present study addresses the main pathophysiology caused by ROS in the female reproductive system.

12.2 Pathological Effect of ROS on Female Reproductive System

ROS and its scavenging system play an important role in reproductive physiology. Reports confirmed the existence of ROS and different antioxidant enzyme transcripts in the female reproductive tract (Sugino 2005; Agarwal et al. 2008). If ROS are kept in adequate concentration in the reproductive apparatus, it acts as an important mediator in steroidogenesis in the ovary, hormone signaling, ovulation, formation of the corpus luteum, luteolysis, oocyte maturation, luteal maintenance in pregnancy, implantation, compaction, blastocyst development, and germ cell function. It is also observed that intermittent ROS generation occurs inside the ovary as a physiological by-product during follicular and luteal phases (González et al. 2006). Macrophages and neutrophils are considered as other sources of ovarian ROS, and its presence is well documented in both corpora lutea and follicles (Nakamura and Sakamoto 2001).

12.2.1 Reduced Growth and Development of Oocyte

Stress is a significant component that affects a healthy person's physical and emotional well-being, disrupting the body homeostasis. The foremost reason of psychological stress is a change in one's lifestyle. Psychological stress may have an effect on female reproduction biology by affecting the follicle, ovary, and oocyte. Increased stress hormone level, such as cortisol, limits estradiol synthesis within the follicle with modifications in the granulosa cell functions, resulting in poor oocyte quality. Modern lifestyle changes can affect female reproduction by production of ROS in the ovary. Neutralization of ROS and balancing antioxidant enzymes concentration are a prior requirement of the ovary for maintaining female reproductive health. The ROS generation at the basal level is necessary for regulation of oocyte activities, but excessive accumulation can be the reason of OS (Agarwal et al. 2012).

The major causes that induce ROS accumulation can be environmental and lifestyle changes, pathological conditions, or drug treatment, which imparts negative effect on oocyte physiology by promoting apoptosis which can lead to OS (Tripathi et al. 2011; Sharma et al.

2013). Apoptosis of granulosa cells triggered by OS leads to reduction in levels of estradiol 17ß, quality of oocyte, and rate of ovulation (Tripathi et al. 2013). A recent report suggested that granulosa cell apoptosis by ROS lowers the granulosa cell-oocyte communication, which impacts nutrition availability and decreases the quality of preovulatory oocytes (Chaube et al. 2014). Furthermore, OS induces disorders in chromosomal segregation, telomere shortening, oocyte fragmentation, and failed fertilization resulting in age-related fertility decline (Ishii et al. 2014; Tatone et al. 2015). High ROS level (beyond physiological range) may trigger mitochondria-mediated cell cycle arrest by maturation-promoting factor (MPF) destabilization and apoptosis in oocyte (Tiwari et al. 2016). An in vitro study defended the probability of transitory increase in intracellular ROS facilitating resumption of meiosis from diplotene arrest, while further enhancement caused OS leading to arrest in cell cycle followed by apoptosis (Chaube et al. 2005; Tripathi et al. 2009). Similar reports explain rise in ROS level triggering cell cycle arrest in embryos of humans and mice (Tripathi et al. 2009). Despite the fact that immature and mature oocytes both encounter cell cycle arrest and cell death induced by OS. Although, immature oocytes are more prone to OS-mediated morphological alterations by apoptosis like membrane blebbing, cytoplasmic granulation, shrinkage, and degeneration (Men et al. 2003; Chaube et al. 2005). Another study suggested that frequent stimulation of exogenous gonadotropin hormone also induces ovarian OS and ovulation of poor-quality oocytes with reduced growth (Chao et al. 2005). Oocyte apoptosis is facilitated both by death receptor and mitochondria-mediated pathways. Especially OS-induced mitochondrial caspase-mediated pathway takes an important part in eliminating germ cells from the ovarian cohort which have the capability to impair oocyte quality even after ovulation (Tiwari et al. 2016).

12.2.2 Ovarian Steroidogenesis

ROS are the preordained end product of normal aerobic metabolism, and hence, steroidogenic cells can be served as one of the primary sources of ROS. Some other potential intracellular sources of ROS are endoplasmic reticulum, plasma membrane, and electron transport systems of mitochondria and nuclear membrane (Freeman and Crapo 1982). Evidence suggested that there is a substantial correlation between Cu, Zn-SOD, and progesterone concentrations in serum. However, the amount of lipid peroxide rose during the regression phase in the corpus luteum in rat models and showed an opposing trend in progesterone concentration from serum (Sugino et al. 1993; Shimamura et al. 1995). At the time of steroidogenesis, ROS production is normal to restrict the corpus luteum capability for progesterone synthesis (Carlson et al. 1995). During pregnancy, a decrease in the expression of Zn-SOD and Cu-SOD leads to a rise in ROS, which inhibits progesterone production. Therefore, an increase in the capability to scavenge ROS could be linked to the preservations of the integrity of luteal cells and a longer corpus luteum lifespan (Sawada and Carlson 1996). Repoport et al. depicted that progesterone synthesis in the corpus luteum is associated with SOD and catalase in other mammals, such as bovines (Rapoport et al. 1998). It is possible that luteotropic chemicals, which are generally produced by the placental cells during pregnancy, induce the expression of luteal cells protecting molecules from ROS. Finally, placental luteotropins enhance Zn-SOD and Cu, which is a key mechanism for rescuing the corpus luteum and maintaining progesterone synthesis (Behrman et al. 2001). During follicular growth, where superoxide radicals are produced through normal metabolism and steroidogenesis in mitochondria and cytosol, there it also bears the major role among ROS to inhibit steroidogenesis. Cu, Mn-SOD, and Zn-SODs act as scavengers of superoxide radicals and protectors of

granulosa cells and theca interna cells that significantly facilitate steroidogenesis and follicular growth. On the other hand, a unique hypothesis explains that Cu, Zn-SOD may have a role in progesterone biosynthesis by theca interna cells.

12.2.3 Ovulation

The ovulation mechanism has been compared to an inflammatory response (Espey 1980; Behrman et al. 1996). The major factors involved in inflammation during ovulation process are higher level of prostaglandin and cytokine production, along with the proteolytic enzymatic action and enhanced vascular absorptivity (Brannstrom 2004). ROS may act as a significant inflammatory response mediator, and therefore these have been described to be associated with ovulation. Sato et al. demonstrated that in pregnant mare serum gonadotropin-human chorionic gonadotropin (PMSG-HCG) rats, intravenous injection of SOD suppressed the ovulation during in vivo condition (Sato et al. 1992). Using a perfused in vitro ovary model, Miyazaki et al. also reported that ovulation is inhibited in rabbit upon SOD administration stimulated by HCG. In fact, after HCG injection, raised lipid peroxide concentration is the result of ROS in the ovary of rat (Miyazaki et al. 1991). Therefore, these observations strongly indicate that ROS are involved in the rupturing process of the follicle. As per in vitro reports, the perfused ovary also encounters SOD averted ovulation, revealing that ROS sources are localized in the ovary. Residential leukocytes or endothelial cells swarm around preovulatory follicles, infiltrating the granulosa cell layer and that could be the source of ROS during the ovulatory process (Araki et al. 1996). Kodaman and Behrman reported that ROS are generated from isolated follicles (Kodaman and Behrman 2001). According to Shirai et al., the polymorphonuclear leukocytes in the peripheral circulation secrete LH from the LH receptors present in it and also increase superoxide radical generation (Shirai et al. 2002). Administration upon monoclonal antibody (Mab) depleting neutrophil results in reduction in rate of ovulation in rats (Brännström et al. 1995; Kodaman and Behrman 2001). When the effect is compared to SOD alone about the ROS species, these factors, like parallel administration of catalase and hydrogen peroxide catalysis, impart no additional effect on the ovulation rate (Miyazaki et al. 1991). Moreover, SOD can fully inhibit ROS generated by follicular cells, but catalase could not do the same (Kodaman and Behrman 2001). These findings divulge that superoxide radical is the radical species involved in ovulation.

12.2.4 Formation of Blastocysts

Blastocysts, like every other actively metabolizing cell in the body, produce ROS. Basically, three enzyme systems regulate ROS production: oxidative phosphorylation, xanthine oxidase, and NADPH oxidase system (Guerin et al. 2001). Participation of other oxidase enzymes in the production of ATP consequently elevates the ROS levels. As demonstrated in rabbit blastocysts 4/5 days after coitus, the embryos can produce O_2, H_2O_2, and OH (Manes and Lai 1995). ROS concentrations have decreased in in vivo culture as compared to in vitro culture in mice. The amount of ROS produced varies depending on the stage of embryo development. ROS is manufactured twice in mouse embryos during the period of fertilization and the G2 or M stage of the second cell cycle (Nasr-Esfahani et al. 1990; Nasr-Esfahani and Johnson 1991). Another study showed that mitochondrion is not the only source of ROS production. In rabbit blastocysts, Manes and Lai (1995) found that cyanide is an irreversible mitochondrial respiration blocker that did not decrease ROS production and suggested that, aside from oxygen metabolism, there are other sources of oxygen radical generation. The NADPH oxidase is another oxidizing system discovered in the preimplantation embryo. As seen in rabbit blastocysts, the NADPH oxidase system can also yield free radicals (Manes and Lai 1995). In two-cell mouse embryos, suppressing the NADPH oxidase system blocks the production of H_2O_2 (Nasr-Esfahani and Johnson 1991). It is

necessary to investigate whether a comparable system in the human embryo exists or not and whether it is responsible for the developmental arrests of embryos.

12.2.5 Implantation

After fertilization, the blastocyst development of the embryo includes ICM (inner cell mass) and trophectoderm stage differentiation which infers cleavage, quick cell division, and compaction (Paria and Dey 1990; Iwata et al. 2014). Cell division in preimplantation of embryos occurs in a quick and regulated manner that demands great energy, through ATP during oxidative phosphorylation in mitochondria, and also produces ROS, namely, H_2O_2. The hydroxyl radical (OH^-) of Fenton reactions is engaged in the production of H_2O_2. In typical conditions, H_2O_2 participates in the mitochondrial respiratory chain. The enzymatic defense system comprises of metalloenzymes such as SOD and catalase (CAT) (Guerin et al. 2001; Dumollard et al. 2007; Levine and Puzio-Kuter 2010; Silva et al. 2010; Migdal and Serres 2011). In mammalian cells, there is a distinct secretory mechanism of H_2O_2. It is manufactured as a consequence of numerous oxidative reactions like peroxisomal enzyme activities, oxidative protein folding, and respiratory chain cascade in the ER. Neutrophils give rise to H_2O_2, which functions adversely to microbial contamination. However, concern is toward its secondary messenger aspect in the course of proliferation and differentiation of the cell (Rhee et al. 2005; Rhee 2006). The transcription factor, hypoxia-inducible factor-1 (HIF-1), is activated and managed by H_2O_2. Numerous growth factors like insulin-like growth factor-1 (IGF-1) and IGF-2 with vascular endothelial growth factor are positively regulated by this transcription factor, influencing normoxia to hypoxia (Ke and Costa 2006). In the course of implantation, the action of HIF-1 is studied to be triggered by follicle-stimulating hormone (FSH) to manifest in granulosa and endometrial cells followed by regulating target genes concerned with angiogenesis and cell survival for implantation of the embryo (Critchley et al. 2006); Ke and Costa 2006; Alam et al. 2009).

12.3 Luteolysis and Luteal Maintenance of Pregnancy

Apoptotic luteal cell death is associated with structural luteolysis as suggested by evidence (Shikone et al. 1996; Roughton et al. 1999; Carambula et al. 2002; Sugino 2005). Reports suggest that different cells are destined to apoptotic cell death due to aggregation of ROS and reduction in SOD parameters (Rothstein et al. 1994; Troy and Shelanski 1994; Greenlund et al. 1995). Exaggerated levels of ROS cause cytochrome c (cyt c) from mitochondria to discharge in the cytoplasm which results in apoptosis that in turn activates caspases in interaction with some cytosolic factors like Apaf-1 and anti-apoptotic factor like Bcl-2. The whole process of releasing cyt c and apoptosis can be inhibited through superoxide generation (Cai and Jones 1998). Daramajaran et al. reported Mn-SOD and Bax are found to express in a higher and lower levels, respectively, where Bax is an apoptotic factor, rescued by HCG in corpus luteum of rabbits and involvement of Mn-SOD in the survival of luteal cells (Dharmarajan et al. 1999). Mitochondrial superoxide radical removal is essential which is demonstrated by the death of neonatal mice that lacks above-described Mn-SOD expression (Li et al. 1995). For instance, when luteal cells get exposed to environment rich in cytokine and Mn-SOD fails to induce rapidly increased ROS production in mitochondria may cause apoptosis. Naturally, few apoptosis may be seen despite the raised ROS level during the regression phase (functional luteolysis) of corpus luteum in pregnancy or pseudopregnancy phases of rats (Takiguchi et al. 2004). It may be due to the well-maintained Mn-SOD levels throughout the ongoing luteolysis in the corpus luteum, which suggests that corpus luteum is still able to safeguard against OS (Sugino et al. 1998). Tanaka et al. reported from the result of an in vitro study

in rats the functional luteolysis inducer PGF2α, which causes apoptosis via ROS in luteal cells (Tanaka et al. 2000). However, it seems insignificant as it affects only 5% loss of viable cells. Hence, this analysis supports the inference deduced by Takiguchi et al. that even the increase in ROS level could not perform a higher level of apoptosis (Takiguchi et al. 2004).

The reason of apoptotic cell death of the regressed corpus luteum during human menstrual cycle is a result of a rise in ROS and fall in Cu, Zn-SOD expression level, where expression of Mn-SOD is consistently higher, which infers the protective ability of luteal cells against OS in mitochondria (Sugino et al. 2000). The outcome of this study opens a possible way of elevation in cytosolic ROS that triggers the reduction in cytosolic Cu, Zn-SOD which collectively facilitates apoptotic death of luteal cells of the corpus luteum in humans. According to the above explanation, Cu, Zn-SOD reduction within a physiological range, like fall in the regression state in pregnant or pseudopregnant rats or decline up to 50% by antisense oligonucleotides of Cu, Zn-SOD, cannot be considered to affect apoptotic cell death. A little depletion in Cu, Zn-SOD actions may not have been sufficient to trigger apoptosis. However, Cu, Zn-SOD activity level in the human menstrual cycle showed a 30% decline compared to the level of mid-luteal phase in the regressed corpus luteum (Sugino et al. 2000). Such a huge drop in Cu, Zn-SOD action might cause apoptosis in the cells of human corpus luteum, because as stated by Rothstein et al. 40% drop in Cu, Zn-SOD expression did not cause apoptosis, whereas a 60% decrease initiated the same in nerve cells (Rothstein et al. 1994). It may be concluded from all of these studies and other reports that apoptosis may be influenced by the ROS level generated upon the decline in the Cu, Zn-SOD level (Rothstein et al. 1994; Troy and Shelanski 1994; Fujimura et al. 2000). For example, Fujiyama et al. found that when the cytosolic release of cytochrome c was obstructed by Cu, Zn-SOD, it inhibited apoptosis in the brain of a mouse (Fujimura et al. 2000). Additionally, some other evidences depict an intimate rapport between ROS and apoptosis of luteal cells in other animals (e.g., bovines or pigs) (Murdoch 1998; Nakamura and Sakamoto 2001).

12.4 Endothelial Dysfunction in the Uterus

Oxidative stress highly impacts the physiology of pregnancy. It is instrumented by placental mitochondrial activity and ROS outcome of normal cellular activity (Roberts et al. 2009). Endogenous ROS is primarily produced by mitochondria, although some amounts are also produced by endoplasmic reticulum and peroxisomes (Snezhkina et al. 2019). Liberation of detrimental mediators into maternal circulation is brought about by excessive ROS generation. This excessive release is distinctly obvious in insufficient placentation that subsequently leads to ischemic placental microenvironment (Wu et al. 2015). Smooth muscle and endothelium are primed by immune cells like uterine natural killer cells (uNK) and macrophages for invasion. Particularly vascular infiltration process of the decidua and myometrium needs extravillous cytotrophoblast (EVCT) as a necessary part (Tannetta and Sargent 2013). Placental insufficiency is treated as an offender in obstetric complications that comprises of preeclampsia and intrauterine growth restriction (IUGR) arises when partial trophoblast invasion occurs in the maternal uterine spiral arteries (Krishna and Bhalerao 2011; Hromadnikova 2012). Conditions such as decreased placentation, OS, ischemia, inflammation, and apoptosis of the syncytiotrophoblast result from impaired utero placental blood flow (Burton et al. 2009; Mifsud and Sebire 2014). In the event of maternal obesity, the visceral adipose tissue mass elevates adipocyte dysfunction, causing increased ROS generation. The adipose and other peripheral tissues both show a raise in insulin resistance which are interrelated to this hyperbolic ROS generation (Aroor and DeMarco 2014).

Numerous vascular conditions such as matrix metalloproteinase (MMP) activation, vascular remodeling, hypertrophy of smooth muscle, and cellular apoptosis are typically the result of ROS

overflow. ROS induces IκB kinase (IKK) complex oxidation followed by nuclear factor kappa B (NF-κB) discharge that promotes transcription of different pro-inflammatory mediators of endothelial dysfunction including intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and inflammatory cytokines like tumor necrosis factor (TNF)-α and interleukin (IL)-6 (Tenório et al. 2019; Sprague and Khalil 2009). Throughout pregnancy, this interaction is aptly maintained. However, in preeclampsia and gestational diabetes mellitus (GDM), it becomes baffled (Powe et al. 2011; Pontes et al. 2015). Endothelial dysfunction in preeclampsia and GDM mainly occurs due to amplified ROS production. This causes potentially permanent vascular damage and modified endothelial phenotype, leading to serious results (Incalza et al. 2018).

12.5 Fertilization of Eggs

Raised levels of ROS lead to OS that acts as a primary cause of male and female infertility. Cell death or senescence is sourced from OS, thereby causing oxidation of biomolecules like DNA, RNA, proteins, and lipids of a cell. During in vitro fertilization (IVF), aiming for assisted reproduction, it is absolutely necessary to reduce OS. Today, we know the issues related to assisted reproductive technology and its importance to address the mechanisms and handle it. On the contrary, the advantageous function of ROS, like intracellular signaling, has become clear for fertilization.

Sperm motility and its potential fusion with an oocyte are reduced because of OS (Agarwal et al. 2008; Aitken and De Iuliis 2009). During IVF, the quantity of ROS generated from oocytes, sperm, and fertilizing processes is estimated (Ishii et al. 2005; Miesel et al. 1993). Testicular atrophy and enhanced susceptibility to heat stress occur due to inadequacy of SOD1 encoding Cu, Zn-SOD (Ishii et al. 2005). Despite the fact that no aberration is observed in male fertility, the sperm lacking SOD1 portrays less capability of fertilization owing to increased oxidation during IVF (Tsunoda et al. 2012). Contrarily, transgenic male mice demonstrating elevated level of mitochondrial Mn-SOD encoded by SOD2 were infertile for some unknown reasons (Raineri et al. 2001). High levels of SOD actions illustrate a negative relation to the motility in human spermatozoa (Aitken et al. 1996). Deficiencies in extracellular SOD3 encoding SOD fail to display any remarkable alterations of phenotype in human male reproductive system but when the former mentioned gene was transferred to the penis showed a betterment of erectile function in mature rats (Bivalacqua et al. 2005). The reason behind it is the rapid reaction of superoxide and nitric oxide to produce peroxynitrite that increases extracellular concentration of SOD in blood plasma so that the half-life of nitric oxide was extended which resulted in improved erectile function.

Somatic cells and oocytes are associated with the extremely differentiated sperm cells through their metabolism and function. Despite having low cytoplasmic content, numerous genes are expressed throughout spermatogenesis in a testis-specific method, whose roles are justified in sperm function. Although ROS affects sperm, some quantity of ROS is generated by sperm itself. Contrary to its damaging effects, sperm functions like capacitation and activation require ROS for their mechanisms (de Lamirande and O'Flaherty 2008). Sperm makes a transient attachment to the lower epithelial cells of the oviduct and swims to the site of fertilization called ampulla. Sperm surface displays cells of oviduct and lectin-like molecules which are basically carbohydrates that are intermingled for better sperm attachment. Since adhesion was altered by reductants, adherence of sperm was decided from the redox status on the external surface of the sperm (Gualtieri et al. 2009). The epithelium of the oviduct and uterus contain glutathione (GSH) reduced along with its recycling enzyme glutathione reductase. Glutathione constitutes the chief low-molecular-weight redox system (Fujii et al. 2011) which is required for fertilization, preimplantation, and development of the embryo

(Nakamura et al. 2011). As a matter of fact, many infertile patients are treated with GSH or its equivalent making the administration of GSH a promising way to enhance fertility (Irvine 1996). GSH can be supplemented for improvement in dyspermia which is a germ-free reproductive tract infection or due to varicocele. The infertile γ-glutamyl transferase knockout mice with reduced testes and seminal vesicles can reinstate entirely to their natural size of the testes upon administrating GSH or *N*-acetylcysteine compared to wild-type mice, and the mutant mice get fertile (Kumar et al. 2000).

Cells are defended from oxidative damages by antioxidative defense systems. However, several physiological functions are also portrayed by ROS such as making improvements in signals of phosphorylation by managing phosphatases (Rhee 2006). It is an established fact that extracellular $O_2^{\cdot-}$ and acrosome reaction of spermatozoa are connected. It has been suggested that both hydrogen peroxide and superoxide participate in management of this activity (de Lamirande et al. 1998). Redox reactions seemingly control the fertilizing capabilities of sperm; however, knowledge is limited about their reactions. For example, PDILT, which is the protein disulfide isomerase homolog, modulates the sperm membrane protein ADAM3 found to be needed for fertility (Tokuhiro et al. 2012). Studies suggest that PRDX is relevant and it has remarkable function in ROS signaling (Rhee 2006). Among the six parts of the PRDX group, PRDX4 holds an important role in spermatogenesis because lacking the same results in delay in sexual maturation and makes testicular cells susceptible to heat stress (Iuchi et al. 2009). PRDX4 is expressed with a testis-specific variation and may take part in the spermatogenesis (Sasagawa et al. 2001; Yim et al. 2011). Reduction in redox condition causes elevation of PRDX oxidation in the sperm, which seems to initiate male infertility (Manandhar et al. 2009; O'Flaherty and Rico de Souza 2011; Gong et al. 2012). Nevertheless, in-depth research is essential to recognize their function in reproduction.

12.6 Diseases Caused by ROS in Female Reproductive System

ROS are a double-edged sword; they serve as key signal molecules in physiological processes but also have a role in pathological processes involving the female reproductive tract. There is growing literature on the effects of ROS in the female reproduction with involvement in the pathophysiology of endometriosis, preeclampsia, hydatidiform mole, maternal diabetes, PCOS, ovarian epithelial cancer, free radical-induced birth defects, and other situations such as spontaneous abortion and recurrent pregnancy loss, intrauterine growth restriction, and fetal death.

12.6.1 Endometriosis

Endometriosis is a widespread gynecological disorder in women of reproductive age. The distinguished feature of this phenomenon is it occurs in the external tissue of the uterine cavity with prevalence of infertility and pelvic ache in patients. The primary cause of the disease is somehow indistinct and said to be founded by three main theories: retrograde menstruation, induction theory, and coelomic metaplasia. Both genomics and epigenomics are crucial for the occurrence of endometriosis with fluctuations in the reactive oxygen stress (ROS) levels and oxidative stress (OS) culminating to inflammation in the peritoneum. ROS regulates inflammatory reactions that balance cell proliferation by apoptosis. Genomic variation and cell survival are the examples of molecular modifications which are impaired parts of the pathogenesis of endometriosis. Various factors have been brought to light by latest research, which connects with oxidative stress, like cell cycle checkpoint sensors, forkhead transcription factor (FOX), hepatocyte nuclear factor (HNF), AT-rich interactive domain 1A (ARID1A), and microRNAs. FOX activity is regulated through ROS-induced post-translational modifications. FOX deprivation

wrecks the capability of cells to halt at checkpoints aiding to lesion formation, and a lower level of FOX expression in endometriosis patients compared to healthy women confirms the FOX action in the disease (Shigetomi et al. 2012). Similarly, recent studies reveal the ROS as a DNA methylation leading to aberrant gene expression. The investigation identified AT-rich interactive domain 1A (ARID1A) gene as a key factor of SWI/SNF chromatin remodeling complex, which could regulate gene expression by changing the structure of surrounding chromatin. ARID1 mutation frequency rate is found higher in cancer patients like liver cancer, breast cancer, and gastric cancer (Wu et al. 2016; Tordella et al. 2016; Jiang et al. 2015) than in endometriosis condition, yet sometimes it completely lost its expression during this clinical stage. Besides that, breast cancer only displays changes in ARID1A gene mutation frequency but does not associate with its expression level (Takeda et al. 2016). In the previous report, ROS could affect ARID1A gene expression level (Kwan et al. 2016). However, H. Xie in 2017 stated the mechanism of ROS associated with ARID1A gene silencing in endometriosis. Further experiments showed ROS regulated ARID1A gene expression by affecting its promoter methylation. HSP family includes heat shock protein 70 B as an inducible part. It occurs insignificantly under normal circumstances and gets amplified under stress. It acts as an escort for proteostatic activity like folding and translocation, with quality assurance. It is recognized to favor cell proliferation by subduing apoptosis, particularly when present in elevated concentration, as found in various tumor cells. When misfolded proteins are found in abundance, there is overexpression of HSP70, leading to a plethora of ROS. OS liberates HSP70, which instigates the function of inflammatory cytokines [93, 99] TNF-alpha, IL-1 beta, and IL-6, present in macrophages by toll-like receptors (e.g., TLR 4), perhaps being the reason of endometriotic tissue (Xie et al. 2017).

12.6.2 Preeclampsia

Human pregnancy associated with hypertension and proteinuria during the second or third trimester of gestation phase leads to preeclampsia (PE). This disease occurs among 3–8% of women worldwide, though its rate differs with geographical area, time duration in year, nutritional condition, and race/ethnicity (Steegers et al. 2010). Basically, PE occurs due to de novo hypertension (>140/90 mm Hg systolic/diastolic blood pressure) and proteinuria (>300 mg/24 h). Mostly PE gets associated with comorbidities like disseminated intravascular coagulation (DIC), edema, hepatic alterations (HELLP syndrome), and eclampsia, in particular targeting the brain (cerebral edema). PE leads to complication in the fetus like growth restriction that may lead to prematurity, loss in birth weight (1/3 of cases), and neonatal death. The disease worsens with time from its onset which may progressively lead to demise of both the fetus and mother. PE remains as a few fatal complications during pregnancy in today's most industrialized countries, and there is no cure for it till date. In most cases, PE leads to premature labor induction which demonstrates the risks for premature neonates (Zabul et al. 2015; Ghosh et al. 2014; Aouache et al. 2018).

At the cellular level, PE is associated with release of free radicals generated by the placenta. Placental-borne free radical stresses are considered as major molecular determinants of maternal disease. Low oxygen tension-induced oxidative stress improves maternal blood flow that leads to normal placentation. At the molecular level, the placenta of PE patients explains imbalanced reactive oxygen species (ROS) generating enzymes and antioxidants. In ex vivo preeclamptic trophoblast, it is observed that ROS-producing enzyme expression and activity are elevated and Wnt/β-catenin signaling pathway is inhibited that promotes trophoblast invasiveness (Many et al. 2000; Zhuang et al. 2015). Oxidative stress also leads to increased transcription of sFLT1(soluble fms-like tyrosine kinase-1),

an antiangiogenic factor (Huang et al. 2013). As compared to women with normal pregnancies, PE patients show impaired placental antioxidation mechanisms as explained by decreased expression of superoxide dismutase and glutathione peroxidase (Vaughan and Walsh 2002). However, treatment with antioxidants such as vitamins E and C did not significantly alter the disease in PE women, suggesting that ROS could be less integral to the pathways of the human syndrome (Poston et al. 2006).

Mitochondrial stress may lead to ROS generation. Zsengellér et al. established the inverse correlation in expression of mitochondrial enzyme cytochrome C oxidase, with expression of sFLT1 in the syncytiotrophoblast cells of preeclamptic placentas (Zsengellér et al. 2016). Based on a study on inhibition of HIF-1α by hydrogen sulfide donors, Covarrubias et al. demonstrated that pretreatment with a mitochondrial-targeting hydrogen sulfide donor AP39 may decrease sFLT1 expression in human syncytiotrophoblasts which brings enhancement in cytochrome C oxidase activity in a dose-dependent manner in both normal and PE placentas, which prevents the release of ROS and subsequent stabilization of HIF-1α (Covarrubias et al. 2019). Several other promising studies have also been reported with mitochondrial antioxidants in animal models of PE (Vaka et al. 2018).

Another possibility of elevated oxidative stress is the endoplasmic reticulum (ER) stress that is caused by ischemia-reperfusion injury. ER stress is observed in the deciduas and placenta of patients with restricted fetal growth and PE that also triggers apoptosis of decidual cells and cytotrophoblast by activating UPR (unfolded protein response). Another leading signaling pathway implicated in PE is a transmembrane kinase PERK (PKR-like endoplasmic reticulum kinase) that downregulated translational burden of ER and upregulates proapoptosis (Lian et al. 2011; Fu et al. 2015). Interestingly, a recent study suggested a synergism between ATF4 (activating transcription factor 4), a transcription factor downstream of PERK, and ATF6, a transcription factor regulator of misfolded proteins in ER homeostasis, which negatively regulate the transcription of *PlGF* (placental growth factor), which is a proangiogenic factor central to the pathogenesis of preeclampsia (Du et al. 2017; Mizuuchi et al. 2016).

12.6.3 Maternal Diabetes

Infants born to diabetic mothers have a higher chance of congenital abnormalities and growth disorders than those born to nondiabetic mothers, according to previous research. The cellular mechanisms that cause diabetes in pregnancies remain unclear. The developmental complications are most likely driven by countless factors, and hence, the etiology is presumably multifactorial (Sadler et al. 1989; Eriksson and Borg 1993; Buchanan et al. 1994). One teratological pathway in embryos exposed to a diabetes-like environment involves increased activity of ROS, impaired antioxidative defense, or both (Eriksson and Borg 1991). An increased production of superoxide inside mitochondria of tissues exposed to high-glucose concentrations has lately been proposed as a common mechanism for all diabetic problems, in keeping with the idea of ROS-mediated embryopathy (Nishikawa et al. 2000; Brownlee 2001). Elevated ROS leakage and impairment of the cytosolic glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase could be a result of excessive ROS synthesis in mitochondria (GAPDH). This enzyme has shown sensitivity toward ROS in a number of oxidative stress scenarios. The thiol group of cysteine residue 149 in active site of the enzyme is responsible for this sensitivity (Rivera-Nieves et al. 1999). Reduced enzyme activity is caused by the oxidation of the thiol group by NO or ROS, which may be associated with the development of embryonic dysmorphogenetic alterations (Morgan et al. 2002).

12.6.4 PCOS

Reproductive aged women are prone to frequent multifactorial endocrine disorders of which PCOS is the common one and considered as the primary reason for anovulatory infertility (Joham

et al. 2015). Chereau in 1844 foremost explained it as the variation in ovarian morphology (Chéreau 1844). In 2003, the European Society of Human Reproduction and Embryology (ESHRE) and American Society for Reproductive Medicine (ASRM) established the diagnostic norm for PCOS, based on the detailed research of the last decades, called as the Rotterdam Consensus Criteria. PCOS displays extreme diversities with clinical characteristics like menstrual disorder, secondary amenorrhea, serum hormone abnormality, hairiness, acne, obesity, and infertility (ESHRE and Group 2004). In spite of having an extensive record of research, its specific causal factor still remains unrevealed. At the present time, a pivotal part is played by oxidative stress, not only for PCOS but also for numerous other diseases. It is a known fact that extremely intricate antioxidant enzymatic and nonenzymatic systems manage the generation and distribution of intracellular ROS. However, a thorough knowledge of oxidative stress-induced PCOS mechanisms is required for its prevention and treatment. ROS elevation conducts the discharge of Ca^{2+} ions from endoplasmic reticulum and balance of storage and depletion of intracellular Ca^{2+}. Increased levels of Ca^{2+} impart detrimental effects like imbalance in the mitochondrial membrane and failure of adenosine triphosphate (ATP) synthesis, which cause preliminary necrosis of the cell. According to research, women with PCOS develop follicular arrest because of calcium dysregulation, consequently leading to reproductive and menstrual dysfunction (Mohammadi 2019; Rashidi et al. 2009).

The pathogenesis of insulin resistance in PCOS patient revealed by numerous studies showed that elevated OS leads to various protein kinase activations to trigger serine/threonine phosphorylation of insulin receptor substrate (IRS), which inhibit normal tyrosine phosphorylation of IRS, and directs in degradation of IRS (WANG et al. 1998; Runchel et al. 2011; Brown and Sacks 2009). ROS can activate various pathways including c-Jun *N*-terminal kinases (JNK) which is a component of transcription factor activator protein-1 (AP-1) and p38 pathways. The transcription of various genes like cytokines, growth factors, inflammatory enzymes, matrix metalloproteinase, and immunoglobulins is controlled by activator protein-1 (AP-1). Low-intensity inflammation and increased inflammatory cytokines are related with PCOS, resulting in the pathogenesis of the disease (Diamanti-Kandarakis and Dunaif 2012).

Polyunsaturated fatty acid side chains of the plasma membrane are the site of lipid peroxidation or that of any organelle that contains lipid. Owing to the presence of hydrophobic tail and lipid solubility, vitamin E in these chain reactions can snap and function as antioxidant (Abuja and Albertini 2001; Agarwal et al. 2012). Markers that signify the level of lipid peroxidation like thiobarbituric acid reactive substances, oxidized low-density lipoprotein, and malondialdehyde (MDA) amplify considerably in patients with PCOS in comparison to healthy individuals (González et al. 2006; Nur Torun et al. 2011).

As the oxidation capability of guanine residues is greater than cytosine, thymine, and adenine, DNA oxidation takes place. ROS invasion is highly detrimental to mitochondrial DNA, because of O^{2-} production through electron transport chain (Cooke et al. 2003). DNA damage caused by free radical and failed antioxidant defense has been indicated to be the causative agent for cancer. Dinqer et al. assessed DNA damage caused by increased H_2O_2, which can be used as a marker for DNA detection to oxidation in PCOS women. Ovarian cancer and PCOS connection can be described by considerable spike in DNA damage by H_2O_2 (Dincer et al. 2005).

12.6.5 Hydatidiform Mole

A molar pregnancy (also known as hydatidiform mole) is a form of gestational trophoblastic disease (GTD). Chromosomal anomalies during conception lead to aberrant growth of placental tissues, resulting in this type of pregnancy loss. This condition arises especially when a cluster of fluid-filled cells is developed from a fertilized egg instead of a fetus. Molar pregnancies cannot be sustained till birth and do not result in functional fetus, unless in extremely rare circum-

stances. Although most of the molar pregnancies are not cancerous, the tissue can develop malignancy in certain instances. Molar pregnancies can cause severe clinical complications, demanding months of precautionary supervision following treatment, which generally involves dilation and curettage (D & C), a process that removes conception tissue products from the uterus (Sun et al. 2016).

12.6.6 Ovarian Epithelial Cancer

The fifth major reason of cancer mortality is ovarian cancer, with demise from gynecologic malice being the primary reason and the second most frequently identified gynecologic disease; however, the fundamental pathophysiology remains unclear (Saed et al. 2017; Rojas et al. 2016). Epithelial ovarian cancer is a heterogeneous ailment with reaction to molecular biology, histopathology, and clinical outcome. The top-grade serous ovarian cancer (HGSOC) being the typical and extensively researched progressive levels of tumors for the most part are sourced from epithelial cells. Their origin can be from endometrioid, serous or mucinous cells placed on the surface of the epithelium belonging to the fallopian tube or ovary (Blagden 2015).

Numerous diseases are caused due to the involvement of oxidative stress such as cancer. The initiation, elevation, and advancement of tumor cells are altered as there is modification in the biological redox environment (Reuter et al. 2010). The major cellular processes that manage the stability of cell development and apoptosis are influenced by the constant production of free radicals along with oxidants. It portrays a significant function in the commencement of various cancers. Oxidants initiate and assist the oncogenic phenotype or bring on apoptosis, by considering the level of ROS and RNS in the cellular surroundings, serving as antitumor representatives (Wang and Yi 2008). A number of transcription factors regulate the interpretation of genes important to the growth and development of cancer cells which are known to be managed by oxidative stress. This includes hypoxia-inducible factor (HIF)-1α, nuclear factor (NF)-κB, peroxisome proliferator-activated receptor (PPAR)-γ, activator protein (AP)-1, β-catenin/Wnt, and nuclear factor erythroid 2-related factor 2 (Nrf2) (Reuter et al. 2010).

It is important to note that ROS and RNS produce genetic mutations, altering gene expression along with triggering DNA damage and thus suggesting to be the causative factor of numerous pathologies (Rojas et al. 2016; Reuter et al. 2010; Roos et al. 2016). Flawed DNA owing to ROS and RNS is acknowledged to be a leading factor to develop multiple cancer types (Waris and Ahsan 2006). The DNA bases are revised by oxidative stress by base pair substitutions instead of deletions and insertions of the base. Mutations result whenever there are alterations in GC base pairs; however, AT base pair alteration does not cause the same (Retèl et al. 1993). G to T transversions are the consequences of guanine alterations in cellular DNA that is the most responsible factor to produce ROS and RNS (Waris and Ahsan 2006). The DNA belonging to oncogenes or tumor suppressor genes can establish the commencement of cancer if the modification of G to T in the DNA is not restored. The DNA belonging to oncogenes or tumor suppressor genes can initiate cancer, if the modification of G to T in the DNA is not restored. Thymidine glycol, 5-hydroxymethyl-2′-deoxyuridine, and 8-OHdG are among the few oxidized forms of DNA bases which are recognized sign of DNA impairment caused by free radical (Roos et al. 2016).

Cell migration is amplified by free radicals and oxidants, which leads to increase in tumor invasion and metastasis, resulting in mortality of cancer patients. ROS enables NF-κB to maintain communication of intercellular adhesion protein-1 (ICAM-1), a cell surface protein in numerous cell variants. Owing to trigger in OS, interleukin-8 (IL-8) initiated increased expression of ICAM-1 on neutrophils, amplifying neutrophil movement through the endothelium, which is principal in tumor metastasis (Reuter et al. 2010). Cell migration and resulting tumor invasion are managed by the increase in distinct matrix metalloproteinase (MMPs) enzymes, in the downregulation of vari-

ous factors of the extracellular matrix and basement membrane (Reuter et al. 2010; Westermarck and Kähäri 1999). Free radicals particularly H_2O_2 and NO magnify the function of MMPs, like MMP-2, MMP-3, MMP-9, MMP-10, and MMP-13, due to the enabling of Ras, ERK1/2, p38, and JNK, or the inactivation of phosphatases (Reuter et al. 2010; Westermarck and Kähäri 1999). As a matter of fact, the chief origin of ROS, the NAD(P)H oxidase family of enzymes, is connected to advancement of tumor cells in lung and pancreatic cancers (Rojas et al. 2016; Reuter et al. 2010). Hence, it authenticates ROS to be the major cause in the activation of different cancer types.

12.6.7 Spontaneous Abortion and Recurrent Pregnancy Loss

Recurrent pregnancy loss (RPL) can be termed as the loss of three pregnancies consecutively prior to 20 weeks from the gestational period or fetal weight having less than 500 gms which can affect approximately 30% to 50% of conception before completion of the first trimester. Spontaneous abortion is also a sudden pregnancy loss before 20 weeks of carrying the embryo without intervening any outer factor, and 15–20% of clinical pregnancies are affected from it. RPL can be said as an annoying clinical inconvenience which affects 0.5–3% of fertile group of females from which 50–60% cases are idiopathic. Besides, a primary factor of spontaneous pregnancy loss is due to chromosomal abnormalities, and ROS-generated oxidative stress also might have some probability to participate in fertility dysfunctions like idiopathic recurrent pregnancy loss, spontaneous abortion, defective embryogenesis, hydatidiform mole, and drug-induced teratogenicity. According to research, both systemic and placental oxidative stresses are responsible in the pathophysiologic condition of frequent abortion and RPL. Impaired placental vascularization, oxidant-induced endothelial damage, and immune malfunction are the multiple factors considered for idiopathic recurrent pregnancy loss (Gupta et al. 2007).

As previously stated, there is an oxidative outburst in the placenta between 10 and 12 weeks of pregnancy. After the gush of antioxidant pursuit, the normal level of OS is restored, and the placental cells accustom slowly to the freshly oxidative environment (Jauniaux et al. 2000). In the event of miscarriage, the arrival of maternal intraplacental circulation happens intermittently before time between 8 and 9 weeks of gestation as compared to normal pregnancies (Jauniaux et al. 2000). These placentas show increased concentration of HSP70, nitrotyrosine Hempstock, 2003 #117} (Jauniaux et al. 2003), and markers of apoptosis in the villi, indicating oxidative damage to the trophoblast, thereby terminate the pregnancy (Burton and Jauniaux 2011). During early pregnancy, antioxidant enzymes are not capable to resist the high levels of ROS; rather, a gradual rise in activity occurs with growing gestational age (Jauniaux et al. 2000). If OS happens way too soon in pregnancy, it can damage placental growth and magnify syncytiotrophoblastic degeneration, concluding in the termination of pregnancy (Gupta et al. 2007). Patients with RPL have higher concentrations of plasma lipid peroxides and GSH, as well as lower amounts of vitamin E and β-carotene, which supports the spontaneous abortion process (Şimşek et al. 1998). GSH levels in the plasma of women with a history of RPL were also reported to be significantly higher, reflecting a response to increased ROS (Miller et al. 2000). A different research revealed that patients with idiopathic RPL have extremely reduced levels of the antioxidant enzymes GPx, SOD, and catalase, as well as elevated ROS and MDA levels. Total antioxidant capacity, serum prolidase, and sulfhydryl levels (markers of oxidative stress) have presented significant correlation in women with early pregnancy loss (El-Far et al. 2007).

12.6.8 Intrauterine Growth Restriction (IUGR)

Newborns with birth weight less than tenth percentile are termed as intrauterine growth. 10% of infants are concerned with this state and hence

spike the possibilities of perinatal morbidity and death. Components which majorly cause IUGR include placental, fetal, and maternal factors (Chauhan et al. 2009). A key source of IUGR is preeclampsia which grows in the placenta from uteroplacental inadequacy and ischemic procedures (Scifres and Nelson 2009). Research suggests patients having IUGR progress into OS owing to placental ischemia distress secondary to underdeveloped spiral arteriole. Features of IUGR patients include disproportioned wounds and restoration, along with uncommon progress of the villous tress, making them prone to exhaustion of syncytiotrophoblast, resulting in restricted control of convey and secretory purpose. Hence, in the growth of IUGR, ROS and OS are acknowledged as major components and are produced by potent sources like ischemia and reperfusion trauma (Biri et al. 2007). The controlling apoptotic function of p53 is notably elevated in relation to hypoxic environment in villous trophoblast (Levy et al. 2000); (Levy et al. 2002; Heazell et al. 2008) and signs an increased level of apoptosis secondary to hypoxia reoxygenation than from hypoxia alone. In IUGR placenta, reduction in translation and signaling of proteins sums to overpowering of OS (Yung et al. 2008).

12.6.9 Fetal Death

Fetal death can also be referred to as stillbirth which is described as the unplanned intrauterine death of the fetus occurred at any stage of the pregnancy after 20 weeks of gestation or more. Reports says hypertension, diabetes, multiple gestations, obesity, older maternal age, growth restriction, and preeclampsia like earlier pregnancy complications, history of miscarriage or stillbirth, exposure to alcohol, smoking or any drugs, or any racial group like non-Hispanic black might be some risk factors to induce fetal death. ROS-induced oxidative stress has physiological and pathological function in the placenta, embryo, and fetus. Oxidative stress in the uterus is a consequence of prenatal hypoxia, nutritional deficiency or overnutrition, and excessive glucocorticoid exposure which occurs to the mother (Chen et al. 1999; Morriss 1979; New and Coppola 1970). Among all of these factors, prenatal hypoxia is a condition arises in the early postimplantation phase which is a prerequisite for preliminary organogenesis, and the embryo is utmost reactive to surrounding oxidative stress on account of poorly developed antioxidant defense. As soon as uteroplacental circulation continued, the embryo progresses toward being immune to oxidative stress by amplifying antioxidant defense system (Schafer and Buettner 2001). The measure of oxidative tone and its oscillations is defined as redox switching that modulates the density of the cells in the embryo in the direction of proliferation, apoptosis, differentiation, or necrosis. Altogether, numerous occurrences revealed a major function of ROS in the embryo. Moreover, during embryonic growth, special signaling tracks can be modified by ROS. ROS majorly affects cells and behaves as second messengers by controlling major transcription factors that modulate gene expression in the embryo. Of the numerous transcription factors that are susceptible to redox reactions, nuclear factor jB (NF-kB), hypoxia-inducible factor (HIF-1), redox effector factor-1 (Ref-1), activator protein-1 (AP-1), nuclear factor (NF)-E2 related factor 1 (Nrf-1), and wingless and integration site for mouse mammary tumor virus (Wnt) are important to cell signaling pathways that control proliferation, differentiation, and apoptosis, therefore having a primary function in the embryo's growth (Dennery 2007).

12.7 Conclusion

The delicate balance between ROS generation and cellular antioxidant defense in the elixir of aerobic mode of life and female reproduction is no exclusion. Albeit low levels of ROS are always desirable for maintenance of cellular redox homeostasis and normal physiology, an excess in general leads to pathological states. Both obesity/overnutrition and malnutrition, overexercise, and lifestyle factors such as consumption of alcohol and recreational drugs exert noxious effects of female reproduction. Preeclampsia, gestational

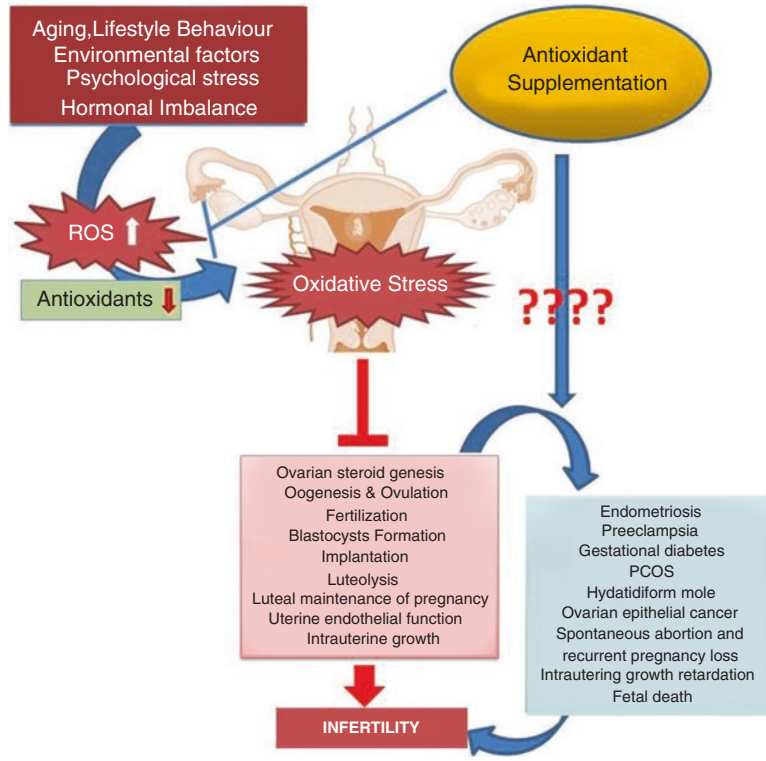

Fig. 12.1 Schematic representation of oxidative stress-induced impairment of female reproduction

diabetes, endometriosis, etc. have oxidative predominance. There are a good number of studies mostly on animals regarding positive impact on female reproduction, while the same on human ailments is controversial (Fig. 12.1). Therefore, future studies may be targeted in understanding the underlying molecular mechanism(s) via high-throughput technologies such as multiomics platforms for personalized medical care.

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13 Impact of Oxidative Stress on Embryogenesis and Fetal Development

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Abstract

Multiple cellular processes are regulated by oxygen radicals or reactive oxygen species (ROS) where they play crucial roles as primary or secondary messengers, particularly during cell proliferation, differentiation, and apoptosis. Embryogenesis and organogenesis encompass all these processes; therefore, their role during these crucial life events cannot be ignored, more so when there is an imbalance in redox homeostasis. Perturbed redox homeostasis is responsible for damaging the biomolecules such as lipids, proteins, and nucleic acids resulting in leaky membrane, altered protein, enzyme function, and DNA damage which have adverse impact on the embryo and fetal development. In this article, we attempt to summarize the available data in literature for an in-depth understanding of redox regulation during development that may help in optimizing the pregnancy outcome both under natural and assisted conditions.

Keywords

Embryogenesis · Oxidative stress · Reactive oxygen species · Hypoxia · Placental remodeling

13.1 Introduction

Growth, differentiation and cell fate of the embryo depend on the integral role of oxygen (O_2) (or the lack thereof) for execution of the developmental genomic program, hence classified as a developmental morphogen in analogy to classical morphogens such as secreted growth factors (Hansen et al. 2020). Oxygen is essential for aerobic mode of life as oxidative metabolism is the primary source of energy. Nevertheless, incomplete reduction products of oxygen, termed as reactive oxygen species (ROS), create havoc by damaging almost all types of biomolecules when their generation is beyond the threshold of cellular antioxidant defense. On the other hand, their controlled release is said to be beneficial. ROS have been established to be implicated in various regulatory cell signaling pathways and regulating important cellular functions (Nathan 2003). Similarly, regulated redox state is important for ensuring a proper embryonic devel-

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opment. Preimplantation embryos were mostly used for study as ethical issues and inaccessibility of conceptus make the implanted embryo difficult to obtain. During early embryonic development, embryos are "reprogramed" on the genomic and metabolic levels to adapt to changing redox state. Specific posttranslational modifications (PTMs) in cysteine proteome would constitute the reprograming of particular redox couples (nodes) to regulate protein function and development (Hansen et al. 2020).

In the current chapter, a more defensible recapitulation of oxygen sensing, metabolism, and developmental regulation through a specific redox interface is discussed. The chapter focuses on how a normoxic embryo/conceptus at fertilization is physically moved to a hypoxic environment and essentially repeat the stages (phylogeny) represented by the evolution of adaption to increasing oxygen level. Besides, the augmented ROS level at pathophysiological state where the quality of the embryo is compromised causing adverse pregnancy outcome under both in vivo and in vitro conditions is also explained.

13.2 Redox Theory of Development

"Redox theory of development" proposed recently (Hansen et al. 2020) has its root at Allen and Balin's "free radical theory of development" (Allen and Balin 1989) which was further extended by Hitchler and Domann (2007). Taking into account the different facets of redox regulation of embryonic development, redox theory of development was put forth which highlighted the relevance of O_2 as a critical morphogen regulating differentiation programs of complex organisms (Hitchler and Domann 2007). According to the free radical theory, O_2-dependent radical generation and free radical-scavenging antioxidant systems constitute the fundamental pathways for development. The differential O_2 supplies in the female reproductive system modulate the developmental metabolic gradients that occur during embryogenesis. Moreover, commencement of certain developmental events is also directed by metabolically generated oxidants implying the involvement of ROS as a critical intermediate to maintain bioenergetics, redox proteome, and supply of nitric oxide, hydrogen peroxide (H_2O_2), oxidized lipids, and other redox signaling systems in the developing embryo. Supplementing the above principles, Hitchler and Domann (2007) postulated the role of glutathione (GSH) production and O_2 sensing for establishment of the epigenetic control of gene expression during development. The availability of S-adenosylmethionine can be limited by regulating the production of GSH. The former is ascribed to act as a cofactor during epigenetic control of gene expression by DNA and histone methyltransferases. Likewise, O_2 is a known cofactor for histone demethylases. Furthermore, oxidative PTMs of cysteine-rich regulatory redox proteins synchronized timely coordinated redox regulation of major developmental events (Hitchler and Domann 2007). On the other hand, the steady state of non-radical redox systems ($NADH/NAD^+$ and $NADPH/NADP^+$) which regulate the bioenergetics and redox proteome equilibrium in the embryo is maintained by relatively stable oxidant pools, comprising of H_2O_2 and other non-radical oxidants. Anomalies in normal redox signaling would cause compromise to the developmental programs in the developing embryo proper and fetus (Fig. 13.1).

13.3 Cross Talk of Life from Gametogenesis Through Fetal Development

13.3.1 Redox Regulation of Gametogenesis

Excessive generation of ROS is well established to negatively affect both spermatogenesis and oogenesis. As the spermatozoa and ova are formed in the testes and ovary, respectively, the multiple steps of gonogenesis/gametogenesis are highly susceptible to oxidative stress insult. Any adverse effect on the quality of gametes would thus compromise the fertilization and postembryonic development. However, controlled levels of

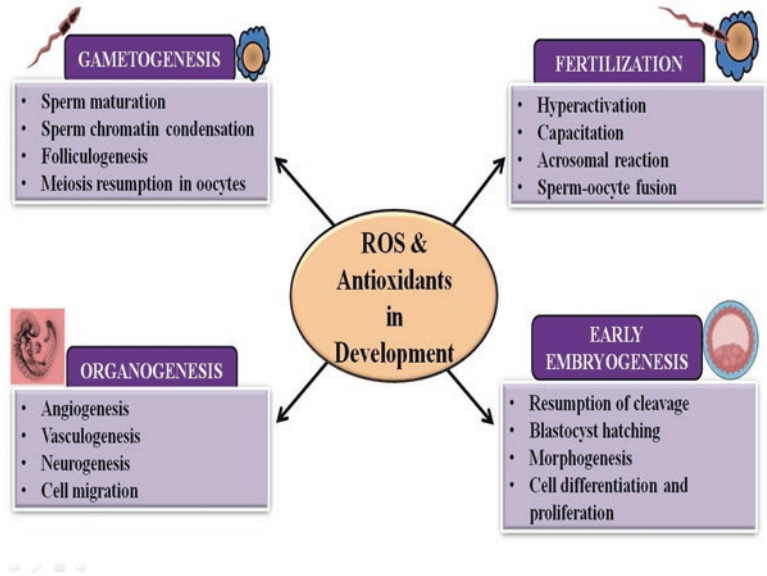

Fig. 13.1 Schematic representation of redox regulation of development

ROS would ensure proper production and functioning of sperm and ovum. During spermatogenesis, a transition in metabolic state of sperm mitochondria from glycolytic to oxidative phosphorylation (OXPHOS) is observed which is associated with regulated formation of ROS in sperm (Zhang et al. 2018). Epididymal sperm maturation marked with sperm chromatin condensation and plasma membrane rearrangements needs ROS as an intracellular signal transducer (Dutta et al. 2019). Moreover, a testis-specific variant of antioxidant protein peroxiredoxin 4 (PRDX4) is reported to modulate spermatogenesis. A lower redox state in the sperm would cause the oxidation of PRDX resulting in male infertility (Yim et al. 2011).

Likewise, metalloproteins in cumulus cells acted as redox buffers in the follicle containing the oocyte. Metalloproteins as antioxidants mainly cytosolic Cu/Zn-superoxide dismutase (SOD1) and the mitochondrial Mn-SOD (SOD2) have been found to be dominant in preantral and antral follicles up to the stage of the dominant follicle (Laloraya et al. 1989; Wang et al. 2017; Matos et al. 2009). An increment in the levels of ROS is observed during final oocyte maturation, inducing apoptosis and breakage of follicular wall and release of the cumulus-oocyte complex (COCs) (Cummins 2002). Moreover, ROS in the follicular fluid is presumed to affect folliculogenesis as well as steroidogenesis and induce apoptosis in antral follicles (Arhin et al. 2018). The glutathione (GSH-GSSG) reductase system in the oocyte is the primary defense against the increasing concentrations of ROS (El Mouatassim et al. 2000; Tsai-Turton and Luderer 2006). Physiological levels of ROS induce meiosis resumption in the growing oocyte. In rodent follicular oocytes, suppression of catalase activity with a concomitant increase in H_2O_2 induces meiotic resumption from diplotene arrest (Tiwari and Chaube 2016). Nonetheless, mitochondrial-triggered apoptosis due to pathological levels of ROS possibly destabilized M-phase-promoting factor (MPF) causing a decline in survival-promoting factors in the oocyte (Matos et al. 2009). An augmented oxidative damage with high ROS and low antioxidant enzyme activity as seen in aged mouse ovaries is known to cause infertility (Choi et al. 2011). Oxidative stress is also demonstrated to induce premature oocyte activation in primordial and dormant follicles by suppression of phosphatidylinositide and PTEN as seen in mice (Leslie et al. 2003; Li et al. 2010). Similarly, PTEN-deficient oocytes have shown an acceleration of maturation under high ROS conditions.

13.3.2 Redox Regulation of Fertilization

Fertilization encompasses highly synchronized and coordinated events marked with phenotypic changes in both sperm and ova. Limited production of ROS by sperm is essential to regulate sperm functions mainly capacitation and activation. Glutathione (GSH), the major low-molecular-weight redox system, is necessary for vital processes like fertilization, preimplantation, and embryogenesis (Fujii et al. 2011; Nakamura et al. 2011). Redox status of the surface carbohydrates of spermatozoa is determined by GSH-GSSG. Its recycling enzyme glutathione reductase found in epithelial cells of the uterus and oviduct is postulated to help the spermatozoa adhere to the epithelial cells in the lower portion of the oviduct and swim up to the ampulla, where fertilization takes place (Gualtieri et al. 2009). Involvement of ROS has been demonstrated in the regulation of hyperactivation, capacitation, and acrosome reaction mainly by phosphorylation of several proteins (Reddy et al. 2008). Adenylate cyclase (AC) is activated by Ca^{2+} and superoxide (O_2^-), producing cyclic adenosine monophosphate (cAMP) and activating protein kinase A (PKA). The above would then activate NADPH oxidase (Nox) augmenting ROS production. Protein tyrosine kinase (PTK) is also correspondingly stimulated, phosphorylating Tyr residues present in the fibrous sheath of the flagellum. In addition, ROS inhibited phosphatases which prevented disintegration of cell membranes, thus increasing fluidity of cellular membranes for sperm-egg fusion (Li et al. 2010).

13.3.3 Oxygen Gradient and the Developing Embryo

The adaptation to the oxygen concentration of the milieu determines and modulates both early and late embryonic developments in utero. Inferring from other relatable rodent species, it is well understood that the conceptus is exposed to varying levels of O_2 depending on the location and the state of implantation. A physoxic environment with physiological O_2 levels is maintained in the ampulla at around 4.2–5% (32–38 mmHg) during oocyte maturation, ovulation, and fertilization in the ampulla/fallopian tubes of oviduct as seen in mouse. The concentration increases up to 6.8% (52 mmHG) by day 3 post-coitum (Fischer and Bavister 1993). However, at around day 4, the O_2 concentrations drop to 1–5% O_2 (0.5–38 mmHg) exposing the implanting blastocyst to a hypoxic environment (Okazaki and Maltepe 2006). This change in O_2 concentration by physical transfer of the conceptus from a physoxic environment to hypoxic placental environment instructs expression of the developmental programs. This severe hypoxia is the ideal platform to maintain totipotency and pluripotency facilitating colony expansion of the inner cell mass of the blastocyst, the source of embryonic stem cells (ESCs). The extreme hypoxic uterine environment is established up to gestational day 15 (GD 15) by increasing diffusion distance and distinct environmental barrier in between fluid-filled cavities, such as the chorioallantoic and amniotic cavities and embryonic tissue layers (epiblast and hypoblast). As the process of gastrulation is initiated in the implanted embryo, different spatial internal O_2 gradients are regulated during the germ layer formation. A marked transition takes place from an extreme hypoxic milieu (7–12 mmHg) during ectoderm formation through moderately hypoxic (15–17 mmHg) during mesoderm formation to physoxic levels of O_2 (>38 mmHg) as the underlying endoderm gets formed. However, throughout organogenesis during GD 9–15, embryos are exposed to low O_2 levels (Dunwoodie 2009). Hematopoiesis, angiogenesis, chondrogenesis, neurogenesis, myogenesis, and most of the embryogenesis and fetogenesis processes basically require acutely hypoxic conditions with hypoxia-inducible factor-1 (HIF)-mediated regulation for proper development (Hansen et al. 2020; Fraisl et al. 2009; Simon and Keith 2008). As the fetus approaches parturition, hypoxic conditions are limited to focal, punctuate pockets which are opined to be rich in stem cells that would require low O_2 levels for colony expansion and proliferation.

13.3.4 Oxygen Consumption by Preimplantation Embryo

Although fertilization and early embryonic development occur in vivo under relatively low O_2 conditions, a steady state of oxygen utilization is maintained in the preimplantation mouse embryo from the zygote to morula stages with a marked increase in the blastocyst at GD 6.5–7.5 (Houghton et al. 1996). A metabolic shift from aerobic respiration during early preimplantation stages to both oxidative phosphorylation and anaerobic glycolysis at the blastocyst stage exposes later to higher ROS. Blastulation rates can be regarded as a basic indicator of embryonic viability. It is seen that nonviable human embryos had increased rate of ROS production and reduced antioxidant capacity which created a more peroxidative environment. Proapoptotic genes Bax and Fas expression increased with suppression of antiapoptotic gene Bcl-2 stimulating apoptosis in those embryos (Liu and Foote 1995). On the other hand, an unexpected positive correlation was observed between ROS levels in the hydrosalpingeal fluid and normal blastocyst development rate (Bedaiwy et al. 2002).This implies that the impact of oxidative stress on normal functioning of embryonic development especially preimplantation is rather uncertain.

13.3.5 Role of ROS in Postimplantation Embryo

For establishment of a healthy maternal-fetal interface, blastocyst needs to be implanted properly postfertilization in the maternal decidua, with subsequent superficial invasion of trophoblast into the myometrium. Conferring to "two-wave invasion" theory, the preliminary invasion in the decidual layer is paused, and a second wave of trophoblast is resumed at around week 12 of human gestation. During the first trimester, the embryo proper develops more rapidly than the placental mass. In early pregnancy (first trimester), extravillous trophoblast (EVT) cells plug the intervillous space in the developing placenta where the maternal blood flows. This constrains the flow of oxygenated blood that causes low oxygen tension in the intervillous space (Burton and Jauniaux 2011). Since the developing fetus is deficient in ROS-scavenging mechanisms, maintaining low ROS levels is essential for proper embryogenesis and organogenesis. At week 12 (second trimester), EVT plugs are dissolved, and a second wave of deep and diffuse trophoblast invasion occurs infiltrating into the endometrium, myometrium, and maternal spiral arteries. Both the interstitial and endovascular infiltration processes ensured proper rooting of the fetus with large-caliber, low-resistance maternal-fetal circulation, establishing a continuous low-flow perfusion of oxygenated blood into the placental intervillous space. Villi around gestational sac composed the definitive discoidal placenta, while the opposed villi regress forming the placental chorionic membrane. A distinct rise in partial pressure of oxygen in the placenta from <20 mmHg (2–4%) at week 8 to >50 mmHg (10%) at week 12 induces augmented oxidative stress in trophoblasts preferentially in critical syncytiotrophoblastic layer. This gradual periphery-to-center spread of oxidative stress triggers an apoptotic cascade in the peripheral placenta leading to placental regression (Jauniaux et al. 2003). Thus, ongoing normal pregnancies too require a burst of oxidative stress in the placenta at 10–12 weeks of gestation, which soon gets resolved as the placental tissues express higher amount of antioxidants (Jauniaux et al. 2000). The placenta is the primary organ where the exchange of nutrients and oxygen between the mother and the fetus takes place. With the establishment of maternal circulation at week 12, a sharp rise in ROS is seen in the placenta exposing later to the oxidative challenges. Impaired placental function due to untimely and premature supply of excess ROS would result in placental diseases arresting the fetal growth. In contrast, fetal oxygen levels are more or less gradual. Low levels of oxygen in the embryo proper are demonstrated to favor both angiogenesis and neurogenesis (Burton and Jauniaux 2011). Although fetal tissues of this gestational age are rich in GSH, cysteine, and GSTP1, an important isoform of glutathione S- transferase (GST), the embryo

is still highly vulnerable to oxidative damage (Raijmakers et al. 2001).

13.4 Developmental Processes Controlled by Redox Reactions

13.4.1 ROS and Early Embryonic Development

Regulated embryonic development is a game of phosphorylation-dephosphorylation reactions that regulates the cell cycle progression in fertilized eggs. Activities of M-phase-promoting factor (MPF) are suppressed in the oocytes released during ovulation in aged mice (Tatone et al. 2006). A cyclin-dependent protein kinase Cdc2 (i.e., Cdk1) combines with cyclin B to form MPF. WEE1 kinase phosphorylates Tyr15 and Thr14 sites of Cdc2 rendering Cdc2/cyclin B complex inactive. A dual-specificity phosphatase Cdc25 (particularly Cdc25B) dephosphorylates the above Tyr and Thr residues at the M-phase of the cell cycle activating the MPF during embryogenesis in mice (Lincoln et al. 2002). Commencement of embryonic gene expression postfertilization is marked with release of developmental arrest of mammalian embryos due to an increased production of antioxidants. ROS precisely H_2O_2 oxidizes reactive Cys residues in Cdc25, transiently inactivating the later and eventually leading to developmental arrest of the two-cell embryos. In physiological conditions, early embryonic development occurs at very low ROS levels, with GSH forming an intricate part of the redox couple. The oxidized Cys in Cdc25 is reduced back to sulfhydryl by intracellular reductant GSH. Augmented production of GSH during oocyte maturation is maintained during the first cleavage (Luberda 2005). However, a fall in concentrations of GSH is observed during preimplantation embryo in vivo (Gardiner and Reed 1994). Similarly, sustained expression of PRDX during the first cell division reduces at 16-cell embryo stage, followed by an increase in expression at the blastocyst stage (Leyens et al. 2004) . SOD also plays an important role to release the developmental arrest of mice embryo at two-cell stage (Natsuyama et al. 1993). SOD1 deficiency prevented the activation of Cdc2 and promoted expression of Cdk inhibitor genes to maintain the arrest phase in two-cell embryos. The p66Shc protein, a member of the Shc family of adaptors for signal transduction, being triggered by ROS is found to cause permanent developmental arrest in bovine embryos (Betts and Madan 2008). Thus, ROS under oxidative stress conditions inactivates cell cycle progression along with stimulating expression of the genes implicated in cell cycle suppression (Tsunoda et al. 2014). Blastocyst development occurs in hypoxic condition, and exposure to any oxidative insult at this stage would arrest development leading to embryonic cell death (Liu and Foote 1995). However, interestingly, a superoxide burst is seen to be the trigger for blastocyst hatching from the zona pellucida in mouse (Thomas et al. 1997). Treatment of antioxidants in blastocysts prevented the hatching which indicated the importance of ROS in regulating the above developmental process.

13.4.2 ROS and Morphogenesis

Morphogenesis which is a fundamental aspect of embryonic development controls tissue growth and patterning of cellular differentiation. It involves coordinated cell proliferation, growth, migration, and aggregation, secretion of extracellular substances, change in cell shape, and even cell death. Proliferating mammalian cells have very low levels of H_2O_2 or superoxide as seen in proliferating smooth muscle cells (Rao and Berk 1992), fibroblasts (Burdo and Rice-Evans 1989), amnion cells (Ikebuchi et al. 1991), and aortic endothelial cells (Ruiz-Gines et al. 2000). A balanced level of antioxidants and ROS is required to ensure a normal proliferation of cells in the embryo. An increased production of catalase and/or SOD2 arrested Egf-induced proliferation of vascular smooth muscle cell followed with decrease in phosphorylation of Erk1/2 (Brown et al. 1999; Shi et al. 2004). In contrast, higher levels of ROS (particularly H_2O_2) arrested growth

temporarily by pausing the expression of housekeeping genes and inducing the expression of stress-related genes related to oxidant-scavenging and DNA repair (Davies 1999). However, a further increase in H_2O_2 concentrations initiate cellular replicative senescence state where the cells undergo a permanently growth-arrested state marked by normal cell functions but no cell division (Davies 1999). At low pathological levels of ROS, type 1 cell death or apoptosis is induced; at intermediate ROS levels, type 2 cell death or autophagy is triggered; while at extremely high levels of ROS, necrotic cell death is prompted (Scherz-Shouval and Elazar 2007; Bras et al. 2005). Apoptotic cell death involves two main pathways: the intrinsic, mitochondrial pathway and the extrinsic, receptor-mediated pathway. ROS initiates the mitochondrial-dependent intrinsic pathway through the activation of the MAPK pathway and inducing expression of pro-apoptotic Bcl-2 proteins Bax or Bak (Ueda et al. 2002). Similarly, ROS-regulated activation of cell surface death receptors of the extrinsic pathway, is associated with tumor necrosis factor alpha (Tnf-α) receptor activation of caspases through adaptor proteins (Shen and Pervaiz 2006).

ROS is seen to regulate one of the earliest morphogenetic processes in mammalian development, i.e., the formation of the proamniotic cavity by cavitation of ES-derived embryoid bodies through ROS-induced cell death (Hernández-García et al. 2008). During embryonic development, regions marked with abundant cell death are rich in ROS concentrations as seen in the mouse embryo (Salas-Vidal et al. 1998). Limb morphogenesis with interdigital cell death and interdigit regression is regulated by ROS-induced cell death. Developing limbs were seen to have high concentrations of ROS in the interdigital regions and increased expression of antioxidant Gpx4 in the digits (Schnabel et al. 2006). In accord, treatment with retinoic acid induced morphogenesis, while treatment with antioxidants reduced the morphogenesis process in the embryo, implying the regulatory role played by ROS (Salas-Vidal et al. 1998; Schnabel et al. 2006; Cuervo et al. 2002). Likewise, ROS also controls during spinal cord development by promoting natural motoneuron death (Sanchez-Carbente et al. 2005).

13.4.3 ROS and Cell Differentiation

Cellular differentiation is an essential step in developmental process as the proliferating cells lose their potency and become determined and committed to a fate. Neurogenesis, osteoclast differentiation, cardiomyogenesis, and adipocyte differentiation are some of the developmental processes demonstrated to be redox-regulated. Augmented ROS synthesis is also engaged in instinctive differentiation of human embryonic stem cells (ESC). A dramatic variation in mitochondrial and cytoplasmic SOD, catalase, and PRDX is also observed during early differentiation (Cho et al. 2006). In mice embryo, it has been shown that E15 brain cells with low levels of ROS differentiate into large pyramidal-like neurons that maintain high concentrations of ROS. Treatment with antioxidants was shown to proportionately produce more numbers of smaller neurons (Tsatmali et al. 2006). ROS also induces/influences differentiation of PC12 cells (Kamata et al. 2005), mesencephalic (Studer et al. 2000), neural crest (Morrison et al. 2000), and oligodendrocyte type 2 (Smith et al. 2000) precursors. The receptor activator of NF-kappaB (RANK) and its ligand RANKL plays a vital role in differentiation and activation of osteoclasts. The binding of RANKL to its receptor in bone marrow monocyte-macrophage lineage (BMM) cells activates Nox1 causing a transient increase in intracellular level of ROS. It also causes binding of Tnf receptor-associated factor 6 (Traf6) to the cytoplasmic domain of RANK, which activates MAPK signaling cascade involving Jnk, p38, and Erk. All these signaling processes lead to ROS promoting differentiation of BMM cells into osteoclasts. A deficiency in Traf6, treatment with antioxidants, or blocking of Nox1 activity prevented ROS production, activation of MAPK, and subsequent osteoclast differentiation (Lee et al. 2005). A low-level ROS pulse causes differentiation of ES cells toward the cardiomyocyte as well as vascu-

lar cell lineages (Li et al. 2006; Sauer and Wartenberg 2005). The cardiovascular differentiation of ES cells is marked by transient expression of Nox4 and the regulatory subunit p67Phox and enhanced production of ROS (Li et al. 2006; Sauer and Wartenberg 2005). Antioxidant exposure reduced expression of genes essential for cardiomyogenesis and vasculogenesis such as HIF and vascular endothelial growth factor (VEGF) (Schmelter et al. 2006). Similarly, redox-regulated germ line and vulval development have also been reported in *Caenorhabditis elegans* (Shibata et al. 2003). In contrast, adipocyte differentiation is negatively regulated by ROS. Upregulation of the expression of the gene encoding the adipogenic repressor Chop-10/Gadd153 along with enhanced production of mitochondrial ROS inhibited adipocyte differentiation (Carrière et al. 2004).

13.4.4 ROS, Angiogenesis, and Cell Migration During Development

HIF control of hematopoiesis (erythropoiesis), vasculogenesis, and angiogenesis for establishing an active cardiovascular system is critical for regulation of O_2 and distribution of nutrients in the developing embryo. Redox regulation of HIF-1-mediated modulation of vascular development is primarily determined by expression of VEGF. HIF-1-deficient mice embryos had reduced myeloid multilineage, committed erythroid progenitors, and hemoglobin contents along with lower mRNA levels of the iron regulatory genes such as EpoR, hepcidin, Fpn1, Irp1, and Frascati (Yoon et al. 2006). Ultimately, many of the structural aspects of O_2 sensing and developmental programing related directly to the preparation of the conceptus to regulate future increase in O_2 concentrations are established through the genesis and maturation of vasculature within a mature cardiovascular system. Interestingly, endothelial cell-specific factors critical for angiogenesis, like Vegf, Pdgf, and angiopoietin (Ang1), are also redox-regulated (Lassegue and Clempus 2003). Vegf- and Pdgf-induced phosphorylation of Akt, Erk1/2, and p38 MAPKs in endothelial cells is contributed by ROS. Stimulation of Ang-1 by ROS activates Tie2 receptors in human umbilical vein endothelial cells, later causing rapid and transient production of ROS. ROS-regulated cellular migration is observed in endothelial cells which are inhibited by overexpression of antioxidants (Harfouche et al. 2005). Similar to HIF, Nox1 also promotes angiogenesis through induction of Vegf expression (Ushio-Fukai et al. 2002; Arbiser et al. 2002). Thus, both hypoxia regulatory and ROS regulatory networks work intricately for fine regulation of angiogenesis process. Apart from endothelial cells, germ cell migration is also controlled by a redox mechanism. Overexpression of thioredoxin peroxidase gene promoted early transepithelial migration of germ cells into the midgut primordium in *Drosophila* (DeGennaro and Lehmann 2007).

13.5 ROS and Transcription Regulation During Development

Critical developmental events are mediated by ROS directly by regulation of embryonic gene expression. In addition, ROS acts as second messengers and controls expression of key transcription factors vital to cell signaling pathways that dictate proliferation, differentiation, and apoptosis, implying their involvement in embryonic development. These redox-sensitive transcription factors include hypoxia-inducible factor (HIF-1), nuclear factor-kB (NF-kB), wingless-type mouse mammary tumor virus integration site (Wnt), activator protein 1 (AP-1), redox effector factor-1 (Ref-1), and nuclear factor (NF)-E2-related factor-1 (Nrf-1) (Dennery 2007).

13.5.1 Hypoxia, HIF, and Developmental Programing

An environmental O_2 sensor and a regulatory system to acclimatize the embryo to the adaptive changes would constitute an effective response to

the fluctuating O_2 conditions during embryogenesis. Preserved developmental regulators include mTOR (mammalian target of rapamycin)-mediated pathway associated with autophagy and the endoplasmic reticulum stress response (Hansen et al. 2020). Among these, the bHlH (basic helix-loop-helix)/PAS transcription factor superfamily members of HIFs are the most critical regulators. HIF regulates embryonic angiogenesis, erythropoiesis, and brain development gene expression in an oxygen-dependent manner. ROS-related regulations of HIF activity include ROS-sensitive NF-κB signaling, oxidation of protein tyrosine phosphatases and protein tyrosine kinase, hydroxylation, and ubiquitin-proteasome system. In the mouse model, HIF-1a or HIF-1b/ARNT knockout or complete deletion results in embryonic lethality by GD 10.5 and prevents the functional embryogenesis/organogenesis in the conceptus (Dunwoodie 2009; Lin and Wang 2021). The developmental program beginning with the earliest cleavage till birth, critical developmental processes such as maintenance of stemness (OCT4) and control of colony expansion, pluripotency, proliferation, and fate determination (EPO, TGFb3, TERT, VEGF, ID2, IGF2, ADM, PGM) of ESCs in the early blastocyst, neural crest cells, epithelial mesenchymal transition, and numerous other progenitor cells are regulated by HIF transcription factors (Hansen et al. 2020). HIF-2a is restricted to the early embryonic vasculature and neural crest cells and later expressed in the liver, lung, and kidney. HIF-3a is expressed in various other restricted organs and compartments. An additional HIF subunit, HIF-1β (also known as ARNT (aryl hydrocarbon receptor nuclear translocator)), is constitutively and ubiquitously expressed and serves as the heterodimerization DNA-binding partner for HIF-1a-HIF-3a.

Critical developmental events such as erythropoiesis, angiogenesis, cell survival, and embryonic energy metabolism are regulated by HIF-1 transcription factors. Being activated in hypoxic conditions, HIF ensues establishment of a proper cardiovascular system to prepare the conceptus to regulate future increase in O_2 concentrations. Null mutation in the Hif1 gene is lethal to the embryo because it causes cardiac, vascular, and neural malformations by E10.5 in mice. In addition, HIF-1-deficient embryos had decreased levels of myeloid multilineage, committed erythroid progenitors, and hemoglobin contents along with reduced expression of the Epo receptor (EpoR) and iron regulatory genes such as hepcidin, Fpn1, Irp1, and Frascati. These implied the significance of redox regulation of HIF-1 in regulating iron homeostasis and embryonic erythropoiesis (Yoon et al. 2006) as well as undifferentiated lung mesenchyme proliferation (Land and Wilson 2005).

13.5.2 Redox Active NF-kB During Development

Nuclear factor kappa B (NF-κB) transcription factors are group of dimeric transcription factors belonging to the rel family that remain bind to cytoplasmic IκB protein in an inactive state. In response to specific stimulatory redox signals, NF-κB translocates to the nucleus, where it binds to an NF-κB enhancer located upstream of NF-κB-regulated genes and upregulates their expression of genes associated with cell proliferation, apoptosis, morphogenesis, and embryonic development (Nishikimi et al. 1999; Kabe et al. 2005). ROS is seen to have stimulatory effect on NF-κB pathway in the cytoplasm by inducing degradation of NF-κB/IκB complex, while it has an inhibitory effect on NF-κB activity in the nucleus by repressing NF-κB DNA-binding (Morgan and Liu 2011). In preimplantation murine embryos, altered NF-κB activity at the early one-cell stage blocks the progression of mouse embryos beyond the two-cell stage (Nishikimi et al. 1999). Embryonic lethality of knockout mice with disrupted NF-kB pathway implied the pivotal role of NF-kB during embryonic development (Pasparakis et al. 2006). The involvement of NF-kB in regulating embryo developmental processes was clearly indicated by studies on not only in mammals but also on other model organisms such as zebra fish, *Xenopus*, and *Drosophila*. Studies have reported the contribution of NF-kB in controlling expression of key developmental genes involved in dor-

soventral and neural patterning, lung-branching morphogenesis, and mesodermal and ectodermal lineage factors. Taking mammalian murine models, NF-kB pathways have been demonstrated to be required for development of notochord, muscle, lungs, liver, and skeletal and neural development during embryogenesis (Espín-Palazón and Traver 2016). Moreover, NF-kB orchestrates the emergence of hematopoietic stem cells (HSCs), the organizers of the adult hematopoietic system. In hyperoxia situation, NF-kB signaling activated antiapoptotic genes such as the BCL and TRAF family members playing a protective role in newborn mice. Any modification of NF-kB signaling would influence maternal inflammation at key points during gestational period affecting embryonic development.

13.5.3 Redox Regulation of Wnt/β--Catenin Signaling Pathway

Known to be a highly conserved signaling pathway, wingless-type mouse mammary tumor virus integration site (Wnt)/β-catenin forms a vital regulating mechanism which governs the cell proliferation, cell polarity, and cell fate determination during embryonic development and tissue homeostasis (Logan and Nusse 2004). Mutated Wnt pathway has been implicated with human birth defects, cancer, and other diseases (Clevers 2006). Canonical Wnt signaling is regarded as the most critical and most studied Wnt pathway that regulates the stabilization of β-catenin, a core component of the cadherin protein complex, and the later is involved in controlling key developmental gene expression programs. In the absence of Wnt, AXIN/glycogen synthase kinase 3 (GSK3)/adenomatous polyposis coli (APC)/casein kinase 1 (CK1) destruction complex degrades the cytoplasmic β-catenin. The complex phosphorylates the β-catenin protein leading to its ubiquitination and proteasomal degradation (He et al. 2004). Activation of Wnt ligand due to binding of Frizzled (Fz) receptor and low-density lipoprotein receptor-related protein 6 (LRP6) forming Wnt-Fz-LRP6 complex along with recruitment of the scaffolding protein dishevelled (Dvl) inhibits the activity of AXIN/GSK3/APC/CKI destruction complex. Subsequently, stabilized β-catenin translocates to the nucleus to act as a transcriptional coactivator and remove the suppression of Wnt target genes by DNA-bound T-cell factor/lymphoid enhancer factor (TCF/LEF) family of proteins (MacDonald et al. 2009). Interestingly, ROS acts as both positive and negative regulators of Wnt/β-catenin signaling. Thionylation of nucleoredoxin (NRX), a specific member of the thioredoxin-related redox-regulating protein family by ROS, causes dissociation of the bound Dvl. Upon release, Dvl inhibits degradation of β-catenin, resulting in its accumulatio (Funato and Miki 2010; Funato et al. 2010), later then translocate to the nucleus to form complexes with TCF/LEF and activate Wnt target gene expression. Furthermore, activation of canonical Wnt signaling was observed to increase Nox expression leading to overproduction of ROS with decreased production of GSH (Kajla et al. 2012). However, elevated levels of ROS particularly H_2O_2 negatively modulate Wnt signaling pathway through downregulation of β-catenin (Shi et al. 2004), thus impairing embryonic development in oxidative stress conditions. Expression of Wnt target genes is involved in many developmental processes, including gastrulation, anterior-posterior axis specification, organ and tissue development, and homeostasis (MacDonald et al. 2009). Wnt-β-catenin signaling is an important morphogen establishing the embryonic body axes during early embryogenesis followed by regulation of morphogenesis of multiple tissues derived from the three germ layers. Temporal activation of Wnt-β-catenin signaling mediates the establishment of both dorsoventral and anterior-posterior axis (Huelsken et al. 2000) and differentiation of embryonic stem cells (ESCs) into different germ layers for normal gastrulation (Tabar and Studer 2014). Knockout mouse embryos for β-catenin were observed to die before gastrulation (Haegel et al. 1995). Moreover, cross talk between Wnt/β--catenin and NF-κB signaling pathways formed a complex regulatory network through physical interactions of mediators and regulation of target genes (Ma and Hottiger 2016) which is vital to all

aspects of embryonic development from patterning to organogenesis (Wells et al. 2007).

13.5.4 AP-1: The Redox Sensor

Activating protein 1 (AP-1) are well-characterized transcription factor families carrying specific functions implicated with embryogenesis and organogenesis. Synchronized activation of AP-1 and NF-kB by ROS has been proposed (Dhar et al. 2002). Being directly activated by oxidative stress conditions, AP-1 proteins are known to be sensors of the redox state of the cell (Toone et al. 2001). A concomitant inhibition of AP-1 activity was observed with a decrease in ROS production (Aharoni-Simon et al. 2006). GSH is known to be a negative regulator of AP-1 gene. On the other hand, AP-1 has its consensus binding site on the promoter of many antioxidant genes (Dennery 2007). As far as cell proliferation is concerned, c-Jun, JunB, and JunD played a crucial role, the former being a positive regulator while the latter two being negative regulators of cell proliferation. In contrast, c-Fos, FosB, and Fra-1 were dispensable for cell cycle progression (Jochum et al. 2001). With regard to embryonic development, during midorganogenesis, augmented expression of AP-1 subunit proteins c-fos, c-jun, junB, and junD is observed in mice embryo which makes the later susceptible to teratogenic insult (Dennery 2007). Subunits, namely, c-Jun, JunB, and Fra-1, are vital, while others, namely, c-Fos, FosB, and JunD, are unessential for embryonic development (Jochum et al. 2001). Knockout mice for c-Jun died between GD 12.5 and 13.5 of embryonic development (Eferl et al. 1999) developing abnormalities in the cardiac outflow system and liver. Moreover, involvement of c-Jun in regulating sklerotome differentiation, thymocyte apoptosis, and T-cell differentiation has also been shown (Jochum et al. 2001). Likewise, death of embryos lacking JunB between GD 8.5 and 10.0 with reported vascular defects in the extraembryonic tissues implied the critical role of the genes for embryonic development. Moreover, contribution of JunB to the differentiation of naïve T-helper cells into functional subsets of T-lymphocytes has been proposed (Jochum et al. 2001). In contrast, inhibition of JunD expression didn't affect the viability of the embryo, but resulted in altered growth, hormone imbalance, and age-dependent defects in reproduction due to impaired spermatogenesis. Similar to JunB, mice lacking Fra-1 gene were lethal at around GD 10 with anomalies in the placenta and the yolk sac (Schreiber et al. 1995). Fra-1 is also implicated with osteoblast differentiation in the embryo. Although knockout mice with Fra-2 has not yet been reported, its involvement during late embryonic development has been speculated affecting ocular differentiation. Mice embryo lacking both c-Fos and FosB are viable and fertile but had developed impaired osteoclast differentiation and chondrogenesis. The oxidative stress-induced upregulation of several subunits of AP-1 family in rat embryos at E10.5 resulted in malformations, altered development, and apoptosis (Ozolinš et al. 2002).

13.5.5 Ref-1: The Embryonic DNA Guardian

Redox effector factor-1 or Ref-1 (also known as apurinic/apyrimidinic endonuclease or APE) is an important DNA repair protein that protects the embryonic DNA from oxidative insult. Ref-1 endonuclease participates in the DNA base excision repair pathway to remove the apurinic and apyrimidinic sites, a typical DNA damage in cell. In addition, Ref-1 stimulates sequence-specific AP-1 DNA-binding activity through reduction of cys residue located in DNA-binding domain of Fos and Jun (Xanthoudakis et al. 1992). As far as ROS is concerned, oxidative agents such as H_2O_2 have been demonstrated to induce Ref-1 activity, which correlates with an increase of its endonuclease and redox activities (Pines et al. 2005). This transient Ref-1 induction could be attributed to non-mutually exclusive mechanisms, i.e., subcellular localization and PTM. Upon ROS exposure, Ref-1 translocates from the cytoplasm to the nucleus to regulate AP-1 DNA binding. Moreover, ROS-mediated phosphorylation and acetylation would also

influence the functional activity of the protein. The presence of Ref-1 transcripts in oocytes, spermatozoa, and preimplantation blocked embryos has been established by RT-PCR analysis (El-Mouatassim et al. 2007). A dramatic change in expression of Ref-1 during early preimplantation and postimplantation development is observed. Variation in temporal and spatial patterns of Ref-1 in the mouse brain is seen from the period of midgestation through adulthood (Ono et al. 1995). At GD 3.5, Ref-1 expression is elevated in the brain which decreases with progression of embryonic development. The sites of higher levels of Ref-1 in the brain correlate with regions that express Fos and/or Jun in response to specific stimuli (Morgan and Liu 2011). Homozygous mice with one Ref-1 allele develop normally, whereas heterozygous mice with both alleles absent die in utero following implantation at around GD 6.5 (Xanthoudakis et al. 1992). Interestingly, the period of embryonic death observed with the Ref−/− mice is earlier than that observed with the Jun−/− mice or c-fos−/− mice (Johnson et al. 1992; Johnson et al. 1993). Lethality of knockout mice for Ref-1 gene implied the role of the protein in regulating early embryogenesis. This may be a consequence of abnormal DNA repair and inadequate expression of Ref-1-dependent transcription factors (Xanthoudakis et al. 1992).

13.5.6 Nrfs: Protectors Against Oxidative Stress

Nuclear factor (NF)-E2-related factors or Nrfs belong to novel CNC ("cap 'n' collar") subfamily of basic region leucine zipper (bZIP) transcription factors and mediate production of antioxidants by binding to antioxidant-responsive elements (ARE). A cell-specific and developmental stage-specific function of Nrf1 in protecting the embryo from oxidative insult has been reported. The indispensable requirement for Nrf1 for the developing embryo could be derived from the experimental evidence which shows lethality of Nrf1 knockout mouse embryo at midgestation. Genes belonging to CNC have been reported to be implicated with cephalic patterning in *Drosophila* (Leung et al. 2003). Post-72 h of fertilization (hpf), sensitivity to prooxidants increases in embryos. In zebra fish embryo, it has been shown that the fertilized embryo is initially in reduction stage but becomes progressively oxidized between 3 and 48 hpf (blastula-hatching stage) before being restored to a reduced GSH/GSSG by 72 hpf (protruding mouth stage) (Sant et al. 2017). Knockdown of Nrf1a or Nrf1b disturbed the GSH redox state until 72 hpf. Both Nrf1 and Nrf2 paralogs have been proven to be activated by oxidative stress and induce antioxidant response (Sant et al. 2017). However, Nrf2 in particular is known to be more sensitive to prooxidants than Nrf1 (Nguyen et al. 2009). On the other hand, genes regulated by Nrf1 are associated with fetal liver erythropoiesis, and homozygous mutant embryos for Nrf1 died due to severe anemia. Knockout Nrf2 mouse embryos were viable but demonstrated reduced expression of antioxidant enzymes which increases the sensitivity of the embryo to oxidative stress (Chan et al. 1996; Chan et al. 1998). This implies that unlike Nrf1, the presence of Nrf2 is unessential for embryonic growth and development. Double knockout for both Nrf1 and Nrf2 was also found to be lethal arresting embryonic growth. Whereas Nrf1−/− embryos died at middle to late gestation starting at GD 13.5, compound mutants Nrf1−/− and Nrf2−/− died at or before GD 10.5 (Leung et al. 2003). It could be proposed that Nrf2 might functionally compensate for the loss of Nrf1 in regulating genes essential for early embryogenesis which shows the overlapping functions of Nrf1 and Nrf2 during early embryogenesis. However, death of compound mutant embryos indicates that Nrf1 and Nrf2 are functionally redundant in mediating ARE function and oxidative stress defense in cells. Abnormal oxidative defense and suppression of Nrf implicated functions would lead to ROS-induced accumulation of p53 protein and activation of Nox-induced apoptosis (Leung et al. 2003).

13.6 Pathological Role of Oxidative Stress on the Embryo

Orchestration of redox signaling forms the key for establishment of normal developmental processes from the embryo patterning all aspects of embryogenesis (Fig. 13.1). Since the onset of pregnancy, oxidative stress interferes with the normal development of feto-maternal physiological relationship. However, excess and untimely oxidative stress would result in different pathophysiological complications like miscarriage, preeclampsia (PE), intrauterine growth restriction (IUGR) or fetal growth restriction (FGR), and premature rupture of the membranes. Moreover, with the developing embryo itself being highly susceptible to oxidative damage, exposure to prooxidants during this period would have a deleterious effect, leading to embryopathies, teratogenesis, later-life pathological conditions, or lethality (Fig. 13.2).

13.6.1 Spontaneous Miscarriage

The placenta is the only maternal-fetal interface during embryogenesis, any damage to the maternal placenta would cause pregnancy complications, subsequently leading to abortion. Disorganized and precocious maternal intraplacental circulation and incomplete plugging of spiral arteries lead to miscarriages. Higher levels of HSP70 and nitrotyrosine in the central region of placental villi as compared to peripheral region with increased apoptotic index cause degenerative syncytiotrophoblast leading to placental anomalies. Subsequently, the ongoing pregnancy is severely affected leading to spontaneous miscarriages. Defective placental tissues were reported to have higher amounts of lipid peroxides, protein carbonyls and oxidatively damaged DNA in both villous and decidual tissues, as well as in serum (Burton and Jauniaux 2011). In normal pregnancy, after 10–12 weeks of gestation, a burst of oxidative stress in the placenta teaches them how to get adapted to their new oxygen environment by increasing the pool of antioxidant enzymes (Jauniaux et al. 2000). However, in such a scenario, reduced levels of the antioxidant defense would expose the developing embryo to an increased risk of miscarriage. It is also observed that in women with recurrent pregnancy loss, intake of antioxidant *N*-acetyl cysteine improved take-home baby rate.

13.6.2 Preeclampsia

Preeclampsia, characterized by de novo high blood pressure (>140/90 mmHg systolic/diastolic blood pressure) and proteinuria (>300 mg/24 h) appearing during second and third trimester of gestation, is a persistent hypertensive gestational disease (Aouache et al. 2018). Vascular endothelial dysfunction, systemic endovascular inflammation, and abnormal secretion of factors from ischemic placenta are attributed to cause maternal preeclampsia. Cytokine TNF-α found in preeclamptic plasma in higher concentration initiates oxidative stress in endothelial cells, through activation of Nox or Lectin-like oxidized LDL receptors-1 (LOX-1) and OXLDL (receptors for oxidized LDL). In preeclampsia, both high levels of oxidative stress and endoplasmic reticulum stress compromise placental physiology (Burton and Jauniaux 2011). Women with preeclampsia conditions are more likely to suffer from major pregnancy complications such as fetal growth restriction, low birth weight (1/3 of cases), premature delivery, and fetal death.

13.6.3 Intrauterine Growth Restriction (IUGR)

Compromised maternal circulation to the placenta may cause IUGR, apart from genetic or infectious cause. Defective physiological alteration of the spiral artery remodeling in the myometrial segment as seen in preeclampsia or other placental pathological conditions may cause retardation of growth in the fetus as seen in IUGR. Placental ischemia or hypoxia in IUGR because of reduced uteroplacental perfusion is reported to be the primary cause. Increased lev-

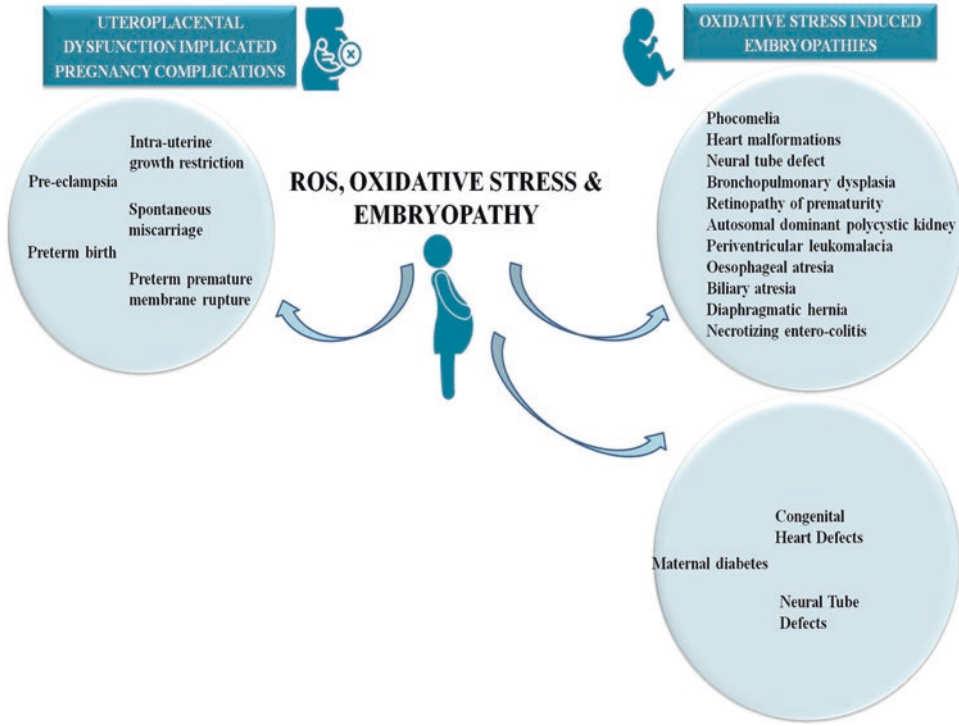

Fig. 13.2 Impaired reactive oxygen (ROS) metabolism in embryopathy

els of malondialdehyde (MDA) and xanthine oxidase (XO) with reduced antioxidant potential were detected in maternal plasma, umbilical cord plasma, and placental tissues of the patients with IUGR when compared to the control group (Biri et al. 2007). Compromised maternal circulation to the placenta would further initiate phosphorylation of eIF2α and suppress protein synthesis, with reduction in cyclin D1 level augmenting the stress conditions. Reduced antioxidant and induced oxidative stress in IUGR cause lipid peroxidation to block the synthesis of prostacyclin and stimulate platelet aggregation causing severe irreversible cellular membrane damage (Burton et al. 2021). Oxidative stress-induced DNA damage, DNA damage response, telomere uncapping, and telomere dysfunction would trigger damage to fetal cells promoting premature fetal cell senescence (aging). Moreover, activation of p53-p21 and p16-pRB signaling transduction pathway causes cell cycle arrest leading to IUGR (Kajdy et al. 2021).

13.6.4 Preterm Premature Membrane Rupture and Preterm Birth

Reperfusion oxidative stress has been known to cause premature rupture of fetal membranes of the placenta leading to preterm labor. Studies have reported that women with oxidative stress and lower serum selenium went to preterm delivery compared to controls. Elevated oxidative stress would initiate proteolytic collagen fiber degradation in chorioamnion fetal membrane to rupture amnion membrane to free amniotic fluids. Abnormal senescing cells with redox imbalance and perturbed p38 mitogen-activated kinase (p38MAPK) signaling pathways would trigger labor process preterm compromising fetal growth (Menon et al. 2017). Furthermore, detection of oxidative DNA lesions, oxidative stress lipid markers, and high levels of aldehyde oxidase enzymes in both late-term and stillbirth placentas also demonstrated the role of ROS as an etiologi-

cal factor in those pregnancy pathologies (Maiti et al. 2017).

13.6.5 Maternal Diabetes-Induced Embryopathy

Maternal diabetic conditions pose as the major etiology of severe embryopathies especially related to congenital heart defects (CHD) such as a defect in the ventricular or atrial septum, valve defects, or abnormal ventricular outflow tracts. Physiological levels of NO and ROS are essential for proper cardiovascular development. Deficiency of endothelial nitric oxide synthase (eNOS) and oxidative stress would lead to CHD and coronary artery malformations (Piccoli et al. 2014). A significant reduction in the activity of endogenous antioxidant enzymes and of vitamins C and E along with increased congenital anomalies and decreased protein content was observed in the malformed experimental embryos of Cohen diabetic rats (Ornoy 2007). In experimental embryos of Cohen diabetic rats when a great reduction in endogenous antioxidant enzymes, vitamins C and E, and protein contents are observed, in one hand, other site shows an augment level of anomalities. Suppressed expression of ROS-scavenging enzymes would be an integral part of a genetic predisposition to embryonic dysmorphogenesis.

Biochemical perturbations due to hyperglycemia in mother induce embryonic oxidative stress eventually leading to embryonic malformations, which is reported to be blocked by increasing SOD or supplementation of N-acetylcysteine to raise the availability of GSH (Wentzel et al. 1997). Supplementation with vitamins C and E also lessened the severity of malformations in diabetic rats (Zaken et al. 2001). Moreover, supplementing the diabetic mothers' diet with the antioxidant butylated hydroxytoluene (BHT) resulted in increased fetal weight compared to diabetic rats fed with a normal diet, surmising diabetic embryopathy to be mediated by ROS (Eriksson and Simán 1996). Embryos of splotch (Sp/Sp) mice homozygous for a mutation in the Pax3 gene are phenotypically similar to diabetic mice. Both the types of embryos develop neural tube defects (Conway et al. 1997; Li et al. 2007). Therefore, it is opined that Pax3 expression affects neural tube development and changes in glucose metabolism affect Pax3 expression. Neurodevelopmental defects in SOD overexpressed animals after induction of diabetes could be prevented by treating diabetic animals with antioxidants. Besides, augmenting O_2^- concentration by inhibiting complex III of mitochondrial electron transport chain leads to inhibition of Pax3 expression and increased neural tube defects (Chang et al. 2003). Therefore, ROS generation as a result of anomalous glucose metabolism in the mother is responsible for embryopathy.

13.6.6 Teratogens, ROS Metabolism, and the Embryo

Exposure to different environmental toxins would compromise the embryonic growth. Early stages of organogenesis are particularly susceptible to teratogen insult causing serious embryonic damage. Failure to repair those damages in the developing embryo may result in embryonic death and/or congenital anomalies. Environmental toxins especially teratogenic xenobiotics pose a serious threat to fetal growth leading to several birth defects (Ornoy 2007). Thalidomide is speculated to be bioactivated by embryonic prostaglandin H synthase (PHS) to generate ROS, disturb the GSH-GSSG redox couple, and induce embryonic DNA oxidation and limb teratogenicity such as phocomelia. Exposure to drug phenytoin, a widely used anticonvulsant in G6PD-deficient dams, caused higher embryonic DNA oxidation, dysmorphogenesis, and more fetal death and birth defects (Kaneko et al. 1991). Chronic cigarette smoking and ethanol consumption also increased ROS levels in maternal circulation, exposing the developing embryo to oxidative stress conditions. Perturbed redox signaling due to lowering of GSH content has been observed in the fetus of alcoholic mothers which could be attributed to several embryopathies (Amini et al. 1996). It could be inferred that these teratogens

might activate common teratological pathways involving altered expression of genes under the control of transcription factors sensitive to oxidative stress. Oxidative stress could be further implicated in the pathogenesis of congenital malformations like heart malformations, neural tube defect, esophageal atresia, biliary atresia, diaphragmatic hernia, and autosomal dominant polycystic kidney (Impellizzeri et al. 2020). Oxidative stress-mediated neonatal diseases such as bronchopulmonary dysplasia, retinopathy of prematurity, periventricular leukomalacia, and necrotizing enterocolitis in preterm newborns could be treated with antioxidant supplementation (Laforgia et al. 2018). Several oxidative stress biomarkers, namely, nonprotein-bound iron (NPBI), isoprostanes, isofurans, neuroprostanes, neurofurans, malondialdehyde, advanced oxidation protein products (AOPP), carbonylated proteins, and 7,8-hydroxy-2′-deoxyguanosine, could be used as diagnostic markers in the fetus and newborn to study the pathogenesis of above neonatal diseases (Perrone et al. 2019).

13.7 Oxidative Stress in Development: Lessons from ART

Assisted reproductive techniques (ART) have surfaced as the most preferred choice of conception for most of the infertile couples. However, exposure of gametes and the embryo to unwanted and unavoidable oxidative stress during procedure diminishes the desired pregnancy outcome. Due to the absence of natural antioxidant system, in vitro system is under the continuous pressure of ROS. In vitro system has a number of cascades of events such as microenvironmental gamete incubation, fertilization, and embryo development. Sperm, oocyte, embryo, blastocyst, media, and each and every component of in vitro system are vulnerable to environmental oxygen. In addition to atmospheric oxygen, exposure to light, oocyte treatment, and general physiochemical parameters also alter the oxidative status of the system. Exposure of the oocyte/embryo to visible light (400–700 nm) especially to blue light (400–500 nm wave length) stimulates increased synthesis of H_2O_2 (Agarwal and Majzoub 2017). More is the exposure of the oocyte to blue light, higher is the detrimental effect on blastocyst production. The use of light filters during microscopic observation and the minimization of the time spent in vitro can help reduce the negative effects of visible light.

Oxidative stress has been well established to negatively affect the success rate of ART outcomes. Most of the studies have shown the detrimental effect of excessive ROS on the pathogenesis of sperm. Damaged sperm and leucocytes are major sources of exogenous ROS generation. Leucocytes synthesize 1000 times more ROS than spermatozoa. In ICSI, the testicular sperm has constructive effect in comparison to the ejaculated sperm in relation to oxidative stress (Alahmar 2019). Oxidative stress-induced sperm damage leads to poor fertility outcomes both in vivo and in vitro. However, removal of antioxidant-enriched seminal plasma during sperm processing makes the sperm more susceptible to an oxidative insult in vitro. An appropriate sperm selection procedure, antioxidant-enriched sperm processing media, and proper sperm storage would reduce the detrimental exposure of sperm to unwanted oxidative stress during ART.

As the developing embryo is sensitive to fluctuating oxygen tension during its transit from the fallopian tube to the uterus, ROS generated during in vitro culture would be detrimental to embryo development particularly for the preimplanting embryos. In vitro, the presence of albumin, glucose, and phosphate in culture media promotes buildup of ROS and arrest of embryo development. To combat it, antioxidant supplementation with the addition of vitamin C, vitamin E, metal chelators, resveratrol, inositol, etc. in culture media would reduce the ROS generation in oocytes/embryos (Budani and Tiboni 2020). There is mounting evidence on supplements of various antioxidant and modifications of physical conditions to reduce ROS production in vitro, while the optimal systems for maturation, fertil-

ization, and fertility outcome remain to be designed. However, surplus antioxidant supplementation in vitro has also been shown to impair the bovine embryo development (Du Plessis et al. 2008). Increasing the estradiol and decreasing the glucose levels in culture media would thus ensue an improved antioxidant counterbalance. Mimicking the microenvironment of the female reproductive tract in an ART setup is difficult, and generation of ROS is inevitable; however, modification of media, gamete processing techniques, and embryo handling procedures would result in ameliorated success rates while maintaining an optimum level of ROS.

13.8 Conclusion

ROS play a pivotal role as primary or secondary messengers by regulating the key transcription factors, thereby impacting the cell signaling pathways during cell proliferation, differentiation, and apoptosis. Therefore, embryonic development that involves all the above process, namely, proliferation, differentiation, and apoptosis, is expected to be affected both positively and negatively depending on the level of ROS inside the embryo and its immediate surrounding in vivo or in vitro conditions. Physiologic and pathologic ROS levels differentially modulate the transcription factor signaling, gene expression, and cell cycle alterations. The susceptibility of an embryo to oxidative stress varies with developmental stages where antioxidant defenses have a significant impact in curbing the noxious effects of ROS and maintaining the redox homeostasis. Hence, an in-depth understanding of the events in embryonic and fetal development at molecular level is vital in ensuring normal development in utero as well as optimizing embryonic culture conditions in ART.

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14 Interplay of Oxidants and Antioxidants in Mammalian Embryo Culture System

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Abstract

One principal purpose of assisted reproductive technology (ART) is to produce viable and good quality embryos. However, a variety of environmental factors may induce epigenetic changes in the embryo. Moreover, laboratory conditions including the culture media may also affect embryo development. Therefore, media change is an important factor in maintaining proper oxidant/antioxidant balance during embryo culture. Alterations in the oxidant/antioxidant balance are related to various cellular responses such as an increase in the level of reactive oxygen species (ROS) and consequent lipid peroxidation (LPO), DNA damage, and apoptosis. The current study focuses on the role of external factors on embryo culture and the ability of antioxidants to enhance in vitro fertilization (IVF) outcomes. Indeed, an optimization of media culture by the addition of enzymatic and nonenzymatic antioxidants in animal models and human embryos in ART has been updated in this study, with an emphasis on comparing the available results and their possible reasons.

Keywords

Assisted reproduction · Embryo culture · Oxidative stress · Oxidation-reduction potential · Antioxidants · IVF outcomes

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14.1 Introduction

In vitro culture has led to thousands of healthy live births around the world. Despite the improvement of technology, several laboratory conditions

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may induce alterations in the gametes as well as embryos (Gupta et al. 2010). Many of these alterations are caused by an increase in reactive oxygen species (ROS) levels. While a basal metabolism of ROS participates in several cellular functions such as cleavage capacity and glucose uptake in early embryos, an excessive increase in the concentration of oxidants is known to diminish ART outcomes (Harvey et al. 2002). Some of the most studied alterations that have been reported include an increase in apoptosis, impairment and delay in embryo development, high fragmented cleavage embryos, and, therefore, poor embryo quality and competence (Hardy 1999; Martin-Romero and Álvarez 2009).

Remarkably, ART has recently started to focus on more physiological techniques in order to ameliorate the adverse consequences of oxidative stress (OS) and an improvement in the outcomes. In vivo embryonic development is supported by multiple antioxidant mechanisms, which help the embryo in modulating ROS production and diminishing OS (El Mouatassim et al. 2000). Several enzymatic and nonenzymatic antioxidants have been found in the reproductive tract such as glutathione (GSH) and ascorbate (Gardiner et al. 1998; Paszkowski and Clarke 1999). GSH is a tripeptide that aids glutathione-S-transferase and glutathione peroxidase to scavenge ROS (Gardiner et al. 1998). Ascorbate is also found there, and it helps inhibit LPO (Sies 1997). Consumption of oral antioxidants has demonstrated some benefits in infertile couples, which may enhance fertility treatment outcomes (Batioglu et al. 2012). Also, addition of exogenous antioxidants to IVF culture media has gained interest, since culture media could be improved to resemble the in vivo environment potentially leading to better-quality embryos and healthy live births.

14.2 Assisted Reproductive Technology (ART)

In the field of medically assisted reproduction (MAR), there is a clear necessity to introduce new ARTs through preclinical safety research, mainly by the use of animal models, and these techniques may also be used to improve and optimize future clinical research and/or outcomes (Verna et al. 2018; Davidson et al. 2019). The ART to treat human infertility has remarkably progressed since the birth of Louis Brown in July 1978 (Cohen et al. 2005). Major trends in ART research have focused on increasing the rate of live births; and minimizing the possibility of development of diseases in the IVF newborns has also been a major concern (Ferrick et al. 2019). However, the outcome of ART still depends on several factors that cannot be controlled in the IVF laboratory. Therefore, it is necessary to consider the widely discussed idea that IVF conditions may never fully match the physiological conditions of an in vivo system (Agarwal et al. 2006a).

Recently, in some countries, up to 5% of children have been reported to be conceived by ART (Sutcliffe et al. 2006; Agarwal et al. 2005). Therefore, it is urgent to continue the search for matching IVF laboratory parameters to physiological parameters. Epigenetic patterns might have a relationship with the process of assisted reproduction and may influence the individual during the various stages of life after birth (Urrego et al. 2014a). During IVF procedures several physical factors may also influence the clinical outcomes and long-term effects on the offspring particularly during the in vitro culture of gametes and embryo culture in mammals (Agarwal et al. 2005). Mechanical manipulation of gametes, embryos, and associated chemical components is considered exogenous sources of supraphysiological levels of ROS in culture systems, which may lead to OS (Wale and Gardner 2016). Several known factors such as mineral oil peroxidation, atmospheric oxygen concentration, visible light, and culture media formulation may induce OS by contributing to ROS generation during in vitro procedures of both animal models and humans (Wale and Gardner 2016; Gardner et al. 1996). Also, the exposure of mammalian gametes and embryos to high concentrations of cryoprotectants during cryopreservation may induce changes in osmolarity of cells (Smith et al. 2004).

ROS represent a broad category of molecules that have one or more unpaired electrons in their last orbital. Due to this electronic configuration, they are also called free radicals, which are highly reactive with other molecules near to it. Radical hydroxyl-ion (OH-), superoxide anion (O_2)-, nitric oxide (NO), the non-radical ozone (O_3), hydrogen peroxide (H_2O_2), peroxyl radical (ROO), and oxygen derivatives are considered ROS molecules (Sies 1997). Cells have an elaborative antioxidative defense system consisting of enzymes. Such as catalase, superoxide dismutase (SOD), and glutathione peroxidase or reductase, and numerous nonenzymatic antioxidants such as vitamin C, vitamin E, vitamin A, pyruvate, glutathione, taurine, and hypotaurine (Guérin et al. 2001). Despite this, ROS can react with biomolecules such as lipids, proteins, carbohydrates, and DNA (Rikans and Hornbrook 1997). A narrow balance between the concentration of ROS and antioxidants maintains the system in equilibrium under normal physiological conditions (Rikans and Hornbrook 1997). However, with a supraphysiological increase of either endogenous or exogenous ROS levels, the exposed cells may suffer from immediate OS. OS occurs when ROS production exceeds cellular defenses and induce oxidative damage (Covarrubias et al. 2008; David et al. 2007).

14.3 Oxidant/Antioxidant Balance and Measurement of Oxidation-Reduction Potential (ORP)

Although direct measurement of ROS is possible by nitro blue tetrazolium test or chemiluminescence, both direct and indirect methods enable the recognition of oxidant/antioxidant balance and potential ROS-related damage to embryonic cells (Agarwal et al. 2018). There are many indirect ways to measure OS in the culture media such as the LPO by thiobarbituric acid-reactive substance (TBARS) assay, oxidation-reduction potential (ORP), or total antioxidant capacity (TAC) (Agarwal et al. 2018). Measurement of ORP is one of the most recent and accurate indirect methods and also considered a composite parameter of oxidant/antioxidant balance. It represents the balance/imbalance among all oxidants and antioxidants in fluids. Different culture media, either for embryo culture or sperm preparation reportedly has different ORP levels (Agarwal et al. 2012; Panner et al. 2018).

To scavenge the elevated levels of ROS produced in the embryo culture system by physical and chemical factors, the administration of antioxidants to culture media during in vitro embryo culture has been tested in animal models and humans. For example, in cattle and mice, there was an improvement in embryo growth, implantation, and birth weight after the addition of antioxidants to the culture medium (Truong and Gardner 2020; Truong et al. 2016; Wang et al. 2014a). However, these approaches have failed to demonstrate a significant improvement in terms of live birth rates in humans (Tarin et al. 1994; Hardason et al. 2018). Naturally, cells have an antioxidant system which plays an important role in scavenging excess ROS to inhibit the oxidation of proteins, lipids, carbohydrates, and DNA (Gardner et al. 1996). In the female reproductive system, the natural equilibrium between oxidants and antioxidants at physiological concentrations involves an essential process of regulation, such as follicular development, ovulation, and fertilization (Gardner et al. 1996). Consequently, organisms contain a complex network of antioxidant metabolites and enzymes that work together to prevent oxidative damage (Sies 1997). Currently, there is a scarcity of information on the antioxidants and enzymes present either in the mammalian follicles, uterine tubes, or uterus, which represent the direct environment surrounding the oocytes in the follicles and embryos into the uterus. The lack of knowledge of the most suitable antioxidants and their optimal concentrations in the reproductive tract presents many complications in the development of culture media that can efficiently protect embryos and gametes against OS during the entire process of in vitro culture (Gardner et al. 1996).

14.4 pH

In IVF, carbon dioxide (CO_2) from incubators is used to stabilize the media pH, and other factors such as temperature, culture time, medium composition, and altitude of the laboratory may also modify the pH in the culture media (Dale et al. 1998; Gatimel et al. 2020). During the different developmental stages of the embryo, changes in pH are demonstrated to induce several functional disorders. Mice oocytes have shown disruption in the meiotic spindle with an increase in pH and consequently increase in aneuploidy (Cheng et al. 2016; Swearman et al. 2018). Also, the sperm-zona pellucida interaction is pH-sensitive and affects the fertilization rate in conventional IVF rather than intracytoplasmic sperm injection (ICSI) procedures (Edwards et al. 1998). pH variations can modify glycolysis in the cleavage stage and may also be responsible for embryo arrest in IVF as demonstrated in mice (Edwards et al. 1998).

14.5 Peroxidation of Mineral Oil

Mineral oil is a standard component of embryo culture that can become toxic over time during culture and is considered as one of the products with high potential for variation in quality from batch to batch (Wolff et al. 2013). Mineral oil might be susceptible to peroxidation and consequently form peroxides – a type of ROS that causes OS to gametes and embryos – leading to poor embryo development and IVF outcomes even after passing mouse embryo testing by the manufacturer (Morbeck 2012; Otsuki et al. 2007; Otsuki et al. 2009).

The use of oil overlay helps to protect the culture media against bacterial contamination and diminishes the fluctuations in pH, temperature, and osmolality. The mineral oil also may contribute to absorbing lipophilic toxic compounds, accumulated in the medium (Miller et al. 1994; Martínez et al. 2017). Avoiding osmotic pressure changes in culture media is critical because osmotic stress can lead to cellular damage including alteration of DNA chain, protein synthesis, and disruption of biochemical reactions and metabolism (Martínez et al. 2017). However, mineral oil remains one of the least characterized and regulated components used in ART (Morbeck and Leonard 2012). Additionally, fatty acids in mineral oil contain a polycarbonate lipid tail, and it possesses unsaturated bonds, which are sensitive to peroxidation and photooxidation (Elder 2015). Peroxides are considered the most dangerous and serious contaminants found in mineral oil and arise from fatty acids in oil, and they also lead to ROS generation (Erbach et al. 1995; Martinez et al. 2017). Peroxide contamination of laboratory grade mineral oil and the degree of peroxidation is known to be dependent on exposure to heat, UV light and extended storage. Additionally, high peroxidation in mineral culture overlay has been reported to be detrimental to fertilization and embryo development because of toxic contamination or deterioration of oil quality (Otsuki et al. 2009). Identifying and knowing how to detect these chemicals present in oil used for ART is essential to prevent further culture media contamination that may negatively impact gametes and embryos (Morbeck and Leonard 2012).

It has been demonstrated that not only mineral oil but even other types of oils such as paraffin oil used in ART can produce peroxides (Morbeck and Leonard 2012; Gardner et al. 2005; Van Soom et al. 2001). Some studies have used cumene hydroperoxide to assess the effects of free radicals and ROS in biological models, since it has been considered a good candidate for studying the negative effects of peroxides present in mineral oil by its stability and lipid solubility (Hughes et al. 2010).

Peroxide-contaminated oil has been reported to adversely affect human embryo development and IVF success (Otsuki et al. 2009). Cleavage rates of porcine zygotes incubated under increased level of oil peroxidation have been found to be lower, and none of the cleaved embryos were able to develop to the blastocyst stage and showed accelerated embryonic degeneration (Martínez et al. 2017). The blastocyst cell number was also affected in a dose-dependent manner with the concentrations of peroxides (Otsuki et al. 2009). Since quality of oil can be affected by several factors, the end users need to

be careful in order to minimize environmental effects on oil, including handling, storage conditions and bottle-to-bottle variations. Although adequate storage conditions to prevent or minimize oil oxidation are advisable, it should be made mandatory across all types of IVF laboratories (Martinez et al. 2017).

14.6 Oxygen Concentration

It has been demonstrated in animal models that embryonic plasticity allows embryos to develop physiological responses to ensure their short-term survival in sub-optimal environments (Duranthon and Chavatte-Palmer 2018a). Exposure to atmospheric oxygen concentrations has been associated with an increased ROS generation thereby compromising embryo development. A low oxygen concentration (~5%) has a beneficial effect on mouse embryo quality by reducing the ROS levels produced in atmospheric oxygen conditions (Bavister 2004; Kovacic 2012). Lower oxygen concentration may allow a better regulation of intra- and extraembryonic environments, by a minimal oxidative damage to mammal embryos (Takahashi 2012).

Oxygen concentration in the oviduct of various mammalian species has been reported to vary between 1% and 9% (Ar and Mover 1994). Many IVF laboratories still culture both animal and human embryos during IVF at 20% oxygen concentrations, which is greatly different from the in vivo environment (Mehta 2001; Pool 2002). Several studies have suggested that in vitro concentrations of 20% oxygen may also affect embryogenesis in mice (Karagenc 2004; Umaoka 1992).

The use of physiological levels of oxygen decreases cellular apoptosis and DNA fragmentation in blastocysts protecting them during the active period of mouse differentiation into trophectoderm (TE) and inner cell mass (ICM) (Morin 2017). Some studies have shown a close relationship between lower concentrations of oxygen and better proliferation of the embryonic cells, besides reducing apoptosis and inhibiting trophoblast cell invasion in both mouse and human embryos (Zhan et al. 2018; Kovacic et al. 2010; Kovacic and Vlaisavljević 2008). Furthermore, embryo culture for at least 2 days under 5% oxygen conditions has shown to improve the quality of embryos (Fischer and Bavister 1993). However, not only biochemical changes have been demonstrated when physiological oxygen tension was adjusted, but DNA methylation patterns were modified, too, exerting a beneficial effect on the embryo and placental epigenome (Ghosh et al. 2017).

14.7 Visible Light

During an IVF procedure, the gametes and embryos are exposed to light during the collection, mechanical manipulation, evaluation, and grading. Excessive exposure to light may compromise in vitro development which depends on the duration of cellular exposure, intensity, and wavelength (Lars et al. 2007; Pomeroy and Reed 2013). For example, blue light has been associated with the generation of H_2O_2 by enzymes applied in the respiratory chain (Agarwal et al. 1978; Hockberger et al. 1999), breaking down DNA chains, and causing oxidative damage in other biomolecules (Ramadan 1978) and affecting embryo development in mammals (Pomeroy and Reed 2013; Oh et al. 2007). White fluorescent light is commonly used in IVF laboratories and is considered a source of ROS in mouse and hamster embryos causing blastocyst apoptosis (Takenaka et al. 2007). Furthermore, mammalian oocytes and embryos exposed to several kinds of light during in vitro manipulation showed impairment of subsequent bovine embryo development and quality (Korhonen et al. 2009; Schultz 2007). The light source used in the time-lapse system is the red light-emitting diode (LED), emitting within a narrow wavelength range of 625 nm. Therefore, this type of light exposure inside the time-lapse systems is much lower than the light used in stereo and inverted microscopes. However, the exposure of mouse, hamster, and porcine embryos to red light did not affect the blastocyst rates and total cell number (Li et al. 2014; Morishita et al. 2018).

14.8 Centrifugation

Centrifugation is one of the common techniques to prepare sperm to fertilize oocytes during both experimental and practical procedures, because it separates motile sperm from immotile as well as other contaminating cell debris (Agarwal et al. 2005). Sperm from different species such as the rat, human, and mouse are more sensitive to mechanical and centrifugal forces, while in other species such as equine and bovine, sperm are more resistant to damage by centrifugation, which indicates that the level of sperm injury after this process may be species specific (Carvajal et al. 2004).

Removal of seminal plasma from sperm is a necessary step to prepare them for fertilization during ART. However, sperm processing techniques also raise the ROS levels in sperm (Lampiao et al. 2010) and cause LPO of plasma membrane and sperm DNA fragmentation (Torres et al. 2019). Therefore, different sperm preparation techniques have been developed (e.g., microfluidic devices, glass-wool filtration, swim-up) to obtain sperm with high motility and DNA integrity (Jayaraman et al. 2012).

14.9 Culture Media Composition

OS inside the gametes or embryos may originate from the cellular metabolism and its surroundings (Agarwal et al. 2006b). An embryo-free culture media may itself be considered a source of ROS for embryos and gametes in IVF procedures, and this may result in an internal modification of the cellular redox balance (Martin-Romero et al. 2002). Antioxidants are frequently added to culture media from the beginning of their production to maintain a prooxidant-antioxidant equilibrium in embryos during their in vitro culture, but it is still unclear for how long the antioxidants added to the culture media can protect embryos during the whole period of culture (Martin-Romero et al. 2002; Maldonado et al. 2018).

Several tests have been used to measure the ROS and reactive nitrogen species (RNS) in the culture media (Martin-Romero et al. 2002; Samhan et al. 2004). Though ROS have been found in embryo culture media, specific compounds in commercial media remain unknown, and little is known so far about the differences between various commercial brands. Essential amino acids such as arginine, isoleucine, leucine, threonine, lysine, and valine as well as nonessential amino acids such as glycine, proline, serine, and asparagine are present in higher concentrations in the media in comparison with other type of amino acids. Interestingly, ion concentrations vary among different assessed brands (Morbeck et al. 2017). Variations of these components among different types of culture media may affect the oxidant-antioxidant balance in each culture media.

During the in vitro embryo culture, some factors can explain the continued formation of supraphysiological levels of ROS in culture media – exposure to pH, atmospheric oxygen, etc. (Cunningham et al. 1985; Michelson 2000; Wells et al. 1995). Furthermore, addition of pH buffers to some types of handling, culture media fosters the production of H_2O_2. Hydrogen peroxide may react with O_2 to produce $OH^{\bullet}$ and ROO and increase the formation of ROS inside the culture media through Fenton reaction, as is shown below (Michelson 2000):

$$\begin{aligned} Fe^{2+} + H_2O_2 &\rightarrow Fe^{3+} + OH^- + OH\bullet \\ Fe^{3+} + H_2O_2 &\rightarrow HO_2\bullet + Fe^{2+} + H^+ \\ \hline 2H_2O_2 &\rightarrow HO_2\bullet + OH\bullet + H_2O \end{aligned}$$

However, certain levels of ROS have been proposed to play a significant role in the regulation of some vital processes of cells (Martin-Romero et al. 2002). Supraphysiological levels of ROS in the culture media could disturb the intracellular ROS balance during in vitro embryo culture, leading to harmful levels of OS in the exposed cells (Martin-Romero et al. 2002). Some of the proposed interventions for ameliorating deleterious effects of ROS in mammalian embryos include lower O_2 tension to avoid the pH changes in culture media and additionally a more efficient form of antioxidant supplementation to culture media, searching a physiological OS status (Fig. 14.1) (Agarwal et al. 2014).

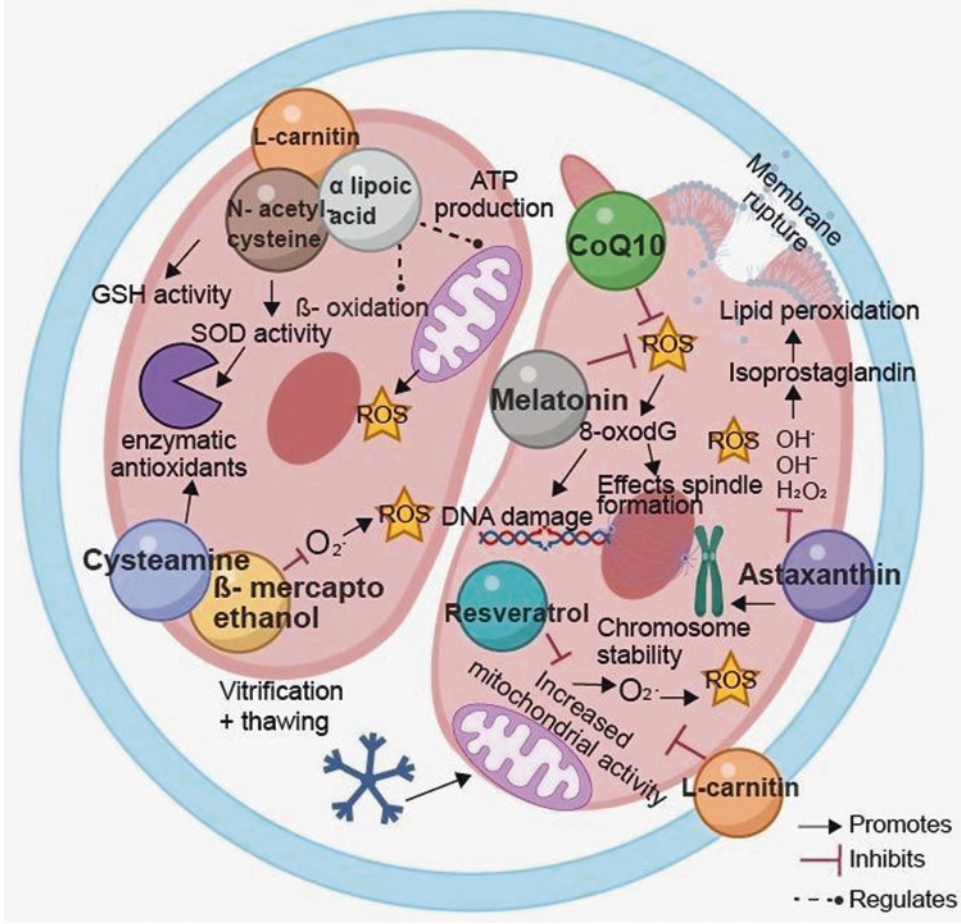

Fig. 14.1 Sources of ROS and ameliorating effects of some antioxidants on IVF outcomes. Embryos are exposed to several lab conditions and procedures that may cause an increase in ROS levels, such as oxygen concentration, visible light, composition of culture media, variations in pH and temperature, as well as gamete and embryo handling, centrifugation, and cryopreservation. One of the leading approaches to lower ROS-related damage is the addition of antioxidants to the culture media. This supplementation has shown to improve ART outcomes not only in terms of embryo development, rates, and quality but also when cellular processes such as apoptosis and DNA damage have been assessed in several models

14.10 Types of Antioxidants Used in Embryo Culture Media

In the in vivo systems, the presence of antioxidants in mammalian reproductive tracts has been studied to understand some compounds that interact with the growing embryo. The presence of ascorbate (vitamin C), cysteamine, tryptophan, cysteine, tyrosine, homocysteine, α-tocopherol, and β-carotene has been reported from the follicular fluid (Paszkowski and Clarke 1999; Guyader et al. 1998; Guerin et al. 1995; Casslen 1987). In tubal fluid the presence of GSH, transferrin, albumin, hypotaurine, taurine, tryptophan, cysteine, tyrosine, homocysteine, and catalase has been observed. Whereas in the oocyte and the embryo, the presence of GSH, as well as some antioxidant enzymes such as glutamylcysteine synthetase (γ-GCS) and SOD (cytoplasmic Cu, Zn-SOD, mitochondrial Mn-SOD) has been reported (El Mouatassim et al. 2000; Gardiner et al. 1998; Castillo et al. 2015a; Lapointe et al. 1998; Noda et al. 1991; Chun et al. 1994). These antioxidants have been studied to replicate physi-

ological conditions in in vitro systems. Antioxidants can be classified into two main groups: enzymatic and nonenzymatic.

14.11 Enzymatic Antioxidants

Family of SOD enzymes catalyzes the oxidation and reduction of O_2^- and H_2O_2 into diatomic oxygen and water through Haber-Weiss reaction:

$$2H^+ + H_2O_2 + 2e^- \rightarrow 2H_2O$$
$$H_2O2 \rightarrow O_2^- + 2H^+ + 2e-$$
$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Different SOD enzymes are categorized according to the location and the type of ion they have in their active site. Cu-Zn SOD is a cytoplasmic two-subunit protein that interacts with a copper ion in its active site. MnSOD is a mitochondrial four-unit protein that contains four manganese ions in its catalytic center. Catalase is another four-unit protein with ferric in the active site of each subunit. It has a cellular role in the direct breakdown of H_2O_2 into oxygen (Covarrubias et al. 2008).

Currently, the addition of different antioxidant enzymes has not fully demonstrated any ameliorative effect in embryo development and pregnancy outcome in animal models, as has been proposed that the transcription of most antioxidant enzymes occurs in the oocyte and it remains inactive until embryo compaction process starts, hence their activity does not change (Covarrubias et al. 2008).

14.12 Nonenzymatic Antioxidants

Most in vitro approaches for ameliorating the deleterious effects of ROS in embryos are focused on the study of nonenzymatic antioxidants. These are small molecules with free radical scavenging properties (Table 14.1).

GSH is a tripeptide that acts both as an antioxidant and a radioprotector. It is a cofactor of several enzymes such as glutathione-S-transferase (GST), some glutathione peroxidases and glyoxalases (Deponte 2013). Few studies have been conducted on in vitro culture of human embryos, and, therefore, animal models were used to demonstrate the ameliorative effect of some of the antioxidants on ART embryos. Coenzyme Q10 (CoQ10) has shown an increase in mice embryo cleavage and blastocyst formation of aged postovulatory oocytes, when added to the culture medium, as well as a reduction of spindle assembly disruption and a decrease of O_2-concentration and DNA damage (Zhang et al. 2019). Blastocyst formation rate also increased after CoQ10 supplementation also showing a decline in ROS, apoptosis, and DNA damage in porcine embryos (Liang et al. 2017). In contrast, another study did not find any positive effect of CoQ10 addition on oocyte maturation nor blastocyst formation. These opposite results could be attributed to the difference in the exposure to the stress (Maside et al. 2019).

Melatonin, as a free radical scavenger has been studied from different perspectives. Its role during the activity of some antioxidant enzymes has been widely assessed (Barlow et al. 1995). Some of the different approaches of the use of melatonin in embryo culture have resulted in a decrease of ROS levels or apoptotic nuclei in bovine embryos exposed to stress by heat shock or herbicide (Cebrian et al. 2013; Pang et al. 2016). In rabbit morulae, addition of melatonin in the culture media showed an increase in blastocyst formation and the activity of GST and SOD, while LPO and NO concentrations were lower (Mehaisen et al. 2015). Although in bovine embryos, it showed an increase in blastocyst rate (Wang et al. 2014b), addition at high concentrations during IVF co-incubation showed a deleterious effect in the bovine model (Cheuquemán et al. 2015).

Some amino acids as L-carnitine have also shown increased blastocyst development rate in mouse embryos and reduced DNA damage (Abdelrazik et al. 2009). Vitamin C has demonstrated diverse effects when added at different concentrations in vitrification-devitrification media. It showed an increase in blastocyst survival in pigs and a decrease in ROS generation, with no changes in DNA fragmentation (Nohalez et al. 2018; Castillo et al. 2015b). Resveratrol has

Table 14.1 Antioxidants used in IVF culture media experiments

Antioxidant	Experimental model	Main findings	Reference
Coenzyme Q10	Porcine	Did not affect 2–4 cell stage rate, and blastocyst formation. Highest concentration negatively affected blastocyst rates	Maside et al. (2019)
Quercetin, vitamin C or resveratrol	Bovine	ROS reduction, higher blastocyst rate, more total cell, no changes in nuclear maturation rates, no changes in GSH levels	Sovernigo et al. (2017) and Guérin et al. (2001)
Cysteamine or carnitine	Bovine	ROS reduction, no changes in nuclear maturation rates, increase in GSH levels	Sovernigo et al. (2017) and Guérin et al. (2001)
Resveratrol	Bovine	Moderate prooxidant effect, decreased inactive mitochondria rate	Gaviria et al. (2019)
L-carnitine	Bovine	Reduced DNA damage, increased embryo quality, better pregnancy rate	Kim et al. (2018)
Astaxanthin	Bovine	Somatic cell nuclear transfer embryos showed decrease in lipid peroxidation levels, increased chromosomal stability	Li et al. (2015)
Melatonin	Mice	Promoted meiotic spindle assembly and increases GSH production in oocytes, better mitochondrial function, lower ROS levels	He et al. (2016)
L-ascorbic acid	Porcine	Increased survival rates and reduced peroxide levels in vitrified-thawed blastocysts. Decreased ROS levels, increased blastocyst post vitrification survival, no changes in GSH levels	Castillo et al. (2015a) and Nohalez et al. (2018)
Co Q10	Porcine	Decreased apoptosis, ROS generation, DNA damage in blastocysts. Improvement in cleavage rate, blastocyst quality and formation rate	Liang et al. (2017)
β-mercaptoethanol + cysteamine	Mice	Improvement of fertilization, cleavage and blastocyst rates	Nikseresht et al. (2010)
Acetyl-L- carnitine + N-acetyl-L-cysteine + α-lipoic acid	Mice	Increased inner cell mass number, increased fetal and placental weight, higher crown-rump length, improvement in limb, eye and ear morphological grades	Truong and Gardner (2020)
α-lipoic acid, α-tocopherol, hypotaurine, N-acetyl-cysteine	Mice	Improvement in blastocyst formation rate in embryos from old animals but not in young ones	Silva et al. (2015)
Anethole	Bovine	Enhanced blastocyst formation rate when added to culture media rather than in vitro maturation media	Anjos (2019)

been one of the most widely studied compounds, as it has demonstrated increases in blastocyst rate quality, number, and total cells, when added to the culture media in bovine, mice, and cat models (Kwak et al. 2012; Li et al. 2018; Liu et al. 2013; Piras et al. 2020), although no such effect was noted for human embryos (Tarin et al. 1994). Blastocyst formation showed an increase in a bovine model when supplemented with GSH (Luvoni et al. 1996; Takahashi et al. 1993). Various concentrations of Cu-Zn SOD addition after fertilization have shown an increase in expanded blastocyst rate and blastocyst formation in mice (Nonogaki et al. 1992). Moreover, the ROS scavenger astaxanthin has demonstrated its role in the reduction of acetylation of several histone proteins leading to a loosened chromatin structure in bovine embryos (Li et al. 2015).

Furthermore, the expression of antioxidant enzyme genes such as GPX1 and SOD1 has been documented as an indicator of ROS scavenging, as they showed detoxification of H_2O_2 and O_2-. It is remarkable that in embryos treated with ascorbic acid, the survival rate increased only when

both vitrification-warming media and culture media were supplemented (Castillo et al. 2015a; Nohalez et al. 2018). However, when it was added only to the vitrification media, it showed no change (Urrego et al. 2014b). This interesting finding suggests that exogenous antioxidants should remain stable during every IVF process to maintain gene expression and biochemical pathways that may increase ART outcomes. In reference to the aforementioned statement, it is proposed to maintain a stable ORP level during the entire duration of embryo culture.

Biochemical processes involved in the functioning of nonenzymatic antioxidants on embryos are presented in Fig. 14.2.

Lately, the perspective of the use of different combinations of antioxidants has gained interest in ART research for its possible benefits in comparison to single antioxidants supplementation. β-mercaptoethanol and cysteamine supplementation of culture media has shown an improvement in embryo formation and number of blastocyst cells in mice (Nikseresht et al. 2010). Acetyl-L-carnitine, N-acetyl-L-cysteine, and α-lipoic acid

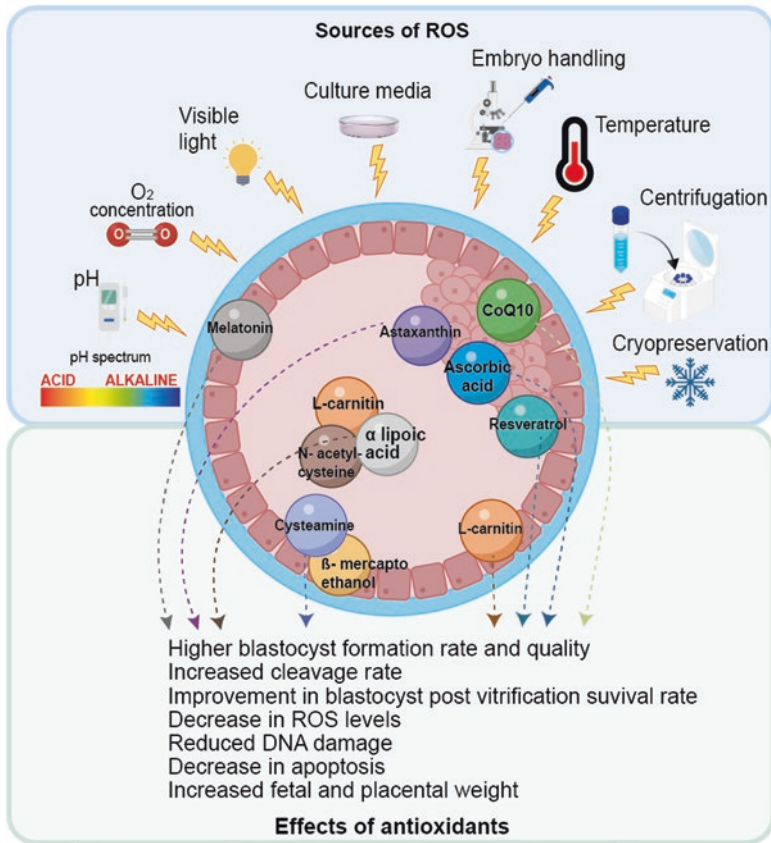

Fig. 14.2 Intracellular effects of some antioxidants on embryo culture. CoQ10 and melatonin inhibit DNA damage, as measured by 8-hydroxy-2′ -deoxyguanosine (8-oxodG) formation. Melatonin prevents spindle damage due to ROS. Astaxanthin diminishes iso-prostaglandin formation, an indicator of LPO, as well as causes an improvement in chromosomal stability. Cryopreservation processes result in an increase of mitochondrial activity, as well as ROS production. However, resveratrol reduces this activity and carnitine lowers superoxide levels. Combination of L-carnitine, α-lipoic acid, and N-acetyl-cysteine helps regulate adenosine triphosphate (ATP) production and transport of fatty acids for β-oxidation in mitochondria. Enzymatic activities such as SOD are increased by these antioxidants and by cysteamine and β-mercaptoethanol and also activity of some thiols such as GSH

have demonstrated enhanced blastocyst quality regarding ICM and TE cell numbers (Gardner and Kelley 2017; Truong and Gardner 2017).

However, it is a matter of concern that even though the culture conditions have been studied widely, specific mechanisms that interfere with embryo development remain unclear. In this regard, recent reports have directed attention to epigenetic modifications during embryo culture (Bomfim et al. 2017; Crosier et al. 2002). Moreover, embryonic DNA damage due to elevated ROS levels has been well documented, but the effect of OS has now been proposed as the source of histone acetylation, diminished skeletal muscle gene expression, and increased amino acid consumption, which could be an indication of an altered metabolism and increased DNA damage (Crosier et al. 2002; Seisenberger et al. 2012).

Furthermore, epigenetic modifications due to antioxidant supplementation have lately been studied since ROS are key regulators in gene expression. Nevertheless, whether there is an effect of antioxidants on these epigenetic modifications is not clearly known yet. Therefore, it is essential to focus future studies on this association to trace back the biochemical, genetic, and epigenetic importance and viability of antioxidant supplementation on embryo culture media. Laboratory factors that contribute to alterations in the epigenome should be diminished as OS reportedly generates IVF culture conditions that may support induction of lower birth weights and subsequent cardiac defects (Agarwal et al. 2014).

14.13 From Animal Models to Human ART

Extrapolation of animal processes for human ART should be carefully analyzed since embryo metabolism is different for each species (Duranthon and Chavatte-Palmer 2018b). Embryos of distinct species have different requirements of biomolecules at each stage of development. For example, rabbit embryos require glucose consumption for early cleavages unlike human and bovine embryos (Duranthon and Chavatte-Palmer 2018b; Kane 1987). Intake of amino acids by human embryos is necessary for embryo culture to blastocyst stage, not so in mice (Kane 1987). Lipid content in bovine and porcine embryos has been reported to be markedly higher than in humans and mice (Kane 1987).

It is possible for several antioxidants to act as prooxidants once they have reacted with other free radicals. This mechanism has been reported as the formation of several metabolites such as adducts by carotenes and tocopherol (Bast and Haenen 2002). These reactions occur due to the ability of antioxidants to reduce transition metals such as Fe^{2+}, with Fenton reaction (described above) and produce ROS (Bast and Haenen 2002; Van Haaften et al. 2001).

Synergistic effect of the combination of antioxidants in media culture is not always assessed when a combination of antioxidants is tested and is required to assure their safety and efficacy (Dorne and Renwick 2005). However, there are some studies that documented an enhancement in antioxidant activity by the addition of another antioxidant (Kamezaki et al. 2015; Kogure 2019; Wang and Day 2002).

Therefore, justified testing in human IVF should be cautiously performed to guarantee the safety of each antioxidant and their concentration in the culture media to achieve successful long-term outcomes.

14.14 Conclusions and Future Perspective

The aim of this review was to elucidate the impact of the OS generated by many factors during in vitro embryo production based on information available on animal models in order to be applied in human ART, particularly comparing the approaches to control OS during embryo culture, through the addition of antioxidants to the culture media. From the enzymatic perspective, it remains unknown whether the enzymatic antioxidants and its cofactors should be added directly to the media to enhance IVF outcomes. Regarding the nonenzymatic compounds, the current research approach seems to be directed toward the use of a combination of different types of

antioxidants that may maximize antioxidant potential effect and limit the exposure of embryos or gametes to toxic antioxidant concentrations. However, the "safe" antioxidant to be added to culture media during the in vitro culture of human embryos remains unknown yet.

Since the purpose of antioxidant addition to the culture media is to simulate physiological conditions during preimplantation embryo development, further studies must establish a physiological parameter to adjust and maintain the oxidant-antioxidant balance in the culture media similar to intrauterine environment to potentially alleviate the negative effects of OS produced during the in vitro embryo culture. Therefore, future research should focus on the possible suitability of the combination of different antioxidants in the embryo culture media used in human ART and safety determination of both, antioxidant concentration and final metabolites derived after the antioxidant neutralization in the culture media that could be potentially toxic to embryos and gametes.

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15

Roles of Oxidative Stress in the Male Reproductive System: Potential of Antioxidant Supplementation for Infertility Treatment

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Abstract

The decline of fertility in modern society is a serious worldwide concern, and the reasons behind it are complex and difficult to unveil. The fact that a big percentage of infertility cases remain diagnosed as idiopathic, turn the strategies to treat such conditions very limited. Nevertheless, one must agree that keeping the oxidative balance of the reproductive tissues should be one of the first lines of treatment for infertile patients. As reported, 30–80% of male infertile individuals present high levels of pro-oxidant species in the seminal fluid. Thus, antioxidant therapies, which consist of dietary supplementation therapy with one or more antioxidant compound, remain the first step in the treatment of male infertility. Nevertheless, the efficacy of such therapies is variable between individuals. The most common prescribed

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antioxidants are carnitines and vitamins C and E, but recently phytochemical quercetin has emerged as a potential compound for the treatment of oxidative stress in the male reproductive system. Although there are several animals' evidence about the great potential of quercetin for the treatment of infertility, clinical trials on this subject remain scarce.

Keywords

Oxidative stress · Male infertility · Antioxidant therapy · Quercetin

15.1 Introduction

Oxidation-reduction reactions are part of the complex signaling network that is responsible to maintain life. In living cells, a delicate balance between oxidant and antioxidant species occurs, in a process involving enzymatic and nonenzymatic mediators. Reactive oxygen species (ROS) are some of participants of the oxidation-reduction signaling pathway of cells. ROS is a collective term that includes oxygen radicals, such as the hydroxyl (OH^-) and the superoxide ion (O_2^-), and nonradical oxygen derivatives such as singlet oxygen (O_2) and hydrogen peroxide (H_2O_2). Although radicals are less stable and more reactive than nonradical derivatives, the latter can be converted into free radicals during several stages of cell metabolism. Nevertheless, cells are naturally equipped with enzymatic and nonenzymatic antioxidant defenses, responsible for neutralizing the harmful oxygen species and keeping the oxidative balance. Yet, when the levels of ROS overwhelm the natural antioxidant defenses of the cell, an oxidative situation is installed, commonly referred to as oxidative stress (OS) (Bisht et al. 2017). High levels of ROS are known to promote severe cellular damage through the oxidation of amino acids in proteins, lipids in membranes, and carbohydrates in nucleic acids. Yet, controlled, and short-lived events of OS are part of the complex cellular signaling pathways. This dual role of OS in the biological systems is largely controlled by the cell's antioxidant defenses, responsible for maintaining the ROS at controlled levels (Gough and Cotter 2011).

15.2 Reactive Oxygen Species as Mediators of Male Reproductive Events

15.2.1 Leydig Cells

Testes are organs with a very efficient antioxidant system since both Leydig cells and developing germ cells are prone to suffer from OS. Leydig cells are the steroidogenic cells of the testis, responsible for producing testosterone. They are present in small groups (up to ten cells) in the interstitial space between the seminiferous tubules. Due to its proximity to blood vessels, Leydig cells are easy targets for testicular macrophages, which are known to produce high quantities of ROS, cytokines, and other pro-inflammatory factors, triggers of an immune response (Agarwal et al. 2014). Furthermore, ROS are naturally produced by Leydig cells, both by the mitochondrial electron chain and by the P450 system (Chen et al. 2009). The P450 system is intimately involved with steroidogenesis. The luteinizing hormone (LH) is produced at the pituitary gland and is the main mediator of the steroidogenic process. In Leydig cells, LH stimulus is responsible for promoting the synthesis of steroidogenic enzymes and promoting the mobilization of cholesterol from the cytosol to the mitochondria. These events, scientifically designated by trophic regulation and acute regulation, respectively, both start with the binding of LH to the LH receptor at the cell surface. This process starts the cyclic adenosine 3′,5′- monophosphate (cAMP) signaling cascade. The rise of intracellular cAMP levels activates the protein kinase A (PKA) and p38 mitogen-activated protein kinase (MAPK) pathways. These signals promote the expression and production of enzymes responsible for the mobilization and transport of cholesterol to the mitochondria, such as the steroidogenic acute regulatory protein (StAR) and the translocated protein

(TSPO). In the mitochondrial matrix, cholesterol is converted into pregnenolone by the C27 cholesterol side-chain cleavage cytochrome P450 enzyme (CYP11A1). Pregnenolone is then converted into testosterone by the smooth endoplasmic reticulum enzymes, such as 3β-hydroxysteroid dehydrogenase (HSD3B), 17α-hydroxylase/17,20 lyase (CYP17A1), and 17β-hydroxysteroid dehydrogenase (HSD17B) (Wang et al. 2017). The aging process of Leydig cells is responsible for the decreased testosterone levels found in older males, and ROS play a crucial role in this multifactorial process. As mentioned, ROS are naturally produced by Leydig cells during the process of steroidogenesis at the mitochondrial electron chain and by the P450 system enzymes. Younger Leydig cells are equipped with an efficient antioxidant system that includes a wide array of enzymatic and nonenzymatic defenses, such as Cu–Zn superoxide dismutase (SOD), Mn-SOD, glutathione peroxidase (GPX-1), microsomal glutathione S-transferase (MGST1), glutathione S-transferase (GSTM2), and glutathione (GSH). However, older Leydig cells present a lower gene and protein expression of antioxidant defenses, leaving Leydig cells vulnerable to oxidative attacks. Close proximity to the testicular macrophages can exacerbate this process, increasing OS and the damage in the steroidogenic machinery. The decrease in the testosterone levels is usually accompanied by a rise or maintenance of LH levels secreted by the pituitary, which means that Leydig cells also became less responsive to the LH stimulus. Nevertheless, the decreased expression of antioxidant defenses and consequent turnover to an OS environment is thought to be the main process of Leydig cells' natural aging [for review (Wang et al. 2017)].

15.2.2 Sertoli Cells

In the seminiferous tubules, ROS are also important for the communication between the developing germ cells and their nursing cells, the Sertoli cells. Sertoli cells, the sentinels of spermatogenesis, are responsible for fulfilling the physical and nutritional needs of the developing cells. Together, they form the blood-testis barrier (BTB). The establishment of the BTB is only possible due to the Sertoli cells' cup shape, which allows them to constantly change their structure. Specialized tight junctions and extracellular matrix components produced by Sertoli cells guarantee the maintenance of the seminiferous epithelium and the BTB (Mruk and Cheng 2004, 2015). The BTB allows Sertoli cells to control the intratubular environment, while also protecting the germ cells against the host immune system (de Kretser et al. 1998). Sertoli cells are the main mediators of the male gamete formation process by providing the critical factors necessary for the success of spermatogenesis (Sofikitis et al. 2008). Since each Sertoli cell can only sustain a limited amount of developing germ cells, signaling pathways are established to limit the expansion of the spermatogonial population and elimination of defective germ cells. In resume, one can say that the homeostasis of the seminiferous tubules depends on a finely regulated balance between apoptosis and regeneration events (Johnson 1984; Johnson et al. 2008). The process of selective germ cell death is essential, not only to keep the balance between germ cells and Sertoli cells but also to preserve the genomic integrity of the germline (Print and Loveland 2000). The process of selective apoptosis is regulated by a complex network of endocrine, paracrine, and intracellular protein signals, many of which are still to be reported. Cell death can occur through many pathways, and in the seminiferous tubules, apoptotic germ cells are usually phagocytized by Sertoli cells or sloughed into the lumen of the tubule. At this stage, apoptotic germ cells can often be identified as multinucleated mass, a clear sign of failed cytokinesis. The B-cell lymphoma (Bcl-2) protein family is a classic family of apoptotic proteins, and it is involved in the process of germ cells apoptosis. Nevertheless, different signaling pathways promote apoptosis at different stages of germ cells development. For example, in immature sperm cells detached from the Sertoli cells, apoptosis is mediated by the Jun N-terminal kinase pathway

(Show et al. 2013). Meanwhile, apoptosis of mature sperm cells is mediated through the p38 mitogen-activated protein kinase (MAPK) pathway (Pulido and Parrish 2003). When the levels of pro-apoptotic signals surpass the levels of pro-survival signals, spontaneous germ cells apoptosis occurs. The levels of spontaneous germ cell apoptosis are low in normal, healthy conditions. Through the class B of scavenger receptor type I (SR-BI), Sertoli cells can detect phosphatidylserine, produced during apoptosis, and rapidly proceed to the phagocytosis of the apoptotic germ cell (Print and Loveland 2000; Murphy and Richburg 2014). Sertoli cells can also induce germ cell apoptosis, which usually gets exacerbated after testicular injury. The Fas signaling pathway is proposed to be one of the main signaling pathways that mediate germ cell apoptosis induced by Sertoli cells. The Fas family is a well-studied apoptosis pathway, based on the interaction of the Fas receptor with the Fas ligand (FasL). It is proposed that after testicular injury, Sertoli cells increase the expression of FasL. The FasL is liberated in the seminiferous tubules interstitial space where it binds to the Fas receptor present in germ cells. Fas-positive germ cells are then eliminated by Sertoli cells (Lee et al. 1999).

ROS has been proposed to participate in this process of Sertoli cell-germ cell regulation, although its role is still debatable. Ranawat and Bansal proposed that a stressful situation of excess and/or depletion of selenium, an essential trace element for cell growth and survival, could promote germ cell apoptosis (Ranawat and Bansal 2009). The authors observed that in changed selenium conditions germ cells presented an increased ROS generation and, consequently, increased lipid peroxidation (LPO). The oxidative damage promotes the expression of pro-apoptotic transcripts, such as p38, caspase 3, and caspase 8. Meanwhile, the expression of Bcl-2 pro-survival factors is decreased (Ranawat and Bansal 2009). The apoptosis process is thought to occur as follows. p38 MAPK signaling promotes the activation of caspase 8 (El Mchichi et al. 2007). Caspase 8 is then recruited to the mitochondria, promoting the activation of BID, a pro-apoptotic Bcl-2 family member. The truncated BID induces the release of cytochrome c from the mitochondria, amplifying the death signal (Yang et al. 1997; Luo et al. 1998). Caspase 8 also activates other downstream caspases, such as caspase 3, which promotes DNA fragmentation (Kruidering and Evan 2008; Larsen et al. 2010). The amplification of pro-apoptotic signals ultimately leads to germ cell death. In this context, ROS appears to contribute to the regulation of the testicular germ cell population under stress situations.

15.2.3 Spermatozoa

After spermatogenesis, sperm cells continue their journey into the epididymides, where they mature and are stored until ejaculation. For 10 days, sperm cells wander the epididymis from the caput to the caudal region. Herein a panoply modification processes occur, reshaping spermatozoon's membrane and nucleus and culminating with the acquisition of motility and fertilization ability. At this point, sperm cells are very prone to suffer from OS, due to their lack of cytoplasm and consequent low concentration of antioxidant enzymes. Nonetheless, the epididymides are equipped with a very efficient antioxidant system, which is mainly mediated by glutathione peroxidases (GPx) and peroxiredoxins (PRDX) antioxidant families (Vernet et al. 2004; O'Flaherty 2019).

Once in the female reproductive tract, spermatozoa suffer a series of physiological and biochemical changes in preparation for penetration in the egg zona pellucida and fusion with the female pronucleus. The process of sperm capacitation, as it is called, is intimately regulated by ROS levels. This process comprises changes in the plasma membrane, such as the removal of cholesterol and the modifications of glycoproteins present on the surface of the membrane (Ikawa et al. 2010). Afterward, sperm cells become hyperactive, a process characterized by changes in the motility and amplitude of the flagellar movement (Suarez 2008). The initial molecular mechanisms behind capacitation and hyperactivation involve the influx of Ca^{2+} and

HCO_3^- and cytosol alkalization. Ca^{2+} along with ROS (particularly O_2^-) stimulates the activation of adenylate cyclase, inducing the production of cAMP, and PKA activation. PKA triggers NADPH oxidase inducing the generation of ROS, in positive feedback signaling. In its turn, PKA promotes the activation of protein tyrosine kinase (PTK), increasing the levels of tyrosine phosphorylation in the fibrous sheath around the flagellum axoneme, an essential event in sperm capacitation to acquire the potential to undergo acrosome reaction (De Lamirande et al. 1997). During the acrosome reaction, the Ca^{2+} generated in capacitation promotes the cleavage of phosphatidylinosital-4,5-bisphosphate (PIP2). The by-products of this cleavage are involved in the fusion of the acrosomal and plasma membranes and the activation of protein kinase C (PKC). These last events prompt the influx of Ca^{2+} and the activation of phospholipase A2 (PLA2) (Kothari et al. 2010; Goldman et al. 1992). ROS also play an important role in acrosome reaction through the possible de-esterification of the membrane phospholipids, thus increasing membrane fluidity (Griveau et al. 1995) and allowing the sperm-oocyte fusion.

15.3 Antioxidant Defenses at the Male Reproductive System

Despite having a highly efficient antioxidant system, numerous internal and external factors can interfere with the redox balance of testicular tissue. Toxicant exposure, inflammation, testicular torsion, and aging are only some of the conditions that are known to cause testicular OS (Turner and Lysiak 2013). Sperm cells are especially vulnerable to ROS attacks. First, its membranes are rich in polyunsaturated fatty acids (PUFAs), essential for membrane fluidity, but also very prone to LPO (John Aitken et al. 1989). Spermatozoa have also a limited capacity of fighting OS. These cells have a limited content of enzymatic and nonenzymatic antioxidants, a direct consequence of their limited amount of cytoplasm. The closed conformation of DNA makes it hard for repairing processes to occur in case of an oxidative attack on the DNA. This means that spermatozoon's antioxidant defenses are mainly extracellular and present the media that surrounds them. As mentioned, both the testes and epididymides are equipped with a very efficient antioxidant system that prevents the oxidative damage of both developing germ cells and spermatozoa. The Sertoli cells can secrete an unusual form of extracellular SOD which protects the germ cell on development (Mruk et al. 2002). Another important extracellular antioxidant enzyme is the epididymal GPX (epGPX or GPX5). This enzyme is exclusively expressed by the caput epididymis, and it is associated with the plasma membrane of the sperm, protecting the acrosome from peroxide-mediated attacks (Taylor et al. 2013). Once ejaculated, the main antioxidant defense system of the spermatozoa resides in the seminal fluid. The latter is rich in α-tocopherol and ascorbic acid (vitamins E and C, respectively), which are the most important nonenzymatic antioxidants present in the seminal plasma. Vitamin E can prevent LPO in testicular microsomes and mitochondria (Aitken and Roman 2008) by reducing alkyl peroxyl radicals and being oxidized in the process. In its turn, vitamin C recycles vitamin E through the reduction of chromanoxyl radicals while it gets consumed in the process. The GSH-dependent dehydroascorbate reductase is responsible for maintaining vitamin C in a reduced state (Paolicchi et al. 1996).

15.4 Oxidative Stress: A Cause of Infertility?

Aitken and colleagues were the first to propose that OS could be a cause of infertility. At first, the authors were investigating the effect of a calcium ionophore (A23187) in the fertilization capacity and motility of spermatozoa from normozoospermic men. The use of divalent cation ionophores has been tested as a way to avoid sperm capacitation during assisted reproduction techniques (ART) (Aitken et al. 1984). In the

presence of extracellular Ca^{2+}, A23187 promoted the formation of a lipophilic complex, which increased the Ca^{2+} through the plasma membrane, promoting the acrosome reaction. The authors reported that in the presence of 50–100 μM of A23187, the fertilization rate increased ($P < 0.01$), with each oocyte being penetrated with an average of four spermatozoa (polyspermy). The sperm motility patterns remained unchanged. However, at higher concentrations, A23187 promoted the decrease of both the fertilization rate and sperm motility (Aitken et al. 1984). Using the calcium ionophore A23187, the authors demonstrated, in the following years, that human spermatozoa could produce ROS and presented this mechanism as a possible cause of male infertility (Aitken and Clarkson 1987). Human spermatozoa were incubated with a Biggers, Whitter, and Whittingham (BWW) medium containing Ca^{2+}, Mg^{2+}, and A23189 (0.05 mg/mL ~ 100 μM). The production of ROS was quantified through the reaction between luminol (a luminescent probe) and the ROS produced. The production of ROS by the spermatozoa boosted in only 5 min. The authors also demonstrated that these ROS were not originated at the mitochondria, since the addition of a mitochondrial inhibitor did not affect the ROS production (Aitken and Clarkson 1987). Regarding the fertilization capacity of spermatozoa, the authors observed an indirect correlation between the luminescence and the sperm-oocyte fusion rate, demonstrating a decreased fertilization capacity (Aitken and Clarkson 1987).

Defective spermatozoa are known to be a source of ROS, although the molecular mechanisms behind the production of ROS are debatable until today. A possible mechanism, as well as one of the most well-accepted, is associated with the enhanced presence of glucose-6-phosphate dehydrogenase (G6PD) in defective spermatozoa, especially the ones with retention of residual plasma (Villaverde et al. 2019). This enzyme catalyzes the regeneration of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), through the reaction between D-glucose-6-phosphate and $NADP^+$. NADPH is the substrate of NADPH oxidases (NOXes), a family of enzymes and the main producers of ROS in cells (Villaverde et al. 2019; Panday et al. 2015). NOXes are commonly found in phagocytic cells, such as neutrophils. Nevertheless, NOX-5 has already been identified in human spermatozoa (Musset et al. 2012), as well as in equine spermatozoa (Sabeur and Ball 2007). Meanwhile, NOX-2 is known to be present in mouse spermatozoa (Shukla et al. 2005).

The subsequent work of Aitken and colleagues proposed that excess ROS levels could severely impair spermatozoa membranes, which are rich in polyunsaturated fatty acids (PUFAs) and very prone to oxidative damage (John Aitken et al. 1989). Human spermatozoa membranes are composed of 50% docosahexaenoic acid, a highly unsaturated fatty acid with six double blonds per molecule. This composition is essential to create the membrane fluidity necessary for the occurrence of the acrosome reaction and fertilization (Jones et al. 1978, 1979). LPO is an oxidative chain reaction, characterized by the attack of ROS to lipids, essentially those that have double bonds, such as PUFAs. The presence of a double bond next to a methylene group makes the methylene C–H bond weaker and promotes hydrogen abstraction (Repetto et al. 2012). Lipid hydroperoxides are the main products of LPO; further several aldehydes are formed as by-products of this process, such as malondialdehyde (MDA) and 4-hydroxinonenal (4-HNE) (Ayala et al. 2014). MDA is a mutagenic by-product and is widely used as a biomarker for LPO (Esterbauer et al. 1990). 4-HNE is another highly toxic by-product of LPO, which is known to promote protein alkylation and DNA damage and interfere with mitochondrial activity, promoting the formation of more ROS (Shoeb et al. 2014). Furthermore, it acts as a signaling molecule, inducing the production of inflammatory markers, exacerbating the oxidative state (Benedetti et al. 1980; Schaur 2003). The most noticeable consequence of LPO is the loss of progressive and total sperm motility, most likely associated with membrane fragility, and midpiece damage (Rao et al. 1989).

Another vulnerable target to oxidation is the nucleic acids. DNA damage is particularly concerning since it can induce health problems that will last for several generations. Furthermore, normal babies (without any evidence of health problems) can be born out of sperm with high levels of chromatin damage (Gandini et al. 2004). Both mitochondrial DNA and nuclear DNA can suffer oxidative attacks and damage. The mitochondrial DNA, due to its physiological position in the cell, is much more prone to suffer an oxidative attack than nuclear DNA. However, since the paternal mitochondrial DNA inherited is much restricted when compared to the maternal mitochondrial DNA, it is believed that these mutations do not present little biological meaning (Aitken and De Iuliis 2009). Nonetheless, the same is not true for nuclear DNA. During spermatogenesis, the sperm chromatin undergoes extensive changes, which included DNA reorganization, and histones replacement (for protamines). In the end, the paternal pronucleus is constituted by a highly compact DNA bound to protamines (~95%), which will remain transcriptionally inactive until the fertilization of the oocyte (Aoki and Carrell 2003). This DNA structure is only possible due to the disulfide bridges formed in the cysteine residues in minor grooves of the DNA coils and protamines, which are formed during the epididymal transit (Aoki and Carrell 2003). Nevertheless, nuclear DNA is still prone to suffer oxidation, which can be caused by a rise in ROS levels at the male reproductive tract but also by a decrease of antioxidant defenses, especially in the epididymides. Herein, epididymis-specific GPX5 plays a crucial role in the maintenance of sperm DNA integrity (Taylor et al. 2013; Chabory et al. 2009), and the impairment of its activity has been found to be associated with DNA abnormalities, which include poor pronucleus DNA condensation and oxidative defects (Chabory et al. 2009) (Fig. 15.1).

As the number of infertile couples rises at a worldwide level, the number of individuals with idiopathic infertility is increasing. Recent evidence has proposed that OS could be a cause of male infertility, being the explanation for several idiopathic infertile cases. Vorilhon and colleagues studied the sperm DNA damage levels of 80 male patients attending a fertility clinic (Vorilhon et al. 2018). Half of these patients were normozoospermic, and the other half had altered sperm quality parameters. As expected, the authors reported significant positive correlations between DNA damage and alterations in sperm quality parameters. This was not the first study to report such correlations, as many authors had reported similar results in previous years (Cambi et al. 2013; Kodama et al. 1997; Ni et al. 1997). Nevertheless, the authors noticed that in the normozoospermic group, 39% of the subjects presented pathological DNA oxidation levels, even though, sperm quality values were at normal values (Vorilhon et al. 2018).

After the overwhelming evidence that OS could be a cause of male infertility, Male Oxidative Stress Infertility (MOSI) was proposed as a term to define infertile men with elevated seminal ROS levels, an indicator of OS, which will attribute a definition to individuals previously diagnosed with idiopathic infertility (Agarwal et al. 2019).

15.5 Antioxidant Therapies for Infertility Treatment

After acknowledging OS as a cause of male infertility, physicians had to propose a way to treat it. Antioxidant therapies have been commonly prescribed to couples undergoing ART. Usually, these therapies are based on the premise that seminal OS is promoted by a deficiency in antioxidant species. However, the efficiency of these therapies is still debatable. Nevertheless, this kind of therapy is still used as a first-line treatment for male infertility, due to its low cost and low risk of toxicity (Agarwal and Sekhon 2010). The use of carnitines and vitamins C and E has been the most prescribed antioxidant supplementation for men undergoing ART. These nonenzymatic antioxidants are consistently present throughout the male reproductive tract. Carnitines play a very important role in the protection of sperm during their passage through the

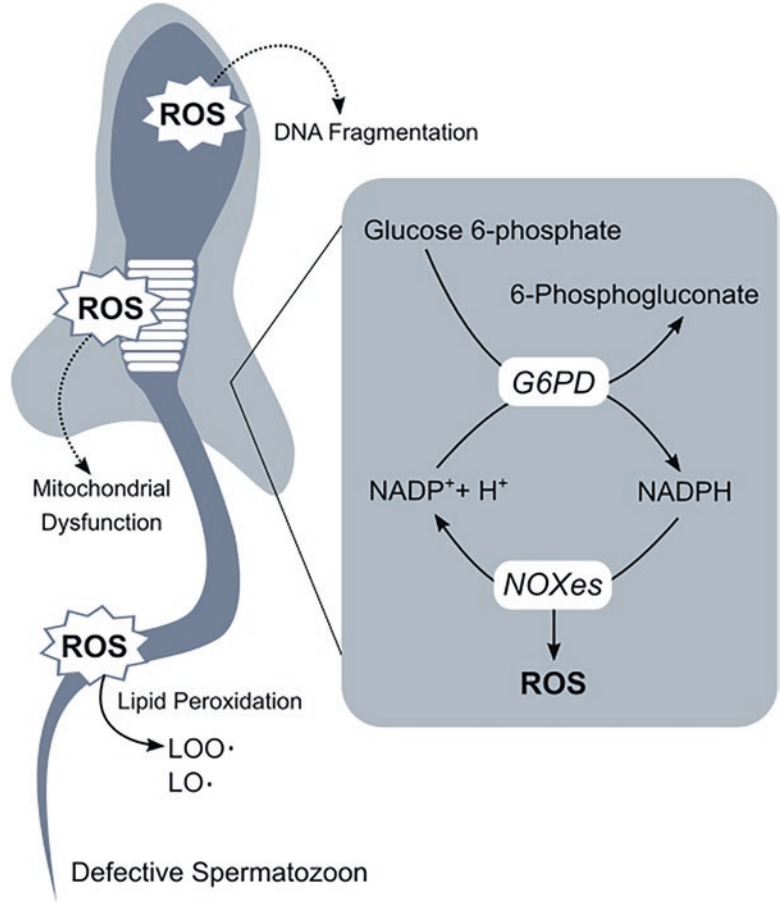

Fig. 15.1 Spermatozoa were very susceptible to OS due to the lack of antioxidant defenses, a direct consequence of its lower cytoplasm level. Yet, residual cytoplasm is thought to further aggravate the oxidative state of the spermatozoon due to the higher quantities of glucose-6-phosphate dehydrogenase (G6PD). Although the activity of this enzyme does not promote OS on itself, it contributes to the availability of NADPH, the substrate of NADPH oxidases (NOXes). This family of enzymes catalyzes the formation of ROS, specifically superoxide (O_2^-) and hydrogen peroxide (H_2O_2). OS affects the spermatozoon physiology at several levels, promoting the fragmentation and mutation of genomic DNA in the nucleus, disruption of mitochondrial activity, and lipid peroxidation. The latter is also responsible for the formation of cytotoxic by-products and peroxyl free radicals, which can further promote oxidative reactions and damage

epididymis (CM et al. 2004) and are an important intracellular factor that appears to be associated with sperm acquirement of motility. Once inside the cell, carnitine suffers a series of modifications, which culminate in the formation of acyl-carnitine in the outer mitochondrial membrane. This compound is then transferred to the inner mitochondrial membrane, where it is re-esterified into acyl-CoA and carnitine once more. Acyl-CoA is then able to participate in the β-oxidation process that generates energy and is essential for sperm motility (CM et al. 2004). Along with its role in cellular energy metabolism, carnitine is a powerful antioxidant. Epididymides are known for their higher concentration of carnitine (2–100 mM in comparison to circulating values of 10–50 μM), which is thought to also participate in the oxidative protection of sperm cells, along with its energetic role. Regarding the popular use of carnitine as a supplement for men under ART, the efficacy of such a practice is still debatable. The large variety of study designs does not

help to draw meaningful conclusions. Vitali and colleagues reported that a daily dose of 3 mg of L-carnitine was able to enhance total and progressive motility of sperm in 37 out of 47 men diagnosed with idiopathic asthenozoospermia (Vitali et al. 1995). Meanwhile, Sigman and colleagues also tested the efficacy of carnitine supplementation in idiopathic asthenozoospermic men. The group recruited 21 idiopathic asthenozoospermic patients, 12 received an oral treatment of carnitine (2 g L-carnitine and 1 g L-acetyl-carnitine per day), and the remaining 9 received a placebo, for 24 weeks. The authors could not find any clinically or statistically significant differences regarding the sperm quality of patient's posttreatment (Sigman et al. 2006). Some years later, Garolla and colleagues reported that oral carnitine supplementation (L-carnitine, 2 g per day for 3 months) was only effective in idiopathic asthenozoospermic patients with normal levels of sperm phospholipid hydroperoxide glutathione peroxidase that catalysis the degradation of lipid hydroperoxide, a toxic radical subproduct of LPO (Garolla et al. 2005). The authors suggested that carnitine supplementation is ineffective in the sperm mitochondria is already damaged, which could explain the large variety of outcomes regarding the supplementation of infertile men with this compound (Garolla et al. 2005). Meanwhile, a consensus opinion appears to exist regarding the efficacy of vitamins supplementation for infertile men.

Vitamin C supplementation was demonstrated to improve sperm count, sperm motility, and sperm morphology of oligozoospermic men (1000 mg of vitamin C twice a day, 2 months) (Akmal et al. 2006). After varicocelectomy, men supplemented with vitamin C (250 mg twice a day, 3 months) demonstrated a better recovery of sperm motility and morphology, although no effects regarding sperm count were found (Cyrus et al. 2015). Vitamin E supplementation has mainly been tested in in vitro studies, where it was demonstrated to improve teratozoospermia motility and viability after 1 h incubation with 40 μM of vitamin C (Keshtgar et al. 2012). This enhancement of sperm motility and viability by vitamin E (40 μM, 1 h incubation) was also reported by Fanaei and colleagues (Fanaei et al. 2011). In oral diet supplementation therapies, vitamin E is usually administrated along with other vitamins and compounds, including vitamin C. However, the efficacy of these treatments is not consensual, most likely due to the large variety of methodologies used, along with the several different combinations of compounds (Piomboni et al. 2008; Rolf et al. 1999; Alahmar 2017).

15.6 Quercetin: A Novel Therapy for Male Infertility?

Nowadays, new antioxidant therapies are constantly emerging, and their applications are vast. Furthermore, the popularization of the usage of natural products has instigated the pharmaceutical industry research to better explore natural compounds. On a worldwide scale, about 50% of pharmaceutical products contain natural products, and about 25% of prescription drugs are derived from natural bioactive compounds (Cameron et al. 2005). Several phytochemicals are being studied for their antioxidant effects, among them flavonoids are one of the most popular. Flavonoids are polyphenolic compounds (characterized by their two benzene rings and heterocyclic pyrene ring) (Kuhnau 1976). Flavonoids' proprieties vary widely, according to their chemical structure modifications, which occur mainly in the heterocyclic pyrene ring (Panche et al. 2016). In plants, flavonoids are involved in several processes, such as pigmentation, aroma, and protection against ultraviolet (UV) radiation, and are often synthesized in response to microbial infections (Griesbach 2005; Takahashi and Ohnishi 2004; Dixon et al. 1983). Flavonoids cannot be synthesized by animals, since the phenylpropanoid pathway is not present in these biological systems (Kumar and Pandey 2013). These plant-derived compounds have gained popularity due to their antioxidant properties, along with cardioprotective, antidiabetic, antiaging, anti-inflammatory, and anticancer

properties (Ullah et al. 2020; Kumar and Pandey 2013).

Flavonoid compounds are proven to exhibit powerful antioxidant properties in vitro. However, their effects are minimized in vivo due to their low water solubility, bioavailability, and weak absorption (Moreira da Silva and Silva 2017). In general, the antioxidant mechanisms of action of flavonoids include the inhibition of ROS formation, the direct scavenging of ROS, and the activation of antioxidant defenses (Dias et al. 2021). Flavonoids can prevent ROS generation through the interaction and inhibition of enzyme functions, such as NADH oxidase, and/or by chelating metal ions involved in free radical formation (Kumar and Pandey 2013; Heim et al. 2002). On the other hand, flavonoids are also efficient in scavenging ROS due to the presence of functional hydroxyl groups which enable hydrogen atom transfer and electron transfer to neutralize powerful radicals, such as hydroxyl, peroxyl, and peroxynitrite radicals (Saini et al. 2017). In addition, flavonoids can also counteract ROS through the upregulation of antioxidant enzymes with radical scavenging capacity (Cordero-Herrera et al. 2015).

Quercetin is one of the most abundant flavonoids, and, through glycosylation, it constitutes the backbone of several other flavonoids compounds. It is present in a wide variety of foods, meaning that the average daily uptake of quercetin by a human can vary from 10 to 100 mg, depending on each person's dietary habits. Thanks to its high availability, it is easily extracted and purified, being commercialized as extracts. The consumption of such products can rise the quercetin daily uptake to 500–1000 mg (Bischoff and Care 2008). After ingestion, quercetin is hydrolyzed in the small intestine, where it is converted into quercetin aglycone. This compound is then rapidly absorbed by the intestinal epithelium. Once in the bloodstream, quercetin can suffer several reactions and interact with several serum proteins, which is probably associated with its multiple mechanisms of action, such as antioxidant, antiallergic, and anti-inflammatory activities (Bischoff and Care 2008). These proprieties are very alluring for ART, with a special focus on the antioxidant proprieties of this compound. Quercetin exerts its antioxidant role by benefiting both enzymatic and nonenzymatic antioxidants. Vásquez-Garzón and colleagues treated rats with 10 mg/kg of quercetin, 2 h before the administration of 200 mg/kg of diethylnitrosamine, an OS inducer (Vásquez-Garzón et al. 2009). The analysis of the rats' liver revealed that quercetin participated in the restoration of reduced glutathione. Meanwhile, it also increased the activity of hepatic SOD, CAT, and GPx. After this, the authors proposed that quercetin's antioxidant mechanism of action is due to its promotion of natural antioxidant defenses (Vásquez-Garzón et al. 2009). In vitro, quercetin (100 and 200 μM, 3 h incubation) was found to counteract the effects of hydrogen peroxide in rat sperm, preserving sperm motility, viability, and morphology (Ben Abdallah et al. 2011). Quercetin (100 μM, 2 h incubation) was also found to significantly improve the sperm motility of men diagnosed with leukocytospermia, where sperm cells are naturally more prone to suffer from OS (Diao et al. 2019). The authors also reported a decrease in sperm mitochondrial DNA damage, along with an increase in the NADH and cytochrome C levels in the leukocytospermic samples (Diao et al. 2019). The addition of quercetin to the sperm cryopreservation medium (50 μM) was also reported to significantly improve post-thaw human sperm parameters, specifically sperm motility, viability, and DNA integrity, in comparison to the control (cryopreserved sperm samples with sperm freeze medium only) (Zribi et al. 2012). However, the cytoprotective role of quercetin during sperm cryopreservation needs to be further explored (Zribi et al. 2012).

Not many authors have explored the in vivo potential of quercetin in regard to OS at the male reproductive system. Yelumalai and colleagues used diabetic rats (diabetes induced by streptozotocin-nicotinamide) to test if the administration of quercetin could protect spermatozoa from the damage usually associated with this disease (Yelumalai et al. 2019). OS is proposed to be one of the pathways by which diabetes promotes male infertility (Barkabi-Zanjani et al. 2020). Animals were treated with quercetin (10, 25, and 50 mg/kg) for 28 days, and spermatozoa were retrieved from the cauda epididymis. The authors

reported an increase of sperm quality in diabetic animals treated with quercetin regarding sperm count, motility, and viability. Abnormal sperm morphology was also decreased upon quercetin treatment. It is worth mentioning that the administration of quercetin at the same concentrations in normal animals did not alter the sperm quality in comparison to the control. Through flow cytometry, the authors were able to observe those diabetic animals treated with quercetin presented reduced levels of sperm DNA fragmentation. The activity of sperm antioxidant enzymes (SOD, CAT, and GPx) also significantly increased in diabetic animals treated with quercetin, in comparison to non-treated diabetic animals. The sperm from these animals also presented an increased level of *SOD1*, *CAT*, and *GPx1* mRNA abundance than non-treated diabetic animals, and nondiabetic animals. After these results, the authors concluded that the administration of quercetin to diabetic rats could help mitigate the deleterious effects that diabetes promotes in the male reproductive system. More specifically, quercetin could have positive effects in the control of OS promoted by diabetes in the animals' testis (Yelumalai et al. 2019). Similar effects of quercetin were found in mice testis where animals were treated with cadmium chloride (Bu et al. 2011). Cadmium is a heavy metal known to completely disrupt testicular oxidative balance. Once more, quercetin was reported to increase the activity of antioxidant enzymes (SOD and GPx), decreasing LPO on the testicular tissue (Fig. 15.2). Furthermore, the authors also detected a decreased expression of pro-apoptotics factors, such as caspase-3 and Bax, followed by an increased expression of Bcl-2, a pro-survival factor (Bu et al. 2011). Quercetin also appears to have beneficial effects in the protection of testis against other toxicants, such as lead nitrate (Abd El-Latief 2015).

Quercetin is commonly present in dietary supplements. Although the serum concentration of quercetin increases after the administration of such supplements, no harmful effects have been detected (Conquer et al. 1998; Egert et al. 2008). It appears that quercetin does not affect the plasma antioxidant capacity nor the inflammation and metabolism state of the organism (Egert et al. 2008). Furthermore, the rise of quercetin levels in the serum is variable between individuals, and no correlations have been found between age, gender, body mass index, and demographics (Jin et al. 2010). In humans, as in rodents (Siti et al. 2020), quercetin appears to have a beneficial effect on hypertensive individuals, by inducing the reduction of blood pressure (Edwards et al. 2007). However, to our knowledge, the potential of quercetin as a therapy for other disorders has not been explored in humans. This also included the usage of quercetin supplementation in infertile male individuals.

15.7 Concluding Remarks

Oxidative events are crucial for the wellbeing of the male reproductive system, being part of the complex signaling network responsible for steroidogenesis, spermatogenesis, sperm capacitation, and fecundity. Nonetheless, these oxidative events should be short-lived to not compromise the cellular integrity of male reproductive tissue and gametes. As reported, 30–80% of infertile individuals present high levels of oxidant species in the seminal fluid (Agarwal et al. 2019). Keeping the oxidative balance of the reproductive tissues should be one of the first lines of treatment for these patients. Carnitines and vitamins C and E remain the most prescribed antioxidant supplementation for men undergoing ART. However, the efficacy of antioxidant therapies in the treatment of infertility is highly variable between individuals. The different methodologies and compound combinations usually found in these kinds of therapies make it difficult the retrieve any meaningful conclusions from the clinical trials (CM et al. 2004; Piomboni et al. 2008; Rolf et al. 1999; Alahmar 2017). Quercetin has recently emerged as a possible candidate for the treatment of infertility. This compound exerts its antioxidant role by benefiting both enzymatic and nonenzymatic antioxidants. It is reported to participate in the restoration of reduced glutathione and increased the activity of antioxidant enzymes, such as SOD, CAT, and GPx. It is also thought to decrease the

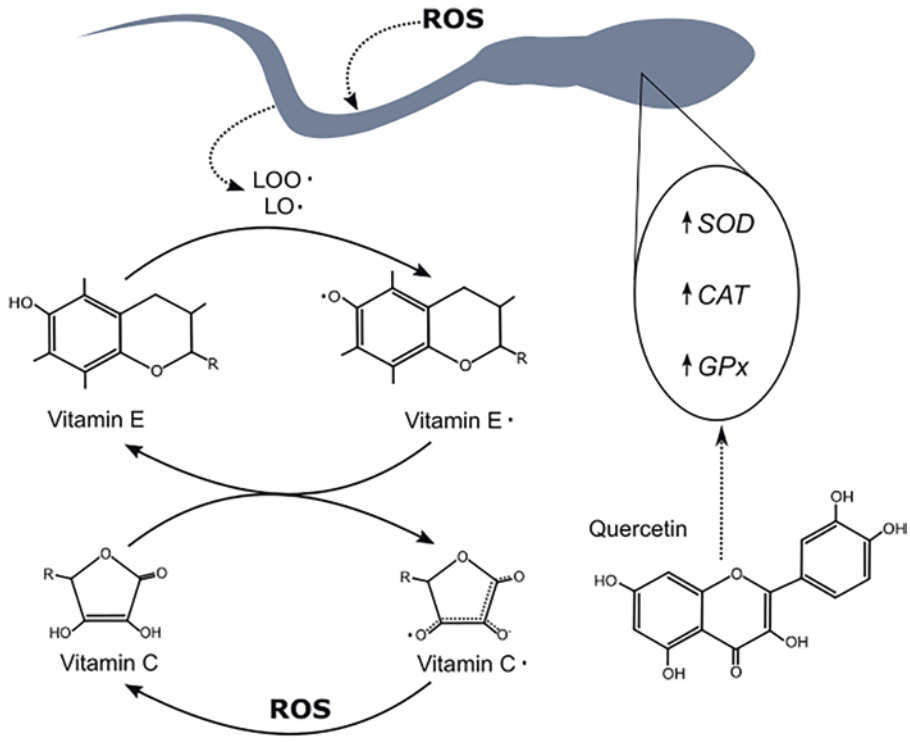

Fig. 15.2 Vitamin C and E are the most available antioxidants in the seminal fluid and crucial for the protection of spermatozoa against OS. Vitamin E is known to react with peroxyl free radicals, which originated during the peroxidation of sperm's cytoplasmatic membrane (LOO· and LO·). The reaction of Vitamin E with such radicals promotes the formation of a radical vitamin E, which is reestablished to its normal form by vitamin C. This reaction results in the radicalization of vitamin C, which is now available to react with other radical species, such as ROS. Although each vitamin can act as a scavenger on its own, the cycle vitamin E and C appears to be one of the principal mechanisms of extracellular antioxidant defense of sperm cells. Meanwhile, quercetin, the novel compound for sperm medium supplementation, appears to exert its antioxidant effects by promoting the activity of sperm antioxidant enzymes, more specifically SOD, CAT, and GPx. Nonetheless, human studies are yet to confirm this hypothesis

expression of pro-apoptosis factors, such as caspase-3 and Bax, followed by an increased expression of Bcl-2, a pro-survival factor (Yelumalai et al. 2019; Bu et al. 2011). Although the antioxidant role of quercetin has been fairly explored in animal studies, regarding the treatment of several conditions (including male infertility), the transition of these trials to human subjects has been slow and gradual. Nevertheless, this flavonoid compound presents a big potential to be used as a first-line treatment of male infertility, promoting the oxidative homeostasis of the male reproductive tissues.

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Conflict of Interest The authors declare no conflict of interest.

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16 Oxidative Stress-Induced Male Infertility: Role of Antioxidants in Cellular Defense Mechanisms

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Abstract

Male infertility is linked to several environmental and mutagenic factors. Most of these factors, i.e., lifestyle, radiations, and chemical contaminations, work on the fundamental principles of physics, chemistry, and biology. Principally, it may induce oxidative stress (OS) and produce free radicals within the cells. The negative effect of OS may enhance the reactive oxygen species (ROS) levels in male reproductive organs and impair basic functions in a couple's fertility. Evidence suggests that infertile men have significantly increased ROS levels and a reduced antioxidant capacity compared with fertile men. Although, basic spermatic function and fertilizing capacity depend on a delicate balance between physiological activity of ROS and antioxidants to protect from cellular oxidative injury in sperm, that is essential to achieve pregnancy. The ideal oxidation-reduction (REDOX) equilibrium requires a maintenance of a range of ROS concentrations and modulation of antioxidants. For this reason, the chapter focuses on the effects of ROS in sperm functions and the current concepts regarding the benefits of medical management in men with diminished fertility and amelioration of the effect to improve sperm function. Also, this evidence-based study suggests an increasing rate of infertility that poses a global challenge for human health, urging the need of health care professionals to offer a correct

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diagnosis, comprehension of the process, and an individualized management of the patients.

Keywords

Male fertility · Fertility impairment · Sperm function · Free radicals · Oxidative injury

16.1 Introduction

Infertility is known as the incapability of a couple to achieve a spontaneous pregnancy after 1 year of unprotected intercourse. By the year 2050, around 8–15% of couples in the world will be diagnosed as infertile (Cardoso et al. 2019). It may be that 48.5–186 million of couples will have infertility issues (Inhorn and Patrizio 2015). Male factor alone can be responsible for up to 20% contributing to 30–50% of all cases of infertility (Cardoso et al. 2019).

Male infertility has multiple causes such as genetic mutations, lifestyle changes, medical conditions, exposure to toxins, use of medications, varicocele, genital tract infections, obstructive and nonobstructive azoospermia, hypogonadism, and autoimmune diseases. Even with advances in medicine, many times a cause is not identified, so the term unexplained male or idiopathic infertility is often used (Hamada et al. 2012; Fainberg and Kashanian 2019).

Currently, many idiopathic cases can be related to OS. Since 1940, a potential role of oxygen in sperm function has been suspected (MacLeod 1943). In 1987, Aitken first reported the presence of ROS in seminal plasma through chemiluminescence assay (Aitken and Clarkson 1987). From that moment on, multiple studies have searched for the relationship between elevated ROS and male infertility.

It is known that small ROS production is required to maintain sperm functions such as capacitation, acrosome reaction, and sperm-oocyte fusion (Du Plessis et al. 2015). But when production exceeds the necessary levels and there is no control by antioxidants, lipid peroxidation (LPO) of cell membrane, damage to the sperm deoxyribonucleic acid (DNA) of the nucleus and mitochondria, as well as apoptosis can occur (Takeshima et al. 2020).

There can be different sources of ROS in semen and sperm, but the higher production is in seminal plasma leukocytes and sperm mitochondria. It is clear that leukocytes especially the macrophages and neutrophils can have a production up to 1000 times greater (Plante et al. 1994). For this reason, agents that can cause inflammation such as infections and varicocele can easily alter the REDOX balance (Agarwal et al. 2018). Therefore, OS can be an important factor for the etiology of male infertility since it can cause malfunction of sperm cells due to damage to their structure or defects in their functional capacity (Venkatesh et al. 2011). After considering these issues, recently a term called male oxidative stress infertility (MOSI) has been coined since OS can be an important factor in some of the known causes and risk factors for infertility with an association of 30–80% (Agarwal et al. 2019).

16.2 General Concepts of ROS and Antioxidants in Spermatozoa

OS has been linked to alterations in male health and infertility for a long time, and variations in the REDOX balance can cause deleterious effects on cells, including spermatozoon (Wagner et al. 2018). This imbalance causes OS when ROS and other free radicals are considerably elevated or antioxidants are substantially decreased causing diseases and cellular damage (Agarwal et al. 2016a).

16.2.1 ROS

ROS are highly reactive oxidizing free radical agents and include superoxide anions ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), peroxyl (·ROO), and hydroxyl (·OH) radicals (Wagner et al. 2018). OS is necessary for fertilization to occur. There must be a balance between the ROS production and the concentration of antioxidants for their normal role in the reproductive process of the sperm. ROS induces cyclic adenosine monophosphate

(cAMP) in spermatozoa that inhibits protein tyrosine phosphatase (PTP) leading to tyrosine phosphorylation (p-Tyr). In particular, H_2O_2 stimulates capacitation via p-Tyr triggering a cell signaling cascade (O'Flaherty et al. 2006). Also, acrosome reaction is promoted by this mechanism. Motility can be increased by ROS exposition facilitating hyperactivation and sperm membrane binding to the zona pellucida ZP-3 protein thereby promoting sperm-oocyte fusion (O'Flaherty et al. 2006).

There are intrinsic ROS sources due to REDOX reactions in aerobic metabolism. In the mitochondria from the creation of adenosine triphosphate (ATP) by the respiratory chain in nicotinamide adenine dinucleotide (NADH) (Di Meo et al. 2016). In addition, in the mitochondrial membrane, there may be production by oxidoreductase enzymes, in peroxisomes due to the presence of urate oxidase (UOX) and xanthine oxidase (XO) (Di Meo et al. 2016). Moreover, the endoplasmic reticulum has an electron transport chain that can be activated by xenobiotic metabolism or production of unsaturated fatty acids or protein folding, also, in lysosomes because they contain a REDOX chain that serves to maintain an optimal pH between acidic hydrolases (Di Meo et al. 2016). In spermatozoa, the plasma membrane is of special importance due to the high content of unsaturated fatty acids making them easy targets for ROS and in the cytoplasm the presence of glucose-6-phosphate dehydrogenase (G6PD) to produce it (Said et al. 2004).

Varicocele is associated with elevated ROS and DNA damage. Elevated temperature has alteration of the REDOX balance and negative effects in the seminal parameters. Infections of accessory glands, urethra, prostate, vas deferens, seminal vesicles, epididymis, and testes have been found to have elevated ROS-induced by leukocytes, which are the cells with the highest production of these (Agarwal et al. 2018). Hyperglycemia has also been found to increase the production of ROS and affect seminal parameters (Takeshima et al. 2020). Age can also be a factor in increased OS. This can occur because of elevation of mitotic divisions and more elevation of DNA fragmentation (Nguyen-Powanda and Robaire 2020).

There are also extrinsic sources of ROS. Smoking can cause high accumulation of ROS at the seminiferous tubules with increased toxic substances and free radicals; the production of H_2O_2 anions is augmented leading to damage to mitochondrial DNA having less production of ATP which can affect motility and damage DNA and methylation patterns (Barati et al. 2020). Excessive alcohol consumption can decrease glutathione (GSH) and superoxide dismutase (SOD) levels. Ethanol causes changes in structure and function of mitochondria, reduces ATP levels, and increases the ROS production through metabolism in the liver. It also increases the activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and changing copper- and iron-free ions ultimately increases production of $O_2^{\cdot -}$ in the seminiferous tubules (Manzo-Avalos and Saavedra-Molina 2010).

Industrial exposures are rare but can cause elevated levels of ROS. These exposures comprise heavy metals like cadmium, chromium, nickel, and mercury (Ong et al. 2002). Also, exposure to pesticides, phthalate, and pollution may induce sperm damage (Sedha et al. 2015). Cancer patients exposed to chemotherapy such as cisplatin, doxorubicin, or cyclophosphamide may present increased OS (Bagheri-Sereshki et al. 2016). Exposure to cell phone radiation can also cause ROS damage through LPO and at low levels, increase NADH oxidase, resulting in sperm death (Desai et al. 2009). Damage from OS has also been related with exposure to non-ionizing radiation such as electromagnetic radiation (Gautam et al. 2021).

16.2.2 REDOX Reactions of Free Radicals

Free radicals are substances that have a single odd electron in their outer orbit. This makes them very reactive and unstable due to the high energy they can produce when reacting with other molecules such as proteins, lipids, carbohydrates,

and nucleic acids (Losada-Barreiro and Bravo-Díaz 2017).

ROS are generated in cells by the following reactions.

Rapid oxygen intake with NADPH activation producing $O_2^{\cdot-}$ (Hogg 1998).

$$2O_2 + \text{NADPH} \rightarrow \text{oxidase} \rightarrow 2O_2^{\cdot-} + \text{NADP}^+ + H^+$$

The $O_2^{\cdot-}$ is converted in H_2O_2 by SOD (Hogg 1998).

$$2O_2^{\cdot-} + 2H^+ \rightarrow \text{SOD} \rightarrow H_2O_2 + O_2$$

ROS can also be generated from the myeloperoxidase-halide-H_2O_2 (MPO) system, mainly in neutrophils because they contain the enzyme MPO. Hypochlorite (HOCl) is produced in the presence of chloride ion (Cl^-) (Hogg 1998).

$$Cl^- + H_2O_2 + H^+ \rightarrow (\text{MPO}) \rightarrow \text{HOCl} + H_2O$$

The Fenton and Haber Weiss reactions also produce ROS through respiratory chain (Hogg 1998).

$$H_2O_2 + Fe^{2+} \rightarrow .OH + OH^- + Fe^{3+}$$

$$O_2^{\cdot-} + H_2O_2 \rightarrow \cdot OH + OH^- + O_2$$

ROS can cause damage at various sites in cells. The most frequent and relevant is the LPO of the membrane since it is more sensitive due to its content of polyunsaturated fatty acids (PUFA). This harmful process can be self-perpetuating (Jenkinson et al. 1999). The following reactions explain this process.

LH are PUFA and R is the initial oxidant radical that produces fatty acid radical R, which rapidly adds oxygen and generates fatty acid peroxyl radical. These radicals are the carriers in chain reactions and can oxidize other PUFA molecules to produce more lipid hydroperoxide (LOOH) and break down in aldehydes that can diffuse and affect other cellular structures (Yin et al. 2011).

$$\text{LH}^{(\text{Lipid})} + \text{R}\cdot \rightarrow \text{L}\cdot^{(\text{Lipid radical})} + \text{RH}$$

$$\text{L}\cdot + \text{O2} \rightarrow \text{LOO}\cdot^{(\text{Lipid peroxyl Radical})}$$

$$\text{LOO}\cdot + \text{LH} \rightarrow \text{LOOH} + \text{L}\cdot$$

$$\text{LOOH} \rightarrow \text{LO}\cdot + \text{LOO}\cdot + \text{aldehydes}$$

OH is considered the main effector of deterioration to the DNA molecule that causes oxidative damage of the heterocyclic ring and the carbohydrate portion in the oligonucleotides by distinct mechanisms. This damage is correlated with various conditions like mutagenesis, carcinogenesis, and aging (Breen and Murphy 1995).

When ·OH reacts with guanine, C-8 hydroxy-adduct radical is produced, which is converted to the 2,6-diamino-4-hydroxy-5--formamidopyrimidine but also by oxidation can be converted to 8-hydroxyguanine (8-oxo-G). Although, when it reacts to other bases like thymine and cytosine in positions C5 and C6, it results in the formation of cytosine glycol and thymine glycol, respectively, and all these reactions further lead to impairment in the double-stranded DNA (Breen and Murphy 1995).

Another deleterious reaction of·OH is with deoxyribose in DNA by abstraction of H-atom in C5 and the addition of C-8 position in the same nucleoside. This cyclization forms 8,5′-cyclopurine-2′-deoxynucleosides. All these reactions on sugar radicals result in the DNA strand breaks and base free sites (Dizdaroglu and Jaruga 2012). The combination of ROS and ions such as Fe_2^+ and Cu_2^+ can oxidize proteins and damage these molecules. The amino acids lysine, proline, histidine, and arginine can be more susceptible to oxidative damage. This damage can be measured by the formation of carbonyls (Stadtman 1990).

Therefore, oxidative damage is important in multiple molecules that can cause disease of neurological, cardiac, renal, and other systems. In the sperm these reactions can cause alterations in motility, morphology, and DNA strand breaks that can lead to infertility.

16.2.3 Antioxidants

Physiological oxidative mechanisms must be kept in balance to minimize tissue damage. This

is achieved by producing and obtaining the correct amounts of antioxidants to control harmful effects derived from excess ROS. Antioxidants are substances that can delay or prevent oxidation by inhibiting chemical reactions or eliminating necessary intermediates for them and protecting against OS injury produced by free radicals. They are divided into enzymatic and nonenzymatic types, and the last one can be obtained by endogenous production or consumption of food or supplements (Alahmar 2019a). Figure 16.1 presents a detailed classification of antioxidants.

In spermatogenesis, extracellular antioxidants are needed to counteract OS. Seminal plasma provides optimal pH and a portion of antioxidants for sperm support. Men who undergo vasectomy have antioxidant levels similar to fertile men suggesting that these are contained in seminal plasma and not directly in the sperm production location (Zini et al. 2000). In men with idiopathic infertility, the antioxidant capacity is lower than in fertile men (Barati et al. 2020). These findings imply that the REDOX environment must be in control for optimal fertility. The most important enzymatic antioxidants in male reproductive tract are SOD, catalase (CAT), and glutathione peroxidase (GPX) (Chen et al. 2003). SOD cleanses the $O_2^{\cdot-}$ and catalyzes the conversion of H_2O_2 and O_2. It is found in high levels in Sertoli cells (SC) and developing sperm (Mruk and Cheng 2000). It can be found in the epididymis and seminal plasma. SOD protects against LPO and decreases DNA damage (Negri et al. 2017). GSH is used by GPX as an electron donor to catalyze H_2O_2 and $O_2^{\cdot-}$. SC are rich in GPX, which is secreted in the head of epididymis and found in seminal plasma. GPX protects against LPO of plasma membrane (Meseguer et al. 2007). CAT is an enzyme found in peroxisomes and converts HO_2 to H_2O and O_2. Although minimal CAT is present in the developing sperm, a continuous low level of activity in the testicle is observed, and there is evidence of a considerable reduction over progressive motility and sperm

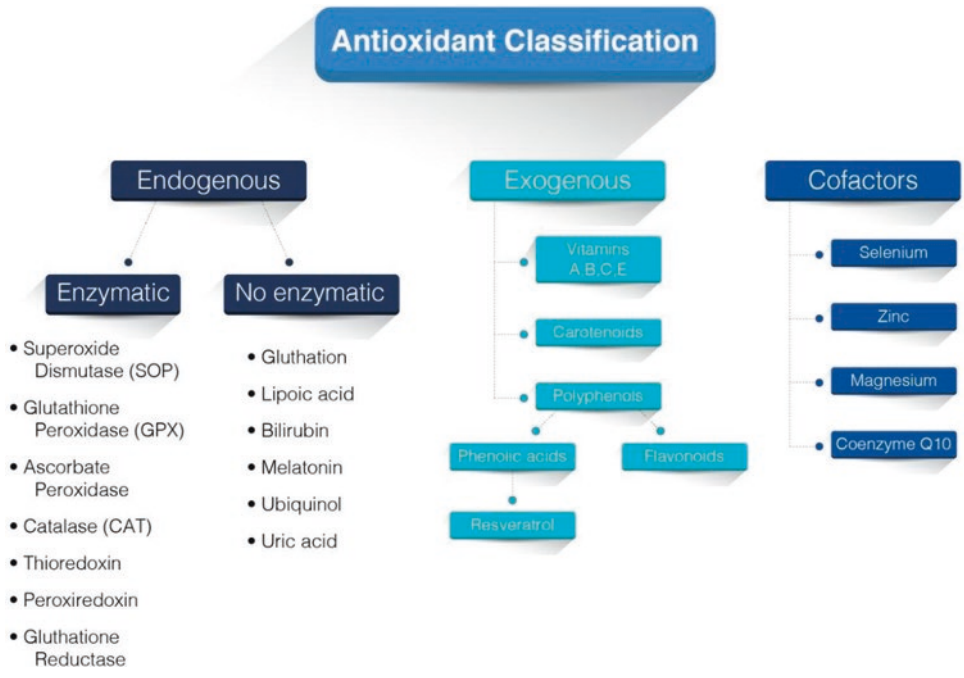

Fig. 16.1 Classification of antioxidants. Endogenous antioxidants are produced within the human body and can be classified as enzymatic and nonenzymatic. Enzymatic agents participate as catalysts for specific reactions with free radicals. Nonenzymatic can have functions as cofactors or react directly with ROS. Exogenous antioxidants are obtained through dietary consumption, and their antioxidant function is varied. There are exogenous substances like minerals that can play a role as cofactors in ROS reduction reactions

viability in infertile men due to CAT reduction (Tavilani et al. 2008). Men with asthenozoospermia have lower activity of CAT in their semen compared to normospermic men, potentially demonstrating the importance of seminal plasma levels of CAT (Tavilani et al. 2008). Nonenzymatic antioxidants can be produced by endogenous metabolism or be consumed in food or supplements. GSH can be produced endogenously. It is a free radical scavenger, and high levels are found in spermatids and developing sperm (Fujii et al. 2003). Different antioxidants as supplementation to treat male infertility are later in the chapter.

16.3 ROS and Sperm Parameters

16.3.1 Sperm Count

Elevated ROS levels have been found in oligozoospermic men (Agarwal et al. 2014). The mechanism that leads to a decrease in the sperm count can be generated by the disruption of the mitochondrial membranes that cause activation of the caspases and finally apoptosis of spermatozoa (Agarwal et al. 2008). Also, during the caspase pathway, a greater amount of ROS is released, which generates sperm DNA damage and can enhance the effect of apoptosis (Agarwal et al. 2008). This mechanism can be especially harmful in freeze-thaw semen samples (Thomson et al. 2009). Sperm count can be directly affected by ROS even though hormone levels may be within normal thresholds.

16.3.2 DNA Strand Break and Apoptosis

DNA strand break and apoptosis are physiological processes necessary for adequate functioning of the cells. In the prophase I during the sperm meiosis, spermatocytes generate double-strand DNA breaks through Spo 11 protein. These breaks are necessary for homologous chromosomes to allow DNA recombination (Keeney et al. 2014).

On the other hand, apoptosis in mammalian spermatogenesis is a necessary mechanism for the regulation of superfluous, senescent, and aberrant cell populations (Shukla et al. 2012). However, its function in ejaculated sperm is not known (Shukla et al. 2012), although a greater number of apoptotic markers have been found in ejaculated sperm, such as externalization of phosphatidylserine to the sperm outer membrane, activated caspase 3, loss of the mitochondrial membrane potential, and DNA fragmentation (Shukla et al. 2012).

16.3.2.1 DNA Strand Break

Mild OS is necessary for sperm functions as in the case of DNA to maintain telomere length, but it has been observed that OS at supraphysiological levels can result in DNA damage (Mishra et al. 2016). Guanine is the most common organic DNA base that is prone to ROS attack and is converted to 8-hydroxy-2-deoxyguanosine (8-OHdG). This derivative is ten times higher in infertility patients than in fertile men. OS leads to the production of 8-OHdG which is an oxidized base found in higher levels at sites with less protamine protecting the DNA (Guz et al. 2013).

Free radicals can also directly affect DNA by attacking the purine and pyrimidine bases and the deoxyribose backbone. Normally sperm DNA is tightly packaged by protamines protecting it from free radical attack, but infertile males showed deficient protamination leaving the sperm more vulnerable to ROS damage (Tremellen 2008).

OS can damage sperm DNA by several mechanisms. It can produce single-stranded, double-stranded breaks, and DNA fragmentation. Also, the introduction of abasic sites, purine, pyrimidine, and deoxyribose modifications and DNA cross-linking can result in arrest or induction of gene transcription (Bauer et al. 2015), as well as the induction of signal transduction pathways, accelerated telomeric DNA attrition, replication errors, genomic instability, and GC to TA transversions (Bauer et al. 2015; Hosen et al. 2015). Peroxynitrite is the result of coupling nitric oxide (NO) and $O_2^{\cdot -}$ and is a strong cellular oxidant that can generate asthenozoospermia, affect the mitochondrial membrane potential, and possibly increase LPO (Uribe et al. 2015). These damages

can cause chromosome breakage leading to mutation, genomic instability, and probably cancer (Raman et al. 2005).

OS can damage both nuclear and mitochondrial DNA. Since mitochondrial DNA has a circular structure, few base pairs, a lack of histones, and an absence of repair mechanisms, it is highly susceptible to ROS damage (Bui et al. 2018). OS can produce mitochondrial membrane instability or can cause the loss of electrons from the transport chain, which increases the production of ROS (Bui et al. 2018). Spermatozoa with high levels of damaged mitochondrial DNA have low potential to natural conception. The consequences of OS on DNA damage are presented in Fig. 16.2.

Nevertheless, mechanisms are not clear since ROS can cause damage to other structures and also to nuclear DNA (Venkatesh et al. 2009). Spermatozoa use glycosylase enzymes for base repair; however, they do not have the compounds to maintain this protective system, so DNA zones without pyrimidine bases pair properly and destabilizes ribose-phosphate, opening the cyclic response of ribose, and the DNA chain is fragmented resulting not only in mutations but also in DNA fragmentation (Bui et al. 2018; Ohno et al. 2014). Sperm DNA damage can be measured by different tests directly or indirectly. The most used sperm DNA fragmentation tests are deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), the COMET assay, and sperm chromatin structure assay (SCSA) (Agarwal and Majzoub 2017). Other options are the measurement of the product of DNA oxidation 8-OHdG or by chemiluminescence assays using luminol or lucigenin (Agarwal and Majzoub 2017).

16.3.2.2 Apoptosis

Apoptosis is the programmed cellular death and is a control mechanism necessary for the development of structures in multicellular organisms. However, ROS can cause pathological activation of apoptosis by the caspase pathway, causing harmful cell death and infertility (Shaha et al. 2010). Also, the hormonal imbalance of testosterone, follicle-stimulating hormone (FSH), and estradiol can cause an increase in the rate of apoptosis (Shaha et al. 2010). Apoptosis is so important that the removal of some genes related to apoptosis in mice, like bcl-2-like protein 1, BAX, growth arrest, and DNA damage-inducible protein GADD45 alpha and cytochrome C show decreased germ cell apoptosis that can lead to infertility (Rucker 3rd et al. 2000). This could be explained since the defective cells cannot be eliminated and will continue to develop into mature cells and will participate in the fertilization attempt (Rucker 3rd et al. 2000; Aitken and Baker 2013).

The DNA damage, mainly DNA fragmentation generated by ROS, causes sperm cell instability increasing apoptosis. Spermatozoa with poorly compacted chromatin are more susceptible to enter to apoptotic cascade, which has been associated with an increased number of testicular apoptotic germ cells, phosphatidylserine exteriorization, higher levels of caspase activation, and DNA damage in infertile males (Aitken and Koppers 2011). Also, the accumulation of free radicals could promote higher levels of tumor necrosis factor (TNF-⟨), resulting in the production of inflammatory cytokines, which inhibits apoptosis and allows sperm with DNA damage to continue maturing and can be found in the ejaculate (Aitken and Koppers 2011; Havrylyuk et al. 2015). It has been hypothesized that during the preparation of seminal samples, OS and apoptosis may occur, which can compromise the outcomes of fertility treatments (Mahfouz et al. 2010).

16.3.3 Hormonal Dysfunction

The balance in the various hormonal axes is important for the correct testicular function. The production of ROS can alter these axes at multiple levels (Fig. 16.3). It is known that various hormones like FSH, luteinizing hormone (LH), prolactin, testosterone, and estradiol can adjust antioxidant capacity in seminal plasma to control the balance between ROS and antioxidants (Mancini et al. 2009). Hormones such as melatonin may act as an antioxidant to protect sperm and testicular cells from free radical damage because it has been reported that infertile men

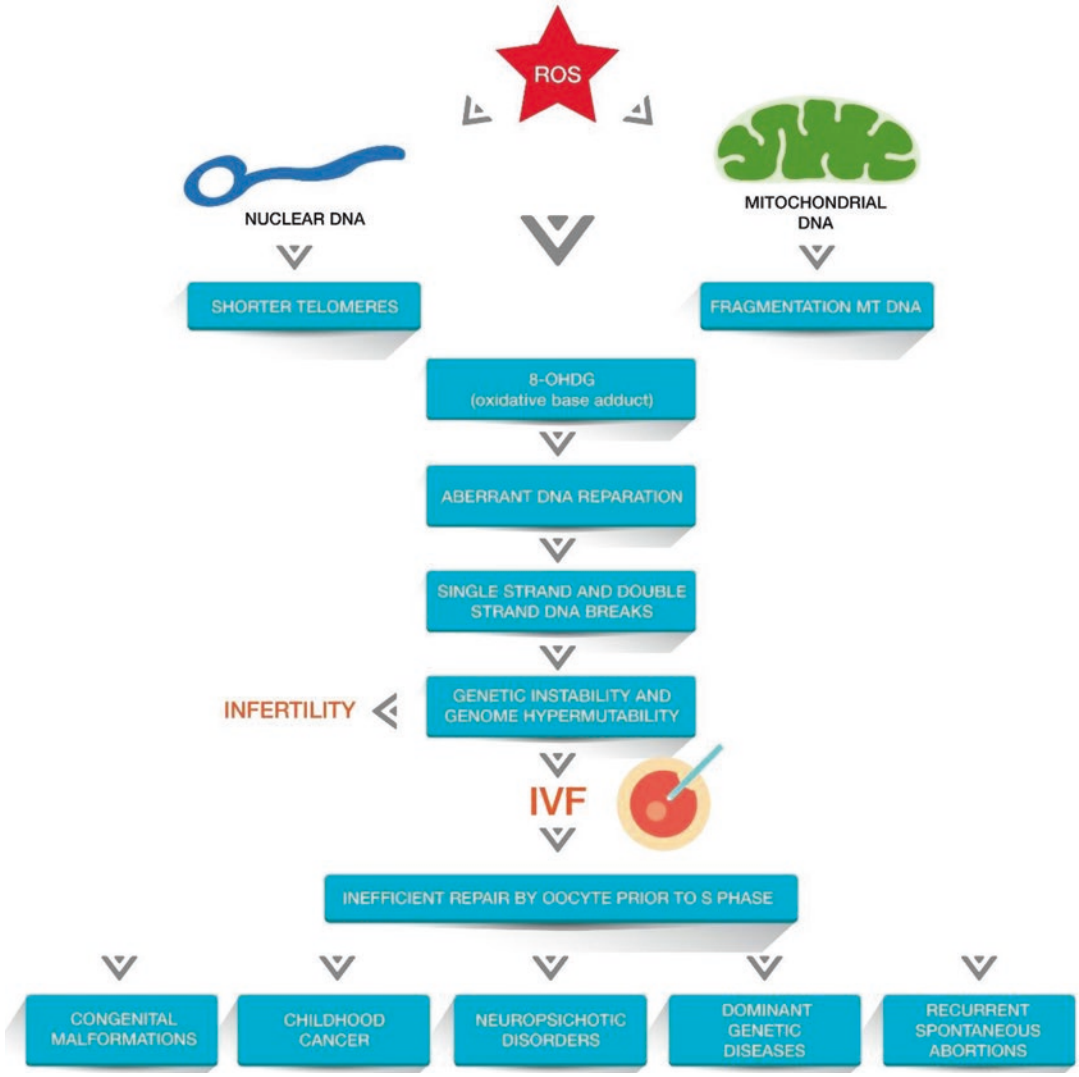

Fig. 16.2 Consequences of OS on DNA damage. OS leads to damage in sperm and mitochondrial DNA which is associated with shorter telomere length, production of oxidative base adduct 8-OHdG, and fragmentation of mitochondrial DNA. The repair system of DNA is limited. Single- and double-strand breaks are generated. This causes genomic hypermutability and genetic instability that can lead to infertility. Due to the high mutagenic load in sperm, every cell of a development embryo can continue with DNA damage by aberrant oocyte repair in the S phase. This perpetuated damage to the embryo is correlated with childhood cancer, neuropsychiatric disorders (such as autism and schizophrenia), and diseases arising from a mutation that can continue in each cell of a developing embryo like dominant genetics (such as Apert syndrome and achondroplasia) (*Adapted from Bisht et al. (2017))

have lower levels of this hormone in seminal plasma (Awad et al. 2006).

ROS can cause decreased testosterone production secondary to direct damage to Leydig cells or other endocrine structures such as the anterior pituitary. The hypothalamic-pituitary-adrenal axis is activated secondary to OS and decreases the production of LH and FSH with a subsequent decrease in the production of testosterone (Spiers et al. 2014). OS decreases the production of triiodothyronine (T3) and thyroxine (T4) with lower production of StAR protein that also reduces the

production of testosterone. Insulin secretion is lowered, also affecting the production of T3 (Manna et al. 1999). So, alteration in the thyroid axis can cause hypogonadism and subsequent alteration in sperm quality. In addition, obesity increases ROS and production of leptin that also decreases the secretion of T3 in the thyroid and reduces GNRH pulses in the hypothalamus with lower production of LH and FSH (Wauters et al. 2000). ROS can cause increased aromatase activity with elevated E2 production that produces less secretion of inhibitory hormones with decreased testosterone production and increased prolactin levels that block GNRH secretion (Darbandi et al. 2018). OS causes not only local damage to sperm or reproductive organs, but it also causes alterations in the hormonal axis with lower levels of testosterone that generates dysfunction in spermatogenesis with subsequent low seminal parameters.

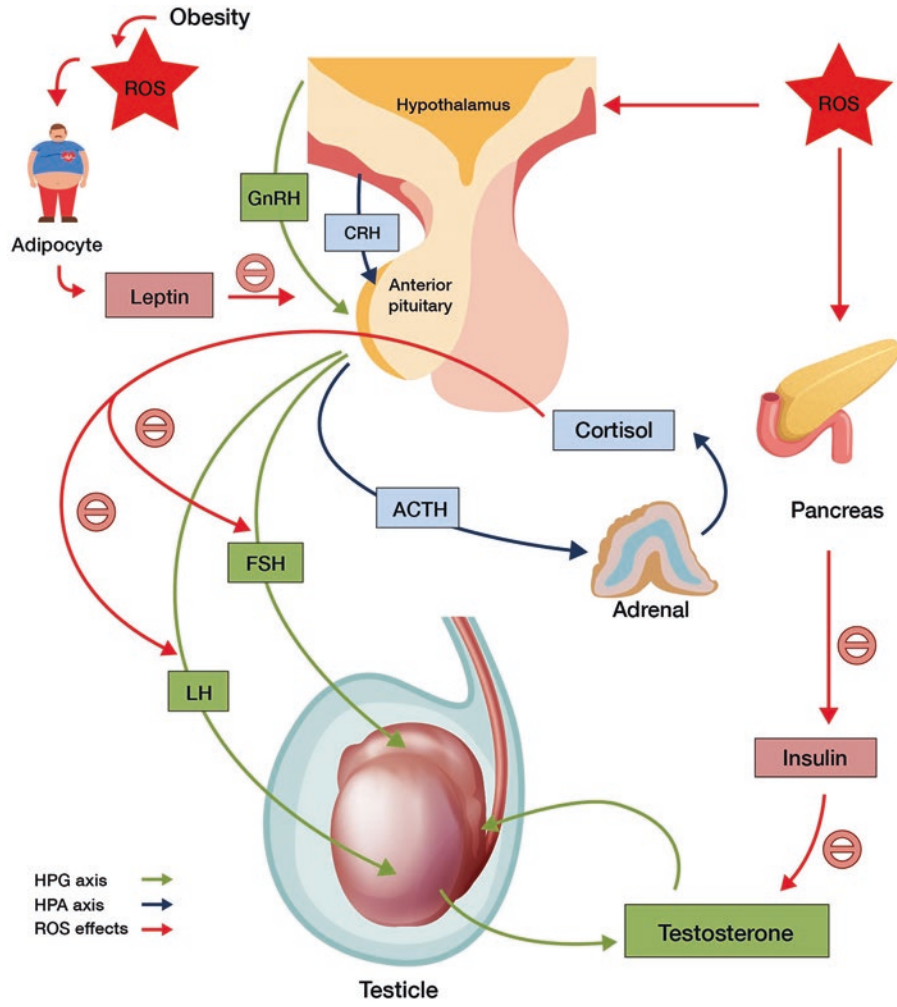

Fig. 16.3 Hormonal alterations derived from OS. ROS generate production of leptin in obesity that blocks GNRH production. Also, ROS induce production of insulin that lowers levels of testosterone and can promote the secretion of CRH with generation of cortisol that can inhibit production FSH and LH with subsequent diminished production of testosterone and altered spermatogenesis

16.3.4 Morphological Changes in Male Reproductive Organs: Human and Animal Models

The relation between ROS and alterations in sperm with subsequent DNA damage, dysfunction in apoptosis, and alterations in hormonal production has been mentioned previously. However, OS can have a deleterious effect on the structure of reproductive organs, mainly at the histological level, some of them a consequence of the impact of toxic agents that cause elevation of OS in the male reproductive organs when it has been applied experimentally.

A substance frequently used in animal models for the induction of OS is tert-butyl hydroperoxide (t-BHP). The epididymis is a large producer of peroxiredoxins which are antioxidant enzymes that do not require cofactors (O'Flaherty 2014). It was observed in an in vivo study that treatment with t-BHP in adult rats promotes an increased OS in epididymal sperm in comparison to testicular sperm. Some studies have shown that ejaculated sperm have higher levels of DNA damage than testicular sperm (Wu et al. 2020). It has also been observed under the induction of OS by TBHP altered expression of microRNAs that could have a negative effect on the antioxidant response and spermatogenesis (Fatemi et al. 2014).

Leptin production, as previously discussed, is increased with obesity. In an experimental study with mice put through an obesity-inducing diet, it was observed that leptin could be related to alterations in hormonal axes, as well as lower testicular weight, apoptosis, and pathological damage to Leydig cells, and decrease in sperm quality and antioxidants besides increased OS (Zhao et al. 2014).

The use of anabolics is known for their deleterious effects on the testicle. Accordingly, in an experimental study boldenone and stanozolol were administered to rats, and a significant elevation of OS with a lower effect in antioxidant systems was found, after prolonged exposure to these drugs. It was proposed that ROS may be one of the factors causing damage to the germ tissues (Bueno et al. 2017). It has also been observed that exposure to other toxins such as iodine, cadmium, and bisphenol A in rats increases ROS production, presenting structural alterations in the testicle and a decreased testosterone production (Chakraborty et al. 2016; Djuric et al. 2015; Kaur et al. 2018).

An event at the cellular level observed by the elevation of OS is autophagy. It is a cellular function in which undesirable components are catabolized. Interpreted as "self-eating," it provides energy and materials for cellular construction and maintenance of homeostasis (Zhu et al. 2019). It has been observed in an experiment with chickens that arsenic poisoning generates autophagy and apoptosis possibly secondary to OS due to increased ROS production and decreased antioxidant enzymes such as CAT and SOD, among other effects (Shao et al. 2018). Heat can increase OS with subsequent structural changes in the testicle. It was found in pigs that the heat-induced ROS could generate changes at the level of tight junction proteins through the inhibition of the Ca^{2+}/CaM-dependent protein kinase B AMP-activated protein kinase (CaMKKb-AMPK) pathway, and this leads to dysfunction in spermatogenesis (Yang et al. 2020). In humans, an example of ROS damage at the level of the testicular structure occurs with the tissue injury that presents the ischemia-reperfusion in testicular torsion secondary to the formation of NLRP3 inflammasome. ROS production levels are increased with damage to the endothelial cell barrier due to the reduction of adenylate cyclase and lower levels of cAMP (Minutoli et al. 2016). As mentioned earlier, OS can generate changes mainly in the microstructure of the testicle, without obvious macroscopic changes. Most of the time, the perceptible variations in reproductive organs may be more of a consequence of hypogonadism than a direct effect of ROS.

16.3.5 ROS-Induced Oxidative Stress in Sperm

Due to the high PUFA content and the low rate of antioxidant enzymes in the cytoplasm, sperm is very susceptible to OS leading to reduced sperm function (Barati et al. 2020). Lipidic

membranes in sperm can produce a large amount of ROS because PUFAs are highly sensitive compounds as the double bonds near the methylene group that weakens H-C bonds with increased likelihood of hydrogen separation (Saleh and Agarwal 2002). These double bonds are susceptible to O_2 peroxidation and create conjugated radicals. Those react rapidly with·ROO forming organic hydroperoxides (ROOH) (Gaschler and Stockwell 2017). H_2O_2 is a powerful promoter of LPO, and it can pass through the membrane of sperm and enter the cytoplasm. It can inhibit G6PD, a regulator enzyme for the entry of glucose with reduction of NADPH. This reduces the activity of GPX enzyme, altering the antioxidant defense of the sperm (Saleh and Agarwal 2002).

Also, during the LPO by ROS, a generation of aldehydes such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and acrolein occurs. These degradation products are very genotoxic and mutagenic and cause ROS production in the mitochondria, higher DNA fragmentation, and apoptosis (Gaschler and Stockwell 2017; Aitken et al. 2012). Damage of DNA also leads to decreased adenosine triphosphate (ATP) and energy availability impeding sperm motility (Wagner et al. 2018).

16.4 Mitochondrial Membrane Potential and Signal Transduction

Mitochondrial primary function is the production of ATP by the respiratory chain to generate energy through oxidative phosphorylation. ATP production is variable depending on the type of tissue, and mitochondria can change in number and structure to meet energy demands. It possesses maternal inherited genome arranged in a circular double molecule that codes for 13 polypeptides. This expression is controlled by nuclear factors that are translocated to the mitochondria (Meinhardt et al. 1999).

Approximately 90% of cellular oxygen is consumed by the mitochondria through oxidative phosphorylation. This pathway produces ROS that are part of normal cellular metabolism, and these can function as second messengers, but when levels are higher than necessary, they can be potentially harmful to cells and their components. The mitochondria are the main ROS generator, converting 1–2% of all oxygen in these products (Orrenius et al. 2007). In mature sperm mitochondria are anchored by the submitochondrial reticulum to the axoneme, and their outer membranes are enclosed in a mitochondrial capsule formed by disulfide bonds between proteins rich in cysteine and proline, including phospholipid hydroperoxide glutathione peroxidase (PHGPx). This structure confers stability but also resistance to hypoosmotic stress (Maiorino et al. 2005). Defects in the mitochondria can cause alterations in sperm function. Genetic defects of mitochondrial DNA can cause alteration in production and motility, among other alterations (St John et al. 2001).

Decreased mitochondrial function in sperm can be assessed by the mitochondrial membrane potential test, also known as the test ⊗¬. This measure is carried out through flow cytometry that allows to discern between high or low level of activity from the mitochondria and may suggest a greater probability of infertility (Troiano et al. 1998). It has been suggested higher OS levels and diminished mitochondrial membrane potential may contribute to cellular death (Marchetti et al. 2002). Decreased mitochondrial membrane potential has also been correlated with sperm DNA fragmentation and ROS production (Wang et al. 2003), while high levels have been correlated with better sperm motility and fertilization capacities (Gallon et al. 2006; Paoli et al. 2011). Mitochondria also participates in calcium (Ca_2^+) signaling for the regulation of various functions of sperm as acrosomal reaction or hyperactivation and can be a reservoir of this ion without its clear utility being known at the time (Publicover et al. 2008). Despite the probable production of proteins in sperm has been found, it is controversial if the synthesis of ribonucleic acid (RNA) and proteins are from mitochondrial or nuclear origin, since these cells are considered to have nonfunctional nuclei and without cytoplasmic transcription machinery. This is unique to all cell types (Diez-Sanchez et al. 2003).

16.5 Plant-Derived Natural Antioxidants and Protective Actions Against ROS Production

Antioxidants have been used for a long time in the treatment of male infertility (Showell et al. 2014). Several formulations alone or combined are reported in literature. There are supplements that have been used for ameliorating OS. Some of them are in food and others from vegetable sources like plants or roots (Smits et al. 2019). It is important to specify that many of these compounds or combinations have been used in trials and practice to improve the parameters of the seminogram in general, since previously there was no accessible and reproducible test to measure their direct effect on REDOX balance (Agarwal et al. 2016b). However, the benefits in live birth rate after the use of antioxidants are still controversial (Smits et al. 2019).

Currently, there are tests such as MiOXSYS® that can measure the static oxidation reduction potential (sORP) and the antioxidant capacity reserve (cORP); these determinations give a complete assessment about the REDOX status of a seminal sample, making the results measurable, reproducible, and comparable between infertile and fertile patients (Agarwal et al. 2016b).

16.5.1 Antioxidant Supplements

Antioxidants with direct scavenging properties actively counteract with free radicals to prevent their reaction, being consumed or chemically transformed, so they must be continuously restored. Antioxidants with indirect action are compounds whose antioxidant effect is not participating as direct ROS scavengers. They can be cofactors for other exogenous antioxidants, increase endogenous antioxidant levels, or even have a faint scavenger action (Dinkova-Kostova and Talalay 2008).

16.5.1.1 Arginine

Arginine is an essential amino acid present in multiple tracts of the human body. Studies on fertility in males demonstrated that it is necessary for spermatogenesis. Polyamines are products of degradation of arginine particularly abundant in seminal fluid and are essential for cell growth and differentiation. It has been reported to play a role in the inflammatory response and is a direct scavenger of ROS. It can be found in relatively high concentrations in seafood, watermelon, nuts, seeds, algae, meats, rice, and soy protein (Wu et al. 2009). In seminal plasma, it may improve the parameters by promoting the synthesis of polyamines and proteins rich in arginine as well as regulation of nitric oxide in sperm motility and capacitation (Balercia et al. 2004a). Studies in Russia and Finland have found that in male subfertility, the combination of L-arginine and pycnogenol can be beneficial for improving seminal parameters. It is suspected that it may increase available nitric oxide since arginine is the direct substrate (Kobori et al. 2015; Stanislavov et al. 2009).

16.5.1.2 Carnitine

Carnitine has long been used for its beneficial effects on the human body (Agarwal and Said 2004). It is found especially in two forms: L-carnitine and acetyl-carnitine. The first one has been used mainly in infertility, and it is a quaternary amine that is highly polar and water-soluble in nature. It functions as a substantial cofactor in the transport of chain fatty acids within the mitochondrial matrix to facilitate the mechanisms of cellular respiration and energy production. The second, on the other hand, modulates the concentrations in the cell of coenzyme A and acetyl coenzyme A. It is synthesized in the liver and can be found in the diet mainly from animal proteins. Elevated levels can be found in the male reproductive system, mainly in the epididymis, which suggests an important function for sperm maturation (Mongioi et al. 2016). In membrane, important oxidative damage can be displayed by arachidonic acid binding to phospholipids, which can be prevented by acetyl-L-carnitine (Agarwal and Said 2004). Low L-carnitine levels might work as an epididymal inflammation marker. An antiapoptotic effect has also been observed due to the inhibition of FAS-FAS ligand and the cas-

pases 3, 7, and 8 (Mongioi et al. 2016). In a recent prospective study, it was found that L-carnitine supplementation alone or in combination with other antioxidants may improve seminal parameters (Lipovac et al. 2016). In 2020, a meta-analysis reported that the combination of L-carnitine and L-acetyl-carnitine vs placebo in seven prospective trials can be effective for men with idiopathic oligoasthenoteratozoospermia (OAT) (Zhang et al. 2020).

16.5.1.3 Carotenoids

These substances are colorful liposoluble pigments found in plants, and more than 600 carotenoids have been reported. β-carotene is a provitamin A and particularly important in metabolism which can scavenge ROS. Lycopene, lutein, and zeaxanthin are also carotenoids but have no conversion to vitamin A. They can be found in leafy green vegetables, fruits, and some vegetable oils. Also, astaxanthin is another carotenoid that can be found in red dye molluscs (Rao and Rao 2007).

Since carotenoids have double bonds that allow them to accept electrons, they act as antioxidants by three different reaction types: electron transfer, hydrogen abstraction, and addition. β-carotene is an excellent chain-breaking antioxidant but less effective at LPO. Zeaxanthin can scavenge both soluble and non-soluble radicals (El-Agamey et al. 2004).

Also, lycopene has been found to be the most powerful oxygen quencher of all carotenoids and has other non-oxidative beneficial effects on testis like gap communication, modulation of gene expression, regulation of cell cycle, and immunoenhancement (Durairajanayagam et al. 2014). Another carotenoid that has been studied for male infertility is astaxanthin. In small studies, it seems to have potential helpful effects on sperm parameters and OS (Comhaire et al. 2005), but another study found no changes between treatment and placebo groups; therefore more research is needed (Kumalic et al. 2020).

16.5.1.4 Coenzyme Q10

This is an endogenously produced fat-soluble antioxidant also known as ubiquinone and is a fundamental part of energy metabolism in the mitochondria. It is found mainly in animal proteins such as meat, fish, as well as nuts and oils (Pravst et al. 2010). In cells, coenzyme Q10 enters a redox cycle to transform from ubiquinone to reduced ubiquinol, which is crucial in the transport of the electron chain in the mitochondria and inhibits peroxidation in the plasma membrane (Littarru and Tiano 2007). It has recently been proposed that cells could have a double stock of coenzyme Q10 production: one that is found in the mitochondrial membrane and another that is found in the Golgi apparatus that may protect the cell membrane from LPO, and, therefore, the exogenous consumption of coenzyme Q10 could give the antioxidant effect against ROS (Santoro 2020).

There are recent studies in literature about the use of coenzyme Q10. Alahmar et al. published a prospective study comparing the use of 200 mg of coenzyme Q10 vs 200μg of selenium and reported a beneficial effect in seminal parameters and antioxidant levels in the coenzyme Q10 group (Alahmar and Sengupta 2021). Along with it, another prospective study compared coenzyme Q10 in different doses (400 mg vs 200 mg) finding better results on sperm quality and antioxidant status in OAT patients with higher dosage (Alahmar 2019b)..

16.5.1.5 Cysteine

L-cysteine is a nutritional semi-essential amino acid. There are three sources for its presence in the body, obtained mainly from food, from L-methionine, and finally from protein breakdown (Yin et al. 2015). N-acetyl-cysteine (NAC) is one of its derivatives and has multiple functions in the body, and its benefits as an antioxidant have been proven. Three NAC antioxidant mechanisms have been proposed: the first as a direct antioxidant, and it tends to be stronger than cysteine, and GSH in optimal concentrations helps to eliminate ROS (Samuni et al. 2013). The second mechanism is indirect, as it is a crucial component in the synthesis of GSH, a powerful antioxidant produced in vivo and a substrate for multiple detoxifying enzymes such as glutathione reductase (GR), glutaredoxin, and GPX,

among others. If GSH levels are low, they can be reversed by NAC supplementation and function as a ROS scavenger. Finally, the last mechanism may be because it is a disulfide bond breaking agent (Aldini et al. 2018).

A prospective study comparing the use of 600 mg of NAC vs control in post-varicocelectomy patients found a greater benefit in the pregnancy rate and chromatin integrity when the supplement was administered (Barekat et al. 2016). Another prospective study in patients with asthenozoospermia 600 mg of NAC was administered. Higher levels of expression of NRF2 mRNA and antioxidant enzymes after treatment were reported. This gene mainly protects against OS by activating the transcription of antioxidant enzymes genes (Jannatifar et al. 2020).

16.5.1.6 Micronutrients (Folate, Selenium, and Zinc)

Folate or folic acid also known as vitamin B9 is a water-soluble vitamin that participates in several cellular mechanisms including DNA synthesis, replication, repair, and methylation, as well the transportation of methyl and formyl groups through the cell. It is present in green leafy vegetables and fruit (Naderi and House 2018). There is evidence that folic acid may have antioxidant activity by scavenging ROS with increase of antioxidant capacity and decrease of MDA levels (Aghamohammadi et al. 2011).

Selenium is a mineral micronutrient that is present in 25 selenoproteins. One of the most important for redox environment is GPX, which acts as a direct ROS scavenger. Selenium can be obtained in the diet by consuming cereals and foods from animal origin, but it has many variations in the level of consumption depending even on the soil where the food is grown. Hence, some populations require the consumption of selenium supplements (Rayman 2012). Magnesium is also an essential element. It can protect DNA against ROS by binding to minor groove of B-DNA. Magnesium is found in many foods. Like selenium, deficient soil for agriculture decreases vegetables and fruit content by 20–30% (de Baaij et al. 2015). Selenium has been considered a very important element for normal spermatogenesis and sperm quality (Boitani and Puglisi 2008).

Zinc (Zn) is another micronutrient that has antioxidant properties. It is a cofactor related to metalloenzymes in DNA transcription and the synthesis of important proteins for spermatogenesis (Ebisch et al. 2007). Zn is considered a ROS scavenger being an important transition metal component of the enzymatic antioxidant defense (Mariani et al. 2008).

16.5.1.7 Vitamin E

Vitamin E is the active metabolite of α-tocopherol and has the main function of being the first defense against membrane damage by OS. This family of vitamins cannot be synthesized in the human body, so it must be consumed in the diet and can be found in vegetable oils (Wang and Quinn 1999). The antioxidant effect of vitamin E has been found to be very complex since it does not have a direct reaction with ROS and requires solvents such as ethanol. It inhibits LPO by capturing two ·ROO molecules with only one **α**-tocopherol molecule. These mechanisms are known as the "hydroperoxide theory"; however, there is evidence that there may be other mechanisms involved that generate a polar paradox where vitamin E is lipophilic but has hydrophilic behavior (Miyazawa et al. 2019).

Vitamin E was used for its antioxidant protective effects in a prospective in vitro study in which samples were collected in patients with asthenoteratozoospermia (AT). Preparations were divided in supplementation with vitamin E and controls, and seminal parameters along with MDA levels were evaluated. Improvement of seminal parameters and lower levels of MDA was observed in the treated group (Ghafarizadeh et al. 2021). In a clinical trial, patients after varicocelectomy were randomized to receive 600 mg of vitamin E or placebo for 12 months. After treatment time, the seminal parameters were found better in the therapy group; however, no statistically significant differences were reported (Ener et al. 2016).

16.5.1.8 Vitamin C

Ascorbic acid is one of the most commonly used vitamins around the world. The human body lacks the enzyme L-gulonolactone oxidase. That is the reason to consume vitamin C in the diet which can be found in fruits and vegetables (Kaźmierczak-Barańska et al. 2020). It has a powerful antioxidant effect since it participates in the process of electron donation and transfer to neutralize OS. It can reduce the amounts of ROS as well as nitrogen and sulfur radicals and regenerating antioxidants such as vitamin E (Caritá et al. 2020). Very high concentrations of ascorbic acid have been observed in seminal plasma up to ten times higher than in blood. Low concentrations have been associated with increased altered sperm morphology and increased DNA damage (Colagar and Marzony 2009).

Ascorbic acid has been used in in vivo and in vitro studies to ameliorate the OS for a long time. Recently, in a prospective study, vitamin C (600μM) was added in the preparation of semen samples to freeze and later thawing was done and evaluated for sperm chromatin/DNA integrity status and acrosome reaction and compared with controls. Results suggest that vitamin C supplementation may attenuate the detrimental effects of vitrification on sperm parameters, chromatin quality, and apoptosis rate (Mangoli et al. 2018).

In a double-blind controlled randomized trial, a group of infertile patients after varicocelectomy were randomly allocated into vitamin C 250 mg intake and placebo for 3 months. An increase in motility and normal morphology was noted, but not in sperm count. The conclusion is that vitamin C is a cheap and easy supplement that may be beneficial for some semen parameters (Cyrus et al. 2015).

16.5.1.9 Myoinositol

Inositol is a sixfold alcohol of cyclohexane that can have nine isoforms, but the most common in nature is myoinositol. It is found in corn, beans, fruits, nuts, and animal sources. It can also be produced in the human body (Vazquez-Levin and Verón 2020). This polyol is a precursor of phosphatidyl-inositol (PI) which is necessary for the regulation of sperm motility processes and acrosomal reaction. Its antioxidant role is likely to increase antioxidant enzymes activities and increase the membrane potential in the mitochondria (Condorelli et al. 2017). PI can be accumulated in the male genital tract by secondary production from SC in response to FSH (Monaco et al. 1988).

In a double-blind controlled trial in patients with idiopathic infertility, improvement of semen parameters and hormonal profilewere noted after 3 months of treatment with myoinositol (2 g) (Calogero et al. 2015). In another recent in vitro study, 2 mg/mL or 20 mg/mL of myoinositol was added to semen samples before and after capacitation. In myoinositol preparations, an improvement in motility and oxygen consumption was found, in addition to a decrease in 8-OHdG levels as a marker of OS-induced damage (Governini et al. 2020).

16.5.1.10 Polyunsaturated Fatty Acids (PUFAs)

PUFAs are essential in the composition of sperm cell membrane and give fluidity needed to participate in fertilization mechanisms; however, these molecules are also vulnerable to ROS attack. These are classified into three groups: omega 3, omega 6 that is necessary to consume in diet, and omega 9 that can be synthesized by animal organisms. They can be found mainly in fish and vegetable oils (Wathes et al. 2007). Omega 3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been used for a considerable time for the management of male infertility. Therefore, their possible antioxidant properties besides their potential role in decreasing ROS-induced sperm damage have been observed. This can be due to mechanisms such as increasing antioxidant enzyme levels significantly like GPX, improving the total antioxidant capacity and also by decreasing LPO in terms of MDA concentration (Heshmati et al. 2019). A meta-analysis including three randomized controlled trials found that the supplementation with EPA and DHA in infertile men enhanced sperm motility and concentration of DHA in seminal plasma (Hosseini et al. 2019).

16.5.1.11 Resveratrol

Resveratrol is a natural polyphenol first found in roots of oriental origin and later found in large

quantities in the vine plant. It can also be found in numerous blackberries, but in the skin of the grape where it has been found in greater concentration (Silva et al. 2019). It has been observed in in vitro experiments that it could have an influence in the improvement of sperm quality, in addition to preventing LPO (Collodel et al. 2011).

For several years, this compound has been used as an antioxidant to assess sperm samples in animal models (Ourique et al. 2013). In an in vitro study, different polyphenols and their effects on seminal parameters were analyzed. Along with resveratrol, genistein and tetrahydroxydiphenyl are the only substances from 16 studies that could have a clinical utility in vivo since their deleterious effects on sperm are low (Aitken et al. 2016).

It is well-known that cryopreservation of semen samples increases ROS production. In an in vitro prospective study, cryopreserved semen samples from fertile and infertile men were exposed to resveratrol at different concentrations (0.1, 1.0, and 10 mMol). The addition of resveratrol showed prevention of LPO in thawed samples of both fertile and infertile men (Garcez et al. 2010). In a prospective study, a group of patients with idiopathic infertility were supplemented with 150 mg of resveratrol. In a 3- and 6-month follow-up, an improvement in sperm count and motility was found (Illiano et al. 2020).

16.5.1.12 Vitamin B12

Vitamin B12 is a water-soluble vitamin, also known as cobalamin. It contains cobalt in the core of its molecular structure. Vitamin B12 is involved in the metabolism of DNA, fatty acids, and amino acids (Banihani 2017). It acts as coenzyme with methionine synthase (MTR), and it is involved with methylation of neurotransmitters, lipids, proteins, amino acids, DNA, and RNA (Banihani 2017). The lack of vitamin B12 and folate is associated with symptoms like fatigue, depression, severe anemia, and memory loss (Torres-Arce et al. 2021). The main causes of deficiency of vitamin B12 are associated with drugs, low consumption, or altered absorption. This vitamin is usually found in meat, fish, and dairy products (Banihani 2017). In the male reproductive system, vitamin B12 alone or in combination with other antioxidants eases spermatogenesis, sperm motility, and lowers DNA fragmentation (Majzoub and Agarwal 2018; Hosseinabadi et al. 2020). Furthermore, in oligospermic rat models, it has been associated with positive histopathological changes in the reproductive system due to an increased diameter of the seminiferous tubules as well as an improvement in sperm count (Banihani 2017). Low concentrations of vitamin B12 lead to hyperhomocysteinemia that may reduce sperm function through hyperhomocysteinemia-induced nitric oxide depletion (Stühlinger et al. 2001).

16.5.1.13 Vitamin D

The benefits of vitamin D supplementation on semen quality have been widely described. Vitamin D deficiency in some populations can be between 50% and 90% (Wadhwa et al. 2020). The main source of vitamin D synthesized in the skin by UV radiation and only a small amount is consumed in foods (Blomberg 2012). As calcium is needed for sperm maturation and is two- or threefold higher in human epididymal and prostatic liquid, vitamin D is hypothesized to be important for calcium transfer (Blomberg Jensen et al. 2011). Its supplementation has been reported to be related to a higher chance of spontaneous pregnancies and live birth rates, elevation in inhibin B, and testosterone concentration (Wadhwa et al. 2020; Blomberg 2012; Blomberg Jensen et al. 2011). However, the real benefit of vitamin D supplementation in terms of sperm concentration and motility is still inconclusive. Since 2006 the vitamin D receptor in sperm was found by immunohistochemistry, and it is located predominantly on the head/nucleus of the sperm and mid-piece (Corbett et al. 2006), and it acts as a versatile signaling molecule rather than being only a regulator of bone health and Ca_2^+ metabolism. Vitamin D is also involved in steroidogenesis and control over testosterone concentrations (Blomberg 2012; Wimalawansa 2019). This discovery enhances the need of a correct supplementation in infertile men (Wadhwa et al. 2020).

Vitamin D's molecular and cellular roles are involved in the control of OS. Nevertheless, except from reports of a positive effect over survival and

integrity of fertile sperm after cryopreservation and an improvement in sperm morphology after vitamin D addition, current information available so far show controversial effects about antioxidant properties of vitamin D (Wimalawansa 2019; Taheri Moghadam et al. 2019)..

16.5.2 Herbal Supplements

Multiple herbal remedies are used in alternative medicine, mainly in traditional Asian medicine is where a large number of herbs are used for male infertility. They all contain compounds such as flavonoids or vitamins that may be responsible for scavenging ROS. Tongkat ali (*Eurycoma longifolia*) is a member of the Simaroubaceae family used in the traditional medicine of Indonesia, Thailand, and Vietnam. It contains various phytochemical compounds like eurypeptide that seem to enhance testosterone serum concentration and stimulate synthesis of various androgens (Tambi et al. 2012).

Balloon vine (*Cardiospermum halicacabum*) is commonly used in Sri Lanka for several therapeutic purposes. In a study in male rats, an increase in sperm count in epididymis and testosterone levels was observed after 30 days of administration. It could have an antioxidant effect due to its flavonoid content (Peiris et al. 2015). Grapevine (*Vitis vinifera*) contains a flavonoid called anthocyanin in considerable quantity, and it has been observed to increase the intracellular level of vitamin C and has a greater scavenger effect than vitamin E (Singh et al. 2004). In a study where the induction of testicular dysfunction was caused by aluminum chloride, grape seed extract induced an increase in sperm count and viability, and motility, as well as protection against DNA damage and nitric oxide production was noted (Hala et al. 2010).

Marjoram (*Origanum majorana*) is a flower from the mint family Lamiaceae and has bioactive compounds such as flavonoids and phenolic glycosides. Ethanol-induced OS has been shown to decrease when marjoram oil and grape seed extracts were administered in combination, as well as an improvement was seen in testosterone levels and recovery of weight loss in testis and epididymis (El-Ashmawy et al. 2007). Clove (*Syzygium aromaticum*), belonging to the the Myrtaceae family is native to Indonesia and is used primarily as a seasoning. In a study with Wistar rats where manganese chloride was administered, a group with application of this herb presented a protective effect against degeneration and decreased sperm count (Boudou et al. 2013). Other herbal components that have been studied in experiments with rats and mice that could reduce OS are black seed (*Nigella sativa*), wolfberry (*Lycium barbarum*), puncture vine (*Tribulus terrestris*), *Hygrophila* (*Asteracantha longifolia*), and Pallipoondu (*Polycarpaea corymbosa*) since they also present increased levels of testosterone and protective effects at the level of testicular microstructure and spermatogenesis (Adewoyin et al. 2017). In fact, one study with 37 different herbs from traditional Chinese medicine showed that many of them can have a different range of antioxidant effects similar to vitamin C when they were applied to the ferric reducing antioxidant potential. 15 of these herbal teas have been reported with a strong antioxidant activity, and the most potent to this was Cortex Phellodendri (Tempest et al. 2008).

16.5.3 The Antioxidant Paradox

However, there are no clear guidelines on the use of antioxidants. Although a beneficial effect of prevention and repair of oxidative damage is expected, unregulated or exaggerated use can lead to a pathological elevation of antioxidants, which causes an imbalance between oxidation and reduction, known as the antioxidant paradox (Lei et al. 2016). In this effect, greater tissue damage and elevated ROS are observed. CAT and excessive SOD can prevent acrosomal reaction and the inhibition of necessary oxidation (Aitken et al. 1995). Hence, the treatment should be careful when it comes to use of antioxidant therapy and assessment of the patient's REDOX status could be made for better clinical management.

Table 16.1. The table shows the most important clinical trials that have used different antioxidants to improve male fertility.

Table 16.1 Articles describing the use of antioxidants and fertility outcomes

Reference	Clinical trial design	Antioxidant formulation dosage and length of treatment	Study population	Reproductive outcomes after antioxidant treatment
Suleiman et al. (1996)	RCT blinded	Vitamin E 300 mg per day for 6 months	Oligoasthenospermic (OA)-(n = 74), azoospermic (n = 38), oligospermic (n = 30) patients High viscosity (n = 22), oligospermic with high viscosity (n = 6), asthenospermic with high viscosity (n = 12), OA with high viscosity (n = 10)	Improved sperm motility. Reduced MDA levels. Higher pregnancy and live birth rates.
Rolf et al. (1999)	RCT blinded	Vitamin C (1000 mg) and vitamin E (800 mg) daily for 56 days	31 asthenozoospermic patients	No changes in semen parameters
Comhaire et al. (2000)	Uncontrolled (open label)	Acetylcysteine (600 mg) or capsules providing a daily amount of β-carotene (30 mg) and α-tocopherol (180 mg)/daily. In addition, capsules containing essential fatty acids for a daily intake of decosahesaenoic acid (1 g) gammalinoleinic acid (0.25 g) and arachidonic acid (0.1 g) for 6 months.	7 idiopathic patients 11 varicocele patients History of cryptorchidism (n = 2), patients with male accessory gland infection (n = 7), immunological infertility (n = 4) and endocrine cause (n = 1)	Improved sperm concentration and acrosome reaction. Reduced ROS and 8-OH-dG levels.
Vicari and Calogero (2001)	Uncontrolled (open label)	Carnitine twice/day for 3 months followed by a treatment-free period for 3 months	54 OAT patients with prostato-vesiculo-epididymitis	Improved sperm progressive motility and viability. Reduced ROS production. Higher pregnancy rate.
Gupta and Kumar (2002)	Uncontrolled (open label)	Lycopene 4 mg daily for 3 months	30 idiopathic patients	Improved sperm concentration and motility. Higher pregnancy rate.
Keskes-Ammar et al. (2003)	RCT unblinded	Vitamin E 400 mg or selenium 225 mcg for 3 months	54 infertile men	Improved sperm motility. Reduced MDA levels.
Balercia et al. (2004b)	Uncontrolled (open label)	CoQ10 400 mg/daily for 6 months	22 asthenozoospermic patients	Improved progressive motility after treatment, which reduced after 6 months of wash out. Pregnancy rate = 2.4% with 3 of 22 patients achieving spontaneous pregnancy.

(continued)

Table 16.1 (continued)

Reference	Clinical trial design	Antioxidant formulation dosage and length of treatment	Study population	Reproductive outcomes after antioxidant treatment
Balercia et al. (2005)	RCT blinded	(a) L-carnitine (3 g) daily (b) L-acetyl-carnitine (3 g) daily (c) L-carnitine (2 g) + L-acetyl-carnitine (1 g) daily (d) Placebo	60 idiopathic patients	Improved sperm motility, total oxyradical scavenging capacity of semen
Greco et al. (2005a)	Uncontrolled (open label)	Vitamin C (1 g) + vitamin E (1 g) daily for 2 months	Oligo-terato (n = 6), OAT (n = 26) patients, 6 unexplained infertile men	Improved semen parameters and SDF. No change in fertilization and cleavage rates. Higher implantation and pregnancy rates.
Greco et al. (2005b)	Uncontrolled (open label)	Vitamin C (1 g) and vitamin E (1 g) daily for 2 months	64 unexplained infertile men	No difference in semen parameters.. Reduced SDF.
Tremellen et al. (2007)	RCT blinded	Menevit 1 capsule per day for 3 months	Not clearly reported. Men with OS stress in semen and SDF >25% by TUNEL.	No differences between treated and placebo for fertilization, implantation, pregnancy and miscarriage rates. Live pregnancy rate higher in treated patients.
Ménézo et al. (2007)	Uncontrolled (open label)	Vitamins C and E (400 mg each), B-carotene (18 mg) zinc (500 mmol), selenium (1 mmol) for 3 months	58 patients with 2 previous failures of IVF or ICSI, and DFI and chromatin decondensation >15%	Reduced SDF but higher sperm decondensation
Ahmad et al. (2008)	Uncontrolled (open label)	Mucuna pruriens seed powder 5 g/daily for 3 months	60 infertile men	Improved volume, sperm concentration, count, motility. Reduced MDA levels.
Piomboni et al. (2008)	Uncontrolled (open label)	Fattore M two tablets/day for 3 months	51 AT patients	Improved semen parameters and leukocytospermia. Reduced SDF
Heidary et al. (2008)	Uncontrolled (open label)	Saffron 50 mg 3 times weekly for 3 months	52 idiopathic patients	Improved normal morphology, total and progressive motility
Tunc et al. (2009)	Uncontrolled (open label)	Menevit 1 capsule per day for 3 months for maximum of 3 months	50 infertile men with high OS	No difference in semen parameters. Reduced SDF, ROS and apoptotic markers. Improved DNA protamination.

(continued)

Table 16.1 (continued)

Reference	Clinical trial design	Antioxidant formulation dosage and length of treatment	Study population	Reproductive outcomes after antioxidant treatment
Ciftci et al. (2009)	RCT unblinded	N-acetyl-cysteine 600 mg daily for 3 months	120 idiopathic patients	Improved volume, motility, semen viscosity, semen and serum OS (TAC, total peroxide, OS index)
Shukla et al. (2010)	Uncontrolled (open label)	Mucuna pruriens seed powder 5 g per day for 3 months	120 infertile men	Improved sperm count and motility, seminal plasma lipid, peroxide levels, SOD, CAT, GSH and ascorbic acid
Ghanem et al. (2010)	RCT blinded	Clomiphene citrate (25 mg / day) and vitamin E 400 mg/ day for 6 months	60 OAT patients	Increased sperm concentration and motility. Higher pregnancy rate.
Ahmad et al. (2010)	Uncontrolled (open label)	*Withania somnifera* 5 g/daily for 3 months	Oligo ($n = 25$), astheno- ($n = 25$) and normozoospermic ($n = 25$) patients	Improved sperm count and motility, SOD, CAT and GSH levels
Nadjarzadeh et al. (2011)	RCT blinded	CoQ10 capsules 200 mg/ daily for 3 months	60 OAT patients	No changes in semen parameters. Reduced MDA and improved TAC.
Shukla et al. (2011)	Uncontrolled (open label)	*Withania somnifera* 5 g/daily for 3 months	Oligo ($n = 25$), astheno- ($n = 25$) and normozoospermic ($n = 25$) patients	Decreased intracellular ROS and apoptosis. Increased levels of Cu^{2+}, Zn^{2+}, Fe^{2+} and Au^{2+}
Safarinejad (2011)	RCT blinded	Eicosapentaenoic (1.12 g) and docosahexaenoic daily for 8 months	211 OAT patients	Improved total sperm count, concentration, motility, normal morphology, seminal SOD and CAT
Moslemi and Tavanbakhsh (2011)	Uncontrolled open label	Selenium (200 mg), vitamin E (400 UI) daily for 100 days	690 AT patients	Improved semen parameters. Higher spontaneous pregnancy.
Safarinejad et al. (2011)	RCT blinded	*Crocus sativus* 60 mg/daily for 26 weeks	260 OAT patients	No changes in semen parameters, SOD and CAT like activity, LH, FSH, PRL, TSH or testicular volume
Safarinejad (2012)	Uncontrolled (open label)	CoQ10 300 mg daily for 12 months	287 OAT patients	Improved semen parameters. No change in pregnancy and miscarriage rates.
Safarinejad et al. (2012)	RCT blinded	CoQ10 200 mg daily for 26 weeks, followed by a treatment-free period of 12 week	228 unexplained infertile men	Improved semen parameters. Seminal CAT and SOD
da Silva et al. (2013)	RCT blinded	Folic acid 5 mg per day for 3 months	70 infertile men	No differences in semen parameters

(continued)

Table 16.1 (continued)

Reference	Clinical trial design	Antioxidant formulation dosage and length of treatment	Study population	Reproductive outcomes after antioxidant treatment
Abad et al. (2013)	Uncontrolled (open label)	Androferti (Laboratorios Q Pharma, Alicante, Spain) one capsule daily for 3 months	20 AT patients	Improved sperm concentration, motility, vitality, morphology, DNA integrity Pregnancy rate = 5%
Ajayi et al. (2013)	Uncontrolled (open label)	Vitamin C (200 mg), Vitamin E (200 mg), folic acid (1 mg), zinc (50 mg), selenium (200 mcg), n-acetyl-L-cysteine (100 mg, L-carnitine (600 mg), glutathione red (100 mg), CoQ10 (30 mg)/daily for at least 2 months	Oligo (n = 20), astheno (n = 33), OAT (n = 42) patients 65 healthy men	Improved semen parameters
Khani et al. (2013)	Uncontrolled (open label)	Sesame 0.5 mg/kg body weight for 3 months	25 unexplained infertile men	Improved sperm concentration, motility. Pregnancy: 3 out of 25 patients. Live birth rate: 3 out of 25 patients.
Bejarano et al. (2014)	Uncontrolled (open label)	Melatonin 6 mg per day for 45 days	30 infertile men	Improved semen parameters, urinary and semen TAC. Reduced SDF. Improved embryo quality.
Nadjarzadeh et al. (2014)	RCT blinded	CoQ10 200 mg/daily for 3 months	60 OAT patients	No changes in semen parameters. Increased seminal levels of CoQ10, CAT, and SOD activity. Reduced level of seminal plasma 8-iso-prostane.
Raigani et al. (2014)	RCT blinded	Folic acid (5 mg) and zinc sulfate (220 mg)/ daily for 4 months	83 OAT patients	No difference in semen parameters. Increased sperm chromatin integrity.
Kobori et al. (2014)	Uncontrolled (open label)	CoQ10 (120 mg), vitamin C (80 mg), vitamin E (40 mg)/ daily for 6 months	169 OAT patients	Improved sperm concentration and motility. 48 (28.4%) pregnancies achieved, of those, 16 were spontaneous and 32 by ART.
Thakur et al. (2015)	Uncontrolled (open label)	Ubiquinol 150 mg daily for 6 months	60 OAT patients	Improved sperm concentration, total and progressive motility. Testosterone unchanged.

(continued)

Table 16.1 (continued)

Reference	Clinical trial design	Antioxidant formulation dosage and length of treatment	Study population	Reproductive outcomes after antioxidant treatment
Hadwan et al. (2015)	Uncontrolled (open label)	Zinc sulfate 440 mg daily for 3 months	60 AT patients 60 healthy men	Improved volume, progressive motility, total sperm count and CAT activity
Martinez et al. (2015)	RCT blinded	Resveratrol 25 mg daily SG 1002 750 mg daily for 75 days	54 OA patients	Improved sperm concentration and motility
Gvozdjáková et al. (2015)	Uncontrolled (open label)	Carni-Q-Nol 2 softsules for the first 3 months, 3 softsules for the last 3 months	40 OA patients	Improved sperm concentration. Reduced concentrations of α-tocopherol and γ-tocopherol in seminal fluid as well TBARS. Pregnancy in 45% of couples.
ElSheikh et al. (2015)	RCT unblinded	(a) Vitamin E (400 mg) daily for 3 months (b) Clomiphene citrate (25 mg) daily for 3 months (c) Vitamin E + Clomiphene citrate daily for 3 months	90 OA patients	Improved sperm concentration in groups b and c, while total sperm motility improved in all groups
Haghighian et al. (2015)	RCT blinded	a-Lipoic acid 600 mg daily for 3 months	44 idiopathic patients	Improved sperm concentration and motility. TAC reduced MDA levels
Martínez-Soto et al. (2016)	Uncontrolled (open label)	1.5 g capsules of docosahexaenoic acid oil daily for 10 weeks	57 infertile men	No changes in semen parameters Increased seminal TAC, reduced DNA damage
Chattopadhyay et al. (2016)	Uncontrolled (open label)	L-carnitine, acetyl-L-carnitine, CoQ10, lycopene, zinc, folic acid, vitamin B12, selenium, fructose and citric acid (dosage no reported) for 6 months	115 infertile men	Increased sperm count, motility and TAC Reduced ROS levels
Hosseini et al. (2016)	RCT blinded	Ginger powder 250 mg/daily for 3 months	100 patients with SDF ε 15%	No differences in semen parameters Decreased SDF
Montanino Oliva et al. (2016)	Uncontrolled (open label)	Andrositol two capsules daily for 3 months	45 AT patients	Improved concentration, motility, and normal morphology
Singh et al. (2016)	Uncontrolled (open label)	Fertisure M 1 tablet twice per day for 3 months	7 oligozoospermic patients 31 OA patients 2 OAT patients	Improved sperm count, motility and GSH level Reduced MDA level

(continued)

Table 16.1 (continued)

Reference	Clinical trial design	Antioxidant formulation dosage and length of treatment	Study population	Reproductive outcomes after antioxidant treatment
Magdi et al. (2017)	Uncontrolled (open label)	Vitamin C (1 g), vitamin E (400 mg) and L-carnitine (2 g)/daily for 6 months	210 OAT patients	Improved sperm count, total and progressive motility, normal morphology after treatment
Soleimani and Masoumi (2017)	Uncontrolled (open label)	Grape seed extract 600 mg daily for 3 months	29 idiopathic patients	Increased catalase and reduced MDA
Negri et al. (2017)	Uncontrolled (open label)	Fertiplus SOD No component specified and length of treatment reported	55 idiopathic patients	No changes in semen parameters and SDF
Stenqvist et al. (2018)	RCT blinded	Vitamin C (30 mg), vitamin E (5 mg), vitamin B12 (0.5 mcg), L-carnitine (750 mg), coenzyme q10 (10 mg), folic acid (100 mcg), zinc (5 mg), selenium (25 mcg) with maltodextrin, calcium, carbonate, citric acid, steviol glycoside, flavors, B-carotene, silicone dioxide daily for 6 months	77 patients with SDF ε 25%	Improved sperm concentration, no change in DNA damage
Alizadeh et al. (2018)	RCT blinded	Curcumin 80 mg/daily for 10 weeks	60 infertile men	Increased sperm count, concentration, total motility, vitality, and TAC Reduced MDA and inflammatory biomarkers
Alsalman et al. (2018)	Uncontrolled (open label)	Zinc 220 mg daily for 3 months	60 AT patients	Improved volume, progressive motility, normal morphology, total thiol concentration, total disulfide linkage concentration, GPX levels
Busetto et al. (2018)	RCT blinded	Proxeed Plus 2 sachets/daily for 6 months	104 patients with semen abnormalities (of those, 52 with varicocele)	Increased semen parameters, except sperm morphology. 10 spontaneous pregnancies in treated couples vs 2 in placebo
Lu et al. (2018)	RCT blinded	Melatonin 400 mg/daily for 3 months	54 oligozoospermic patients	Improved semen parameters Improved TAC
Salehi et al. (2019)	Uncontrolled (open label)	Vitamin E (50 mg), vitamin C (500 mg) and CoQ10 (100 mg) daily for 3 months	485 infertile men with DFI >27% by SCSA	Improved semen parameters Reduced DNA damage Pregnancy rate = 16.8%

(continued)

Table 16.1 (continued)

Reference	Clinical trial design	Antioxidant formulation dosage and length of treatment	Study population	Reproductive outcomes after antioxidant treatment
Hasoon (2019)	Uncontrolled (open label)	L-arginine (1 g) and CoQ10 (200 mg) daily for 8 months	24 infertile men	Improved volume, sperm count, motility, and normal morphology
Jannatifar et al. (2019)	Uncontrolled (open label)	N-acetyl-cysteine 600 mg daily for 3 months	50 AT patients	Improved volume, sperm concentration, total and progressive motility, normal morphology Reduced MDA, SDF, and protamine deficiency. Improved TAC
Gambera et al. (2019)	Uncontrolled (open label)	Arginine (3 g), CoQ10 (200 mg), vitamin C (240 mg), vitamin B3 (27 mg), *Tribulus terrestris* (60 mg), ginseng (12 mg), inositol (100 mg) vitamin E (36 mg) for 2 months	32 OAT patients	Improved semen concentration, sperm count, progressive motility, normal morphology and vitality after therapy Oxisperm: reduced seminal OS after therapy Unclear capacitation check
Micic et al. (2019)	RCT blinded	Proxeed Plus 2 sachets for 6 months	175 OA patients	Improved semen parameters Increased seminal carnitine and α-glucosidase activity Reduced SDF
Nouri et al. (2019)	RCT blinded	Lycopene 25 mg daily for 3 months	44 oligozoospermic patients	Improved volume, total sperm count, concentration, total motility, and TAC
Nurmawati et al. (2020)	RCT blinded	Astaxanthin 8 mg/daily for 1 month	25 infertile men	Improved sperm concentration, motility, and morphology Reduced MDA and 8-OHdG levels
Hadi et al. (2020)	Uncontrolled (Open label)	L-carnitine 2 g/daily for 3 months	58 infertile men	Improved sperm count, total motility, and normal morphology Serum: reduced FSH and LH levels, increased testosterone and inhibin levels
Busetto et al. (2020)	RCT blinded	Proxeed plus daily for 6 months	104 patients with altered semen quality. Of those 52 showed grades I–III varicoceles	Improved total sperm count, total and progressive motility Higher pregnancy rate

(continued)

Table 16.1 (continued)

Reference	Clinical trial design	Antioxidant formulation dosage and length of treatment	Study population	Reproductive outcomes after antioxidant treatment
Alahmar et al. (2020)	Uncontrolled (open label)	CoQ10 200 mg daily for 3 months	65 OA patients	Improved sperm concentration, progressive and total motility, CoQ10 level, TAC and GPx Reduced ROS levels and SDF
Terai et al. (2020)	RCT blinded	(a) L-carnitine (750 mg), zinc (30 mg), astaxanthin (16.05 mg), CoQ10 (90.26 mg), vitamin C (1 g), vitamin B12 (60.1 mcg), vitamin E (150 mg)/per day (b) Hochu-ekki-to (dosage no reported)	31 OA patients	Increased total motile sperm count after treatment in group A
Steiner et al. (2020)	RCT blinded	Vitamin C (500 mg), vitamin E (400 mg), selenium (0.20 mg), L-carnitine (1 g), zinc (20 mg), folic acid (1 g), lycopene (10 mg), and vitamin D (2,000 UI)/daily for a maximum of 6 months	174 oligozoospermic patients	Improved sperm concentration. No change in SDF No change in pregnancy and live birth rates
Alkumait et al. (2020)	RCT unblinded	(a) Glutathione (250 mg sachets) for 6 months (b) CoQ10 (200 mg) sachets for 6 months	51 OAT patients	Improved semen parameters
Kopets et al. (2020)	RCT blinded	L-carnitine/L-acetyl-carnitine (1990 mg), L-arginine (250 mg), glutathione (100 mg), CoQ10 (40 mg), zinc (7.5 mg) vitamin B9 (234 mcg), vitamin B12 (2 mcg) selenium (50 mcg)/ daily for 6 months	83 idiopathic patients	Increased % of normozoospermia in treated patients after 2 and 4 months in comparison with placebo Higher pregnancy rate
Arafa et al. (2020)	Uncontrolled open label	FH PRO for men twice a day for 3 months	119 idiopathic patients 29 unexplained infertile men	Improved progressive motility and seminal oxidation reduction potential Reduced SDF

Adapted from Agarwal et al. (2021)

Combined Supplements info

1. MENEVIT (Bayer, Sydney, Australia) ascorbic acid (100 mg), zinc (25 mg), d-alpha-tocopherol (E) 400 IU, folic acid (500 mcg), lycopene (6 mg), garlic oil (333 mcg), and selenium (26 mcg)
2. Fattore M 2 (Progine, Florence, Italy). Papaya fermata (100 mg), vitamin C (60 mg), beta-glucan (40 mg), zinc (15 mg), vitamin E (10 mg), and vitamin D (20 mcg)
3. ANDROFERTI (Laboratorios Q Pharma, Alicante, Spain). Vitamin C (60 mg), vitamin E (10 mg), zinc (10 mg), folic acid (200 mcg), selenium (50 mcg), vitamin B12 1 (mcg), L-carnitine tartrate+9 (2.2 g), Citric Acid (1.1 g), calcium carbonate (500 mg), and coenzyme Q10 (20 mg)
4. SG1002 (Nuevas Alternativas Naturales Thermafat S.A. de C.V, Monterrey, Mexico) hydrogen sulfide
5. CARNI-Q-NOL (Tishcon Corp. Westbury, NY, USA) vitamin C (12 mg), vitamin E (75 IU), and CoQ10 (30 mg)
6. Andrositol (Lo.Li. Pharma s.r.l. Rome, Italy) myoinositol (1000 mg), selenium (55 mcg), N-acetyl-cysteine (600 mg), vitamin E (30 mg), L-carnitine (30 mg), L-arginine (30 mg), and folic acid (200 mcg)
7. Fertisure M (Sun Pharma) L-carnitine (340 mg), ubidecarenone BP (50 mg), zinc (5 mg), lycopene (2.5 mg), and astaxanthin (8 mg)
8. Proxeed Plus (Sigma-Tau HealthScience, Utrecht, The Netherlands) L-carnitine (1.725 g), L-acetyl-carnitine (0.5 g), citric acid (50 mg), selenium (50 mcg), CoQ10 (20 mg), vitamin C (90 mg), zinc (10 mg), folic acid (200 mcg), and vitamin B12 (1.5 mcg)
9. FH PRO for Men (Fairhaven Health LLC, Bellingham, WA, USA) beta-carotene (5000 UI), vitamin C (120 mg), vitamin D3 (1200 IU), vitamin E (200 IU), vitamin B12 (1000 mcg), zinc (30 mg), selenium (140 mcg), L-carnitine (2000 mg), L-arginine (350 mg), CoQ10 (200 mg), grape seed extract (20 mg), lycopene (10 mg), and other vitamins and minerals

16.6 Conclusion and Future Perspectives

OS is already known to be an important cause of male infertility. It can be primarily due to lack of consumption of antioxidant substances or secondary to pathologies such as infection, inflammation, exposure to toxins or heat, and varicocele, among others. A balanced redox environment is required to have a normal testicular function in hormonal axis and sperm production, as well for an optimal epididymal function for the maturation of the sperm and finally sperm that have adequate capacities for the fertilization processes. Therefore, an antioxidant treatment can be a fundamental piece in the management of patients with infertility who have a pathology that increases OS or when there is an infertility of unknown origin that may fall within the criteria for the diagnosis of MOSI. Therefore, a complete assessment of the male must be performed to discern between these situations and give a comprehensive treatment.

There are many antioxidants under investigation and in the market for this purpose. However, there is no clear guide as to which is the most recommended for any specific alteration of the seminal parameters. Monotherapy or polytherapy is frequently found in literature without offering a clear superiority in its usefulness. Many studies, as already mentioned, have low statistical quality since controlled randomized studies are expensive and require a considerably high number of subjects to treat. This occurs due to different factors such as the heterogeneity of pathologies, different result variables in studies such as seminal parameters, OS measured by different tests, and even fertility results such as pregnancy or live birth rate. For these reasons, it is very difficult to have studies that are reliable and comparable to each other. The review of the bibliography indicates that any of the different antioxidants can be used by prioritizing the probable deficiencies of a patient by a certain substance, which can occur due to lack of consumption or inappropriate general habits, as well as observing the adverse effects that occur during treatment, with this looking to personalize the management of each patient.

In the future, there should be clear objectives in the research of antioxidant therapy. The determination of the most suitable compounds for better fertility outcomes should be a topic for research. There should also be a consensus regarding the use of a single antioxidant or several in combination. The statistical quality of the studies should also be improved to have stronger recommendations. It would be ideal to standardize OS measuring techniques since results are not easily reproducible and are difficult to compare. This could be crucial in determining which patients are candidates and which have the best response with proper monitoring. We still do not have a statement that clearly satisfies the questions: Who should take antioxidants? How long should they take them? Which antioxidant is better? However, everyday researchers involved in this area are developing new technologies to have the answers.

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Reductive Stress and Male Infertility

17

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Abstract

Male infertility research and clinical advances had vast progress in the last few decades. Strong research evidence underpinned the concepts of oxidative stress (OS)-mediated male reproductive disruptions, which bear answers to several cases of idiopathic male infertility. Antioxidant treatment held the prime solution for OS-mediated male infertility. But excess use of antioxidants is challenged by the research breakthrough that reductive stress also predisposes to male infertility, resolutely instituting that any biological extremes of the redox spectrum are deleterious to male fertility. Superfluity of reducing agents may hinder essential oxidation mechanisms, affecting physiological homeostasis. These mechanisms need to be explicated and updated time and again to identify the fine thread between OS-mediated male infertility treatment and induction of reductive stress. This chapter thus presents the evidence-based concepts pertaining to the antioxidants actions to combat OS-induced male infertility, the mechanism of induction of reductive stress and its impact on male reproduction.

Keywords

Antioxidants · Male infertility · Oxidative stress · Reductive stress

17.1 Introduction

Human infertility has been identified as a disease by the World Health Organization (WHO), and it afflicts about of 7–15% of the world population (Louis et al. 2013; Datta et al. 2016). Male factor reportedly is the only or the prime cause of infertility overall 20–30% of infertility cases (Agarwal et al. 2015; Vander Borght and Wyns 2018). This justifies the strengthened research focus on male infertility over the past few decades, surfacing new concepts regarding the causes, mechanisms, treatment, and management of male infertility (Sheweita et al. 2005; Dutta et al. 2019). However, there are still

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gaps in knowing the exact etiology of male infertility and a huge proportion of male infertility cases remain idiopathic (Aktan et al. 2013). Substantial research has underpinned the concepts of oxidative stress (OS) being the underlying mechanism for most of the idiopathic male infertility cases (Alahmar et al. 2021a; Alahmar and Sengupta 2021). Excess reactive oxygen species (ROS) can override the endogenous antioxidant capacities, approaching pathological levels and disrupting normal male reproductive functions. While excess ROS levels have deleterious impact on male fertility (Agarwal and Sengupta 2020), physiological level of ROS are required for the sperm to execute natural functions. For example, nitric oxide and hydrogen peroxide are required molecules for capacitation, which allows for the acrosome process to take place, which is regulated by ROS (Dutta et al. 2020; Dutta and Sengupta 2021). Furthermore, ROS mediates sperm hyperactivation or contact with the oocyte, making it necessary for them to fertilize the egg (Dutta et al. 2020). Normal sperm activity necessitates a proper balance of oxidant and antioxidant systems.

Antioxidant treatment is the prime regimen for OS-mediated male infertility (Sheweita et al. 2005; Poljsak and Šuput 2013). Nevertheless, excessive use of antioxidants faced a major blow by the revelation that such overuse of antioxidants, "over-the-counter" consumption of antioxidant supplements, or even paucities in antioxidant efficacies are assumed to cause adverse consequences by shifting the endogenous redox balance to the reductive end of the spectrum (Pérez-Torres et al. 2017). Thus, any biological extremes of the redox spectrum, reductive stress, and OS are both deleterious to male fertility. These concepts are yet to be fully revealed and are subjected to be updated time and again to facilitate rapid advances in understanding of reductive stress-mediated male infertility. The present chapter aims to elucidate the concepts of antioxidants used to combat OS-induced male infertility, the evidence-based mechanism of reductive stress induction, and its effects on male reproduction.

17.2 Oxidative Stress: Oxidants and Reductants (Antioxidants)

Oxidative stress (OS) acts as a central key mechanism in the etiopathogenesis of most the human diseases, including aging, cancer, and even reproductive disorders. Antioxidant treatment has attracted a lot of interest in research lately, owing to a rapidly aging population. Antioxidants are appealing because they are regarded "natural" and hence "good" compounds, and they are linked to a healthy diet. Aggressive marketing initiatives of antioxidative products in a multibillion-dollar business support this widespread notion. Reduction of OS is projected as the way to avoid ailments including cancer and cardiovascular pathologies (Halliwell 1999; Willcox et al. 2004). Given that these components may without a prescription be bought over the counter and even foods are supplemented by these supplements, it is essential to have no hazardous effects. While the original antioxidant supplement research revealed that these supplements are effective in disease prevention, more current clinical trials and the usefulness of such therapies were questioned. Antioxidant supplementation for any physiological ailment is not under the regulation of the US Food and Drug Administration (FDA). Moreover, current consensus guideline by the European Society for Human Reproduction and Embryology (ESHRE) has put forth that there is presently insufficient report to underpin the use of antioxidants supplementation (Barratt et al. 2017). Excessive supplementation has been shown in several trials to be harmful (Bjelakovic et al. 2007, 2004; Stanner et al. 2004). Later studies have backed up these concerns (Bjelakovic et al. 2012). Antioxidant therapies have also been shown to boost male fertility in numerous research (Alahmar and Sengupta 2021; Busetto et al. 2018; Sengupta et al. 2018; Torres-Arce et al. 2021; Izuka et al. 2020). Furthermore, excessively high amounts of antioxidants have been shown to be teratogenic to embryos (Wang and Rogers 2007). Current attention of reproductive medicine research has thus been intensive on the use of antioxidants for male infertility therapy.

Reactive oxygen species (ROS) are produced by oxygen metabolism and include the superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl ($\cdot OH$) radical whose outer orbit has unpaired electrons, rendering them unstable and highly reactive (Greabu et al. 2008). During aerobic metabolism, ROS are produced via mitochondrial oxidative phosphorylation, and about 1–5% of it leaks out during the process (Dutta et al. 2020; Bioveris and Chance 1973; Halliwell 2011; Hayyan et al. 2016; Turrens 2003) and secreted as cytotoxic cellular metabolic by-products (Agarwal and Sengupta 2020; Raha and Robinson 2000). ROS may damage numerous biological components, including proteins, lipids, and nucleic acids, resulting in oxidations of lipids and proteins, and DNA damage, due to their super-reactivity and half-life periods in the nanosecond range (Halliwell and Gutteridge 2015). Different pathologies like aging, neurological disorders, cancer, and infertility can be caused due the overproduction of ROS (Agarwal et al. 2003; John Aitken et al. 1989; Li et al. 2013). Although ROS have a negative impact on cellular processes at greater concentrations, they are also required for cellular growth, signalling, immunological response to infections and inflammation, maturation of sperm, capacitation, and embryonic morphogenesis (Dutta et al. 2020; Dröge 2002; Hampton et al. 1998; O'Flaherty 2015; Sengupta et al. 2020). Superoxide scavenging through superoxide dismutase (SOD) catalysis, however, blocked capacitation-related tyrosine phosphorylation in human sperm, which basically makes them incapable of fertilizing oocytes (Cn et al. 2005). Cells normally use multiple intrinsic and extrinsic antioxidant pathways to scavenge excessive ROS. Enzymatic antioxidants like SOD, catalase, and thiol peroxidases, as well as nonenzymatic antioxidants like glutathione, are endogenous antioxidants. Exogenous antioxidants, on the other hand, are micronutrients like vitamins A, C, and E, coenzyme Q10, L-carnitine, and trace elements like selenium and zinc (Halliwell and Gutteridge 2015), which must be supplemented to the body exogenously to preserve a healthy redox balance in every living cell (Valko et al. 2007). Obesity, caused by unhealthy lifestyle may result in a systemic inflammation and increased generation of inflammatory cytokines and result in OS (Kahn and Brannigan 2017; Tsatsanis et al. 2015; Bhattacharya et al. 2020). Furthermore, OS is caused by alcohol consumption, smoking, exposure to radiation, and occupational or environmental toxins (Mathur et al. 2011; Sharma et al. 2013).

17.3 Antioxidant Overdose and Male Fertility: The Concept of "AntioxidantParadox"

Antioxidant supplements are commonly prescribed by physicians to treat OS-related disorders, including male infertility. Antioxidants, by definition, are substances that prevent oxidation by donating electron, in contrast to oxidants which are electron acceptors (Atta et al. 2017). The body's redox potential must be balanced in order for homeostasis to be maintained. ROS, free radical productions, and antioxidant levels must be kept in balance. To accomplish this, human system must absorb antioxidants through nutrition, or antioxidant supplements should be given if this is not adequate owing to exposure to high amounts of oxidants. One must thus differentiate dietary antioxidants from antioxidant supplements, which are strongly publicized and widely accessible over the counter. In several cases patients consume excessive over-the-counter antioxidants since these substances are frequently marketed as health boosters since they may "fight" pathological ailments. Exogenous supplements generally have high quantities of a few refined antioxidants including vitamins A, C, and E, as well as lycopene. In addition, many typical food products already contain several vitamins and antioxidants. As a result, it is likely that patients will unintentionally take very high dosages, or perhaps excessively high dosages, of antioxidants (Poljsak and Šuput 2013). Antioxidant supplementation, on the other hand, has not consistently had positive results. Furthermore, some research have even

documented the negative consequences of antioxidant use. For instance, vitamin E has been demonstrated to increase all-cause mortality in individuals at larger dosages (Miller III et al. 2005). Additionally, it is well-known that the vitamin A supplementation has no anticancer impact, while the opposite impact has been noticed in smokers (Alpha-Tocopherol Beta Carotene Cancer Prevention Study Group 1994). Increased OS and DNA fragmentation have linked to low vitamin C consumption (Fraga et al. 1991). High ascorbate concentrations, on the other hand, are reported to cause the same effects by producing OS (Aruoma et al. 1991). Halliwell (2000) coined this unorthodox impacts of antioxidants as the "antioxidant paradox" (Halliwell 2000). In essence, these paradoxical results underscore the need of ROS formation and the scavenging actions of antioxidants for proper physiological functions, including regulation of male fertility. Therefore, to maintain this "homeostasis," a delicate cellular redox balance is required (De Lamirande and Gagnon 1995; Kothari et al. 2010).

17.4 Antioxidant Paradox: Generation of "Reductive Stress"

Following the above discussion, it can be perceived that uncontrolled, unphysiologically high exposure to antioxidants disturbs the cellular redox balance resulting in "reductive stress" (RS). The term "reductive stress" refers to a shift in the body's redox balance in the reductive direction (Wendel 1987), a situation that has been compared to OS in terms of its harmful effects (Castagne et al. 1999).

The GSH/GSSG and/or NAD/NADH+ ratio elevation or overexpression of antioxidant enzymes can reduce all ROS in the presence of excessive reducing equivalents, and that may cause a H_2O_2 surplus and spillage from mitochondria, propelling the cells to RS (Bjørklund and Chirumbolo 2017) (Fig. 17.1).

Pathological mitochondrial oxidation, breakdown of mitochondrial homeostasis, and protein misfolding in the endoplasmic reticulum may result from NADH excess (Zhang et al. 2012). Mitochondrial ROS and their RS-mediated diminution have crucial role in proper protein folding process as well as in the disulfide bonds formation, which are among the key determinants of normal proteins structure and functions (Murphy 2009). When mitochondrial oxidant generation is suppressed, the amounts of cellular disulfide bonds drop dramatically in many cells (Yang et al. 2007). RS disrupts disulfide bond formation and triggers the "unfolded protein response of the endoplasmic reticulum" (UPR^{ER}) (Walter and Ron 2011). To recover proteostasis in this compartment, the proper folding of proteins must be restored (Walter and Ron 2011). Chronic RS can also cause OS, which, in turn, drives RS via a feedback regulation. When electron acceptors are predicted to be largely reduced during RS, for example, several redox proteins can transfer electrons to O_2, boosting ROS generation (Korge et al. 2015). Thus, this is double-edged sword. Excessive reducing equivalents hinders cellular growth responses, diminish mitochondrial function, affect disulfide bonds formation in proteins, and reduce cellular metabolism (Pérez-Torres et al. 2017). The notion of RS has previously been accepted in various other medical fields to explain pathological ailments including carcinogenesis, cardiomyopathy, cerebral microvasculature, malfunction of blood-brain barrier, and neurodegenerative diseases (Klein et al. 2011; Brewer et al. 2013; Mentor and Fisher 2017). Male fertility is also not an exception.

17.5 Reductive Stress, Antioxidant Paradox, and Male Fertility

To reduce the consequences of OS, physicians have begun treating patients with an excessive antioxidants for various causes for infertility (Greco et al. 2005; Abad et al. 2013). In this context many groups have demonstrated a considerable good impact on sperm parameters, with sperm DNA damage, chromatin packing, with additional intakes of antioxidants including

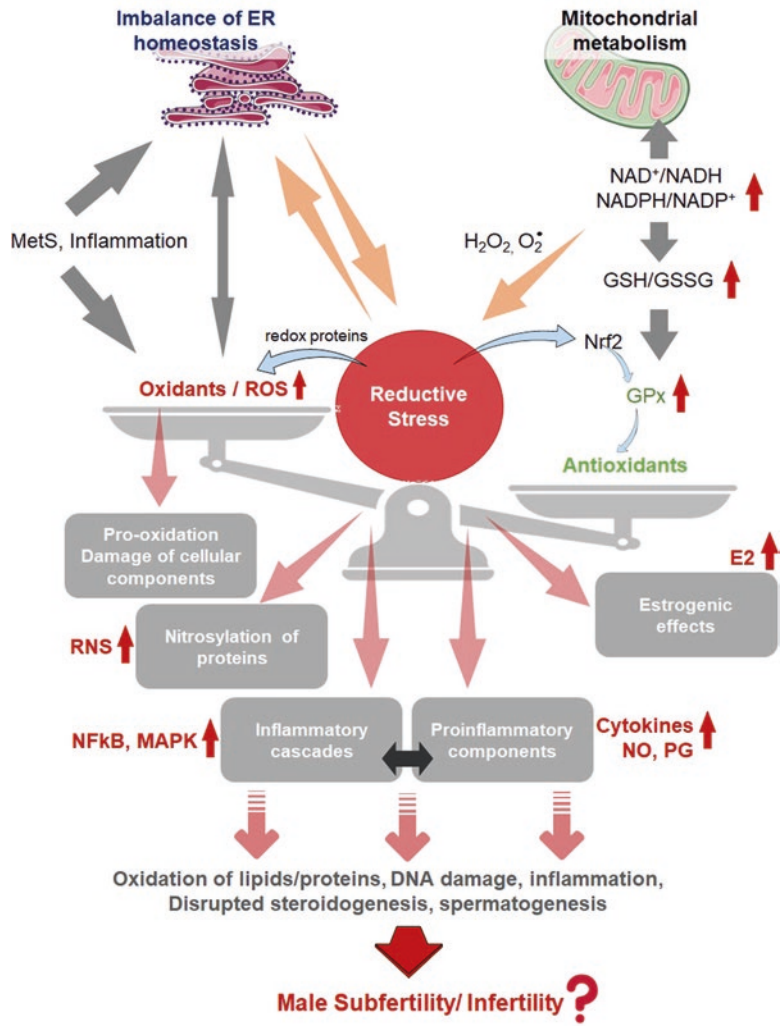

Fig. 17.1 The mechanism of generation of reductive stress and its consequences on male fertility. ER endoplasmic reticulum, MetS metabolic syndrome, GSH reduced glutathione, GSSG oxidized glutathione, ROS reactive oxygen species, RNS reactive nitrogen species, GPx glutathione peroxidase, Nrf2 nuclear factor-erythroid factor-2-related factor-2, E2 estradiol, NFkB nuclear factor kappa B, MAPK mitogen-activating factor kinase, NO nitric oxide, PG prostaglandin

L-carnitine, vitamins C and E, and/or coenzyme Q10 (Alahmar et al. 2021a, b, c; Abad et al. 2013; Ahmadi et al. 2016). According to a Showell et al., supplementation of antioxidants to infertile males may enhance pregnancy outcomes and minimize live birth rates (Showell et al. 2014). Huang and colleagues have established that seminal OS caused by lower levels of antioxidants is linked to male infertility. Specifically, antioxidant therapy has been found to be more beneficial for idiopathic male infertility and varicocele (Garg and Kumar 2016; Alahmar 2018). Although it is obvious and the findings are distinct that antioxidants are essential for the mitigation of OS, some scientists find little or even harmful effects of such therapies on particular sperm parameters (Silver et al. 2005; Stenqvist et al. 2018). Thus, the truth remains that while ROS play a significant part in the etiology of various human diseases, higher dosages of antioxidant supplements have had contradictory outcomes, leading to the coining of the phrase "antioxidant paradox" by researchers (Halliwell 2000). This underlines the limited understanding of the mechanisms of antioxidant treatment. Current antioxidant treatments to treat male infertility seems to be founded on an erroneous premise

that antioxidants are generally good, resulting in the misuse of the antioxidant composition.

As overproduction of ROS and thus OS is a primary cause of male infertility (Agarwal et al. 2008), unphysiologically high antioxidant concentrations, on the other hand, have considerable negative impacts on cells, and male fertility appears to be no exception (Fig. 17.1). Preserving the redox balance is important not only for the body but also for sperm since vital sperm activities such as capacitation, acrosome reaction requires modest amount of ROS, and excessive antioxidants will quench these effects and prevent sperm from fertilizing oocytes (De Lamirande and Gagnon 1995; Kothari et al. 2010). Mammalian embryos have been found to require strict regulation of the cellular redox system (Ufer et al. 2010); in this context, high antioxidant levels have been linked to the development of teratogenicity (Wang and Rogers 2007). As these early embryos are normally exposed to a relatively hypoxic environment in utero, glycolytic energy production appears to be favorable for cellular compaction and blastulation (Harvey et al. 2002). According to the scientists, alterations in redox-sensitive transcription factors and gene expression may occur as a result of this metabolic shift. This can disrupt critical embryonic development processes such as the process of fertilization, genome activation, or cellular differentiation. In general, an examination of the current literature reveals that reproductive redox biology is poorly understood. There are substantial evidences on OS in respect to male infertility, and despite the evidence that excessively high levels of antioxidants or the improper composition of antioxidant mixtures may have harmful effects on male infertility, its counterpart, RS, is often misunderstood and underappreciated (Bouayed and Bohn 2010). In this respect, Menezo and coworkers investigated daily supplementation with a mixture containing vitamins C and E, β-carotene, zinc, and selenium improved sperm DNA fragmentation (Ménézo et al. 2007). Sperm nuclear decondensation, on the other hand, increased, probably as a result of vitamin C reducing the disulfide bonds in the protamines (Giustarini et al. 2008), due to which, the chromatin becomes destabilized, resulting in unsuccessful fertilization.

Indeed, as discussed earlier, several of the normally suggested antioxidants like selenium and vitamins C and E have documented certain unfavorable effects (Ménézo et al. 2014). In the case of selenium, no deficit has yet been shown in the scientific literature. As reported in some studies, higher levels of seminal plasma selenium (≥80 ng/ml) is related with reduced sperm motility, asthenozoospermia, and high rates of abortion, whereas 40 and 70 ng/ml of selenium are found to be ideal in reproductive efficiency (higher pregnancy outcomes and lower rates of abortion) (Bleau et al. 1984). Moreover, there are growing evidences that antioxidant or prooxidant actions of antioxidants ultimately depend on their concentration even if they come from natural sources (Bouayed and Bohn 2010). Furthermore, given the synergistic action of many antioxidant plant chemicals, antioxidant therapy will not only fail but can also be harmful when the synergistic molecule is lacking. This may be demonstrated in smokers with 20 mg/day β-carotene or 30 mg β-carotene and 25,000 IU retinyl palmitate per day, since in 29,133 and 18,314 participants, the risk of pulmonary carcinoma was considerably raised in a placebo efficacy study (Alpha-Tocopherol Beta Carotene Cancer Prevention Study Group 1994; Omenn et al. 1996). Furthermore, many animal research and human findings have shown that high antioxidant levels might have clinical consequences. These effects might be the consequence of redox imbalances in the body, which can result in OS (by the "antioxidant paradox") or also "reductive stress" (RS) (Wendel 1987), which, by causing cellular dysfunction, can be just as harmful to cells or the body as OS (Castagne et al. 1999).

In reproductive biology research, it has been documented that the appropriate balance between oxidation and reduction is essential for normal embryogenesis hence the cellular redox system must be strictly maintained (Ufer et al. 2010). As a result, excessive antioxidant levels

may cause teratogenic alterations (Wang and Rogers 2007), such as alterations in the redox-sensitive transcription factor activities and, therefore, alterations in gene expression (Harvey et al. 2002). Subsequently, deformities and developmental retardation may ensue. The growing embryo, on the other hand, is exposed to an essentially anoxic environment during implantation, showing the limited range of this redox balance for live cells (Leese 1995). Particularly, in andrology, Menezo et al. (2007) had analyzed antioxidants treatment given orally in daily basis to men whose female partners failed to become pregnant after IVF/ICSI, and the antioxidants consisted of vitamins C and E (400 mg each), zinc (500 μmol), β-carotene (18 mg), and selenium (1 μmol) (Ménézo et al. 2007). Sperm DNA damage decreased following the treatment, while sperm DNA decondensation increased, perhaps leading to asynchronous chromosomal condensation. In IVF and ICSI patients, DNA decondensation rates of more than 28% have been demonstrated to induce unsuccessful pregnancy outcomes. The capability of vitamin C to break disulfide bonds in protamines in sperm DNA may be causing greater DNA decondensation (Giustarini et al. 2008; Donnelly et al. 1999). Reduced protamine levels will eventually lead to nuclear decondensation issues and diminished fertility (Ménézo et al. 2007). Vitamin C has been shown to have both positive and negative dose-related effects on sperm membrane lipid peroxidation (LPO) and motility in an earlier in vitro study. Vitamin C at concentrations below 1000 μM resulted in increased sperm motility and decreased LPO, while concentrations above 1000 μM have shown the complete opposite effect with complete sperm immobility at concentrations above 4000 μM (Verma and Kanwar 1998). These studies reveal contradictory antioxidants impacts in the therapy of male infertility, which is dose-dependent [Table 17.1; (Greco et al. 2005; Ménézo et al. 2007; Gual-Frau et al. 2015; Tunc et al. 2009; Schisterman et al. 2020; Moilanen et al. 1993; Kessopoulou et al. 1995; Rolf et al. 1999; Sigman et al. 2006)]. However, one can believe that the makeup of a certain antioxidant combination, such as that found in various antioxidant supplements, has an impact. Finally, physicians would need to know the exact unique cellular redox level of each patient in order to treat them properly. The problem is that (a) no universally accepted method is available to test the bodily or seminal redox status of individuals, (b) one does not know what the normal redox level is, and (c), therefore, no generally accepted cut-off values are available. As a consequence, patients are not only taking antioxidants because they have been persuaded that antioxidants are excellent for antiaging and the body in general, but they are also being treated by physicians for male infertility since OS is regarded to be harmful in this case. Because of our contemporary lifestyle, which includes a lack of vitamin consumption, exposure to environmental toxins, and/or smoking, the body is subjected to OS. As a result, antioxidant therapy can assist to change this situation. Uncontrolled antioxidant consumption, on the other hand, may cause RS and infertility by implosion of antioxidant therapy. As a result, greater research into redox state, its impact on the fertilization process in general, and sperm functional capability in particular is required. Meanwhile, in the absence of recommendations, physicians should do a more comprehensive examination and interview of patients before prescribing more antioxidants to prevent the risk of RS caused by overdose of antioxidants.

17.6 Conclusion

The perception of antioxidants usage to treat OS-induced male infertility seems vastly oversimplified, while the excessive use of widely available antioxidants remains under-discussed. Few studies have emphasized upon the so-called antioxidant paradox phenomenon, and it is perceived that the nonprescription antioxidants supplements and over-the-counter antioxidants consumption might constitute the major reason for antioxidants overuse. Recently, male infertility research has laid

Table 17.1 Individual or combination antioxidant treatments with no significant effects on semen quality

Study	Antioxidant regime	Study population	Sperm parameters
Moilanen et al. (1993)	100 mg of vitamin E daily for 3 months	Unexplained infertility IUI	No change in SC, S_{mot} and S_{morph}
Kessopoulou et al. (1995)	600 mg of vitamin E daily for 3 months	Infertility with high ROS	No change in SC, S_{mot} and S_{morph}
Rolf et al. (1999)	1 g vitamin C and 800 mg vitamin E daily for 56 days	Asthenozoospermia	No change in SC, S_{mot} and S_{morph}
Greco et al. (2005)	1 g of vitamins C and E daily for 2 months	Idiopathic infertility (38 infertile men with previous IVF/ICSI)	No change in SC, S_{mot} and S_{morph}
Sigman et al. (2006)	1 g carnitine and 500 mg L-acetyl carnitine daily for 24 weeks	Asthenozoospermia	No improvement in S_{mot}
Menezo et al. (2007)	400 mg each vitamins C and E, 18 mg β-carotene, 500 µmol zinc, 1 µmol selenium daily for 90 days	38 infertile men with at least two failed IVF or ICSI	Increased chromatin decondensation
Tremellen et al. (2007)	Menevit (lycopene, vitamins E and C, zinc, selenium, folate, garlic oil) daily for 3 months	60 infertile men	No significant difference in DNA fragmentation index (DFI)
Hawkes et al. (2009)	300 mg selenium daily for 48 weeks	Normozoospermia	No improvement in S_{mot} and S_{morph}
Tunc et al. (2009)	Menevit (lycopene, vitamins E and C, zinc, selenium, folate, garlic oil) daily for 3 months	50 infertile men with elevated OS	No change in SC, S_{mot} and S_{morph}
Gual-Frau et al. (2015)	500 mg L-carnitine, 60 mg vitamin C, 20 mg coenzyme Q10, 10 mg vitamin E, 200 µg vitamin B, 91 µg vitamin B12, 10 mg zinc, 50 µg selenium once daily for 3 months	Prospective observational study with 20 men with grade 1 varicocele and primary or secondary infertility	No change in SC, S_{mot} and S_{morph}
Stenqvist et al. (2018)	30 mg vitamin C, 5 mg vitamin E, 0.5 µg vitamin B12, 750 mg L-carnitine, 10 mg coenzyme Q10, 100 µg folic acid, 5 mg zinc, 25 µg selenium twice daily for 6 months	7 infertile men with sperm DNA fragmentation index (DFI) >25% treatment group (37 patients) placebo group (40 patients)	No change in SC, DFI
Schisterman et al. (2020)	5 mg folic acid, 30 mg zinc once daily for 6 months	1773 men planning to undergo infertility treatment with spouse	No improvement in S_{mot} and S_{morph}

SC sperm concentration, S_{mot} sperm motility, S_{morph} sperm morphology

attention to the biochemical characteristics of the oxidant vs. antioxidant equilibrium. Excessive antioxidants exposure drives the endogenous system toward RS, which is as harmful to sperm health as OS. Thus, pre-treatment redox status should be assessed and may be recommended before providing any antioxidants therapy. The exact mechanism by which RS mediate male reproductive disruptions remains largely unexplained; however, it is presumed that either the RS curb sperm functions by reducing ROS below physiological level that is essential for normal sperm functions, or it hinders normal oxidation mechanisms facilitating oxidative damage. There are great scopes for future interventions to reveal the underlying deep-rooted mechanisms of RS-mediated male infertility.

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18

In Silico Analysis of *CatSper* Family Genes and APOB Gene Regulation in Male Infertility

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Abstract

Sperm concentration and sperm motility are the two major causes of male infertility. Having spermatozoa in semen without motility or flagellum tail defect is a major concern needed to be investigated. The *CatSper* genes are the novel family of four sperm-specific Ca^{2+}-permeable channels which plays an important role in sperm motility, acrosome reaction, sperm, and oocyte fusion. *CatSper1*, *CatSper2*, and *CatSper3* are very well-studied genes for their role in asthenozoospermia, but the association of these genes with metabolic genes is still unstudied. Another unrevealed aspect is how ROS alter the function of *CatSper* genes. Among the *Catsper* family genes, the role of *CatSper4 gene* must be explored more. In this study, we have used the in silico approach to find the connection between the *CatSper* family gene with glycolytic genes and also the involvement of CATSPER4 protein in sperm flagellum using the STRING database. Connection of CATSPER1 protein with lipid metabolic gene is also found in Reactome database, and after that gene ontology of these genes was done by using DAVID and Enrichr databases. This analysis showed a strong interaction between CATSPER1, CATSPER2, and CATSPER3 protein with glycolytic protein (i.e., GAPDHS and PGK2), and CATSPER4 protein shows strong relation in the function of sperm flagellum. We also found a novel gene, i.e., APOB contributing to sperm motility. Gene ontology showed the role of APOB and glycolytic proteins in sperm motility. Enrichr analysis showed the association of APOB and glycolytic proteins in asthenozoospermia and CATSPER4 protein with sperm flagellum. Elsevier Pathway Collection also showed proteins involved in male infertility (i.e., GAPDHS). Therefore, we report the role of the *CatSper4* gene in sperm tail function and the APOB novel gene involved in sperm motility. Understanding the molecular mechanism(s) of regulations of the *CatSper* family gene will

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help us to develop new therapeutic approaches in infertile males.

Keywords

Sperm motility · Sperm flagellum · *CatSper* genes · Glycolytic genes · Lipid metabolic genes · Asthenozoospermia · Male infertility

18.1 Introduction

In the past few years, male infertility has increased rapidly by each year because of advanced age marriages, bad lifestyle, fast food consumption, smoking, drinking alcohol, environmental pollutions, psychological hazards, and many more factors (Kumar and Singh 2015). According to the World Health Organization (WHO), 15% of the couples of childbearing age are suffering from infertility worldwide, in which the male partner contributes 50% of overall cases. Infertility stands third most difficult disease globally after cardiovascular disease and cancer (https://www.who.int/news-room/fact-sheets/detail/infertility). Reduced sperm count and low sperm motility are the two most common causes of male infertility. According to WHO manual sixth edition, sperm motility is divided into four-category classification, i.e., rapidly progressive, ≥25 μm/s or at least half tail length per second (normal sperm); slowly progressive, 5 to <25 μm/s or at least one head length to less than half tail length/sec, and nonprogressive, <5 μm/s, or less than one head length (asthenozoospermic sperm). Immotile: no tail movement and also called sever asthenozoospermia.

Calcium ion plays an important role in sperm motility, capacitation, and acrosomal reaction. The influx of calcium ion is regulated by *CatSper* channels which is situated in plasma membrane of sperm mid-piece (Lishko et al. 2012). Ca^{2+} further helps in generation of cAMP which leads to activation of tyrosine phosphorylation which causes hyperactivated progressive motile sperm (Jin and Yang 2017). But due to aging, reactive oxygen species (ROS) start generating in spermatozoa which not only oxidize cholesterol but also inhibit the activity of tyrosine phosphorylation which lead to immotility or less motility in sperm flagellum (Fig. 18.1) (Pereira et al. 2017). Defects in the mammalian flagellum (sperm tail)

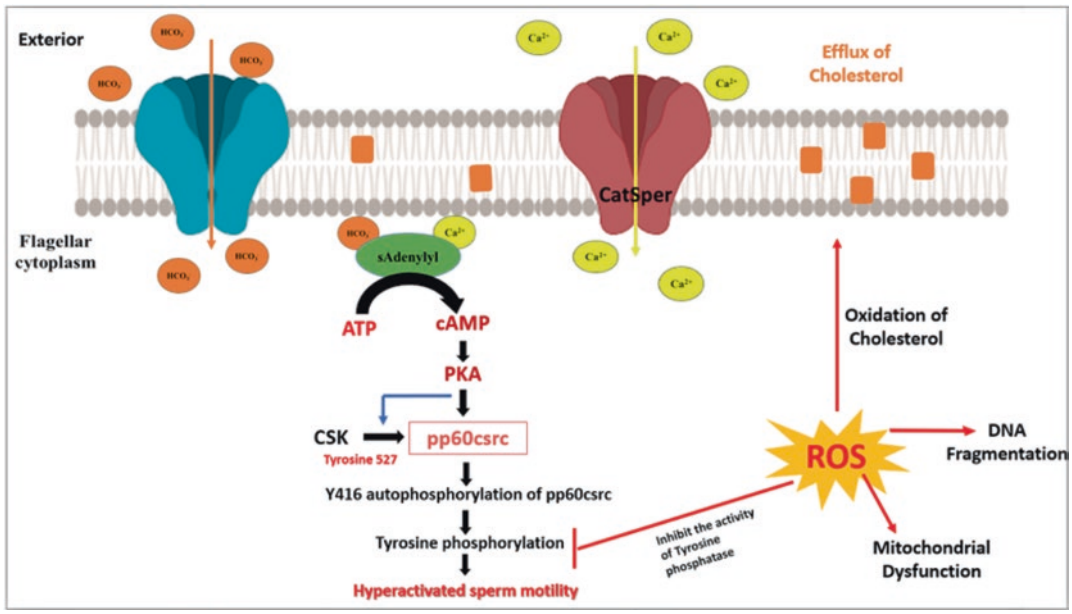


Fig. 18.1 Signaling pathway shows ROS to induce tyrosine phosphorylation in the fibrous sheath of the sperm tail that reduce sperm motility or cause immotility of sperm

which is a specific type of motile cilium result in reduced sperm motility or immotility. Therefore, understanding the mechanism involved in the formation of the sperm tail and knowing its function are needed for solving the issues regarding male infertility (Aprea et al. 2021). At the last phase of spermatogenesis, haploid round spermatids differentiate during spermiogenesis. Spermiogenesis is a process where the nucleus is condensed, the acrosome and sperm tail are formed, and excess cytoplasm is discarded. It involves 16 steps in which the first 8 (1–8) stages include the appearance of a round nucleus, flattening of acrosome, and beginning of elongation of the axoneme from the distal centriole (O'Donnell 2015).

As flagella contain a 9 + 2 microtubule structure similar to motile cilia, therefore, they require the same molecular mechanisms for their formation. At the later (9–14) stages, a transient microtubular platform, the manchette, surrounds the distal part of the sperm head, participating in shaping the head and delivering the proteins to the developing tail (Lehti and Sironen 2016). During the last steps of spermiogenesis, mitochondria are assembled helically around the outer dense fibers (ODFs) in the mid-piece of the sperm tail. While dynein arms in the axoneme provide the motor force for sperm tail motility, all accessory structures are required for the efficient fertilization capacity of sperm. They stabilize the long axoneme and provide support for the sperm tail movement and metabolic pathways for energy production (Linck et al. 2016).

Recent studies on human spermatozoa revealed the association of more than 1000 proteins with the sperm tail structure. This kind of study tells us about the complexity of flagellum and about the genes that cause asthenozoospermia. 26% of identified proteins are found to be associated with lipid metabolism and energy production (Amaral et al. 2014). Therefore, in this study, public database resources and computational tools were used to investigate the genes involved in the energy-driven pathway and lipid metabolism, which led to sperm tail defect in males. This research also aims to elucidate the molecular mechanism(s) of these genes to develop therapeutic approaches in infertile males.

18.2 Materials and Methods

18.2.1 Identification of Asthenozoospermia Gene Targets

Genes associated with "asthenozoospermia," "low sperm motility," "no sperm motility," and "dysregulated sperm motility" were collected from existing databases and literature. Databases like GeneCards (https://www.genecards.org) and DisGeNET (http://www.disgenet.org) were used. The GeneCards database provides comprehensive, user-friendly information on all annotated and predicted human genes (Safran et al. 2010). The DisGeNET database is a discovery platform containing one of the largest publicly available collections of genes and variants associated with human diseases (Piñero et al. 2020).

18.2.2 Protein-Protein Interaction (PPI) Network Analysis

The common genes that were collected from GeneCards and DisGeNET were put into the STRING database (https://string-db.org/,version 11.0) for PPI network analysis. The interacting proteins with a confidence score of $\geq$0.900 were chosen for PPI network visualization construction (Szklarczyk et al. 2019). The proteins that show positive correlation with glycolysis gene, sperm flagellum, and spermatogenesis were chosen for pathway analysis and gene ontology.

18.2.3 Pathway Analysis and Gene Ontology (GO)

Reactome Pathway database (https://reactome.org) was used for understanding the metabolic pathways in which selected genes are involved (Haw et al. 2011). For gene ontology Enrichr tool

(https://maayanlab.cloud/Enrichr/) and DAVID bioinformatic database (https://david.ncifcrf.gov/tools.jsp) were used to get the information on the functions of genes and to understand the biological meaning behind the gene set (Kuleshov et al. 2016; Dennis Jr et al. 2003; Huang et al. 2007). According to a high count and $P < 0.05$, the GO terms and pathways were selected that validate the association of chosen genes with male infertility.

18.3 Results

Total nine genes CatSper1, CatSper2, CatSper3, CatSper4, GAPDHS, PGK2, LDHC, PGM4, and PGM2 were selected from literature review and databases that show its association with asthenozoospermia or with severe asthenozoospermia.

18.3.1 PPI Network Analysis

The selected nine potential targets were input into the STRING database to construct a PPI network. In the network, the nodes and edges represent proteins and protein-protein associations, respectively. In this CATSPER1, CATSPER2, and CATSPER3 show their interaction with glycolytic proteins (i.e., GAPDHS and PGK2), and CATSPER4 protein shows its involvement in sperm flagellum (Fig. 18.2).

18.3.2 Pathway Analysis

In Reactome database CATSPER1, CATSPER2, and CATSPER3 protein were selected to elucidate their role in metabolic pathway. It was found that CATSPER1 protein was involved in three metabolic pathways (Fig. 18.3), and further analysis also showed the interaction of APOA2 gene involved in lipid metabolic pathway with CATSPER1 protein with score 0.781 (Fig. 18.4).

18.3.3 PPI Network Analysis of Selected Genes with APOA2 Genes Involved in Lipid Metabolism

After finding the interaction of CATSPER1 protein with lipid metabolic gene, again PPI analysis was done, and it was found that APOB (gene involved in lipid metabolism) shows its association with flagellated sperm motility and spermatogenesis in human (Fig. 18.5).

18.3.4 Gene Ontology

To validate the findings from PPI network and pathway analysis that gene ontology was done, Enrichr and DAVID databases showed the association of APOB with asthenozoospermia (Fig. 18.6) and sperm motility, respectively (Fig. 18.7), and in Enrichr tool it was also found that GAPDHS (glycolytic protein) is involved in male infertility with *p*-value 3.502e-^{8} (Fig. 18.8) and CATSPER4 protein is found in sperm flagellum with *p*-value 0.0030 (Fig. 18.9).

18.4 Discussion

Functional spermatozoa are important in male fertility and are produced by going through complex processes like meiosis, mitosis, spermatogenesis, and spermiogenesis (Lehti and Sironen 2017). They are highly specialized cells with distinguishing functional region; therefore the expression of key genes is required to regulate the completion of spermatogenesis successfully (O'Donnell et al. 2017; Cheng and Mruk 2012). We hypothesized that alternative expressions of the selected genes *CatSper1*, *CatSper2*, *CatSper3*, *CatSper4*, GAPDHS, PGK2, LDHC, PGM4, PGM2, APOA2, and APOB would contribute to dysregulated sperm motility and defective sperm tail. This study performed using bioinformatics data mining

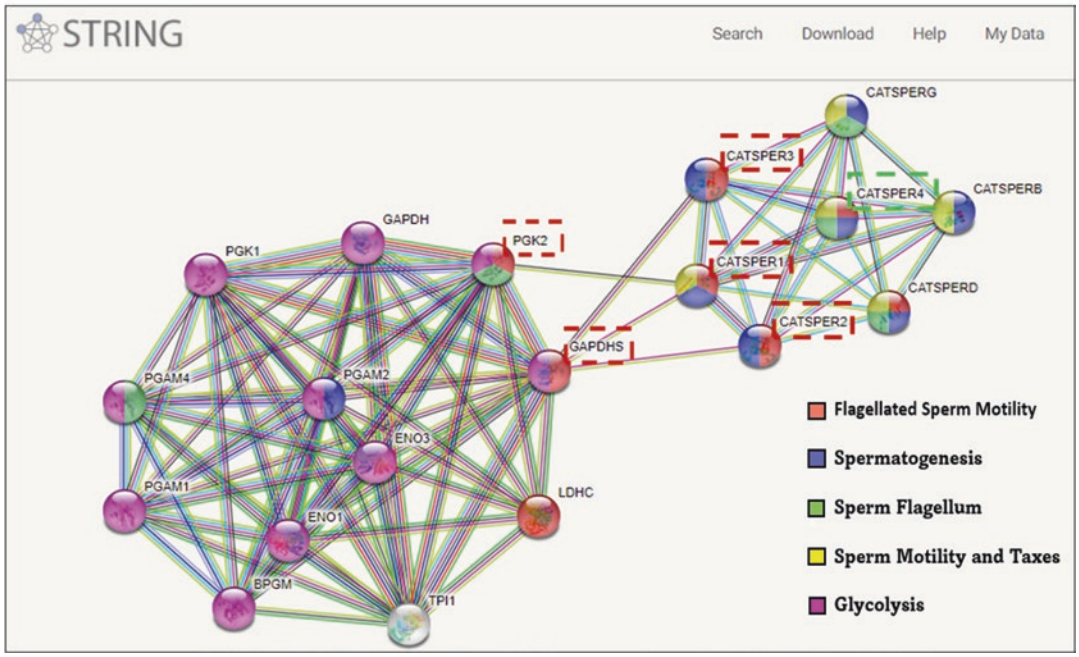


Fig. 18.2 PPI network analysis of nine selected genes, in which red color indicates flagellated sperm motility, the blue color indicates spermatogenesis, green color indicates sperm flagellum, yellow color indicates sperm motility and taxes, and pink color indicates glycolysis

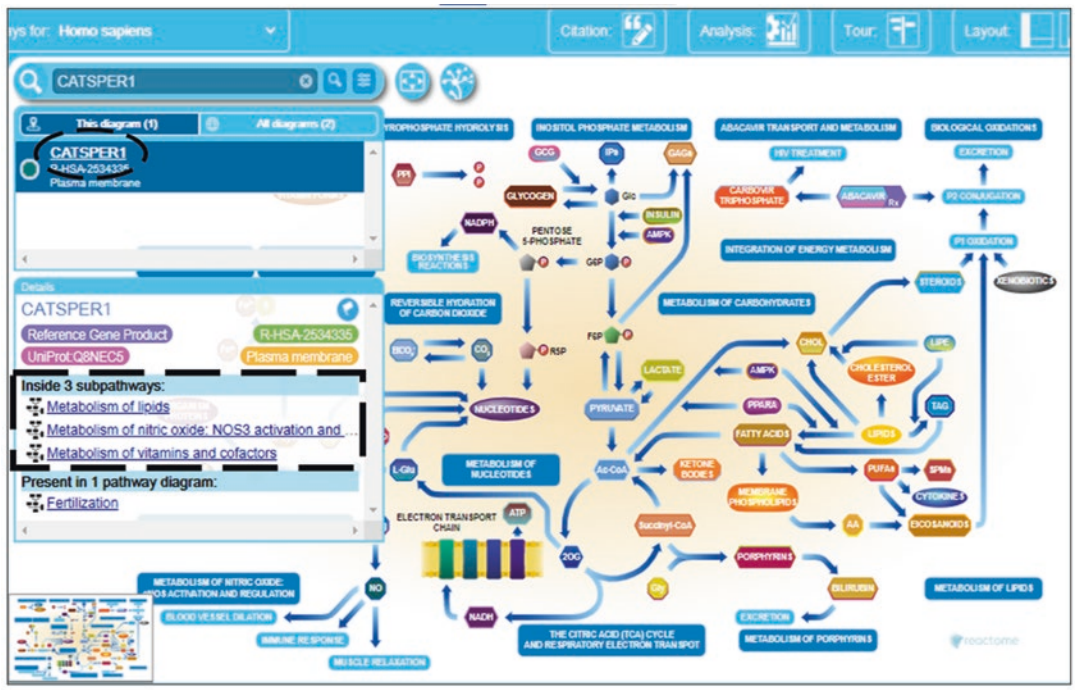


Fig. 18.3 Expression of CatSper1 gene in three different metabolic pathways. (1) metabolism of lipids; (2) metabolism of nitric oxide: NOS3 activation; (3) metabolism of vitamin and cofactors

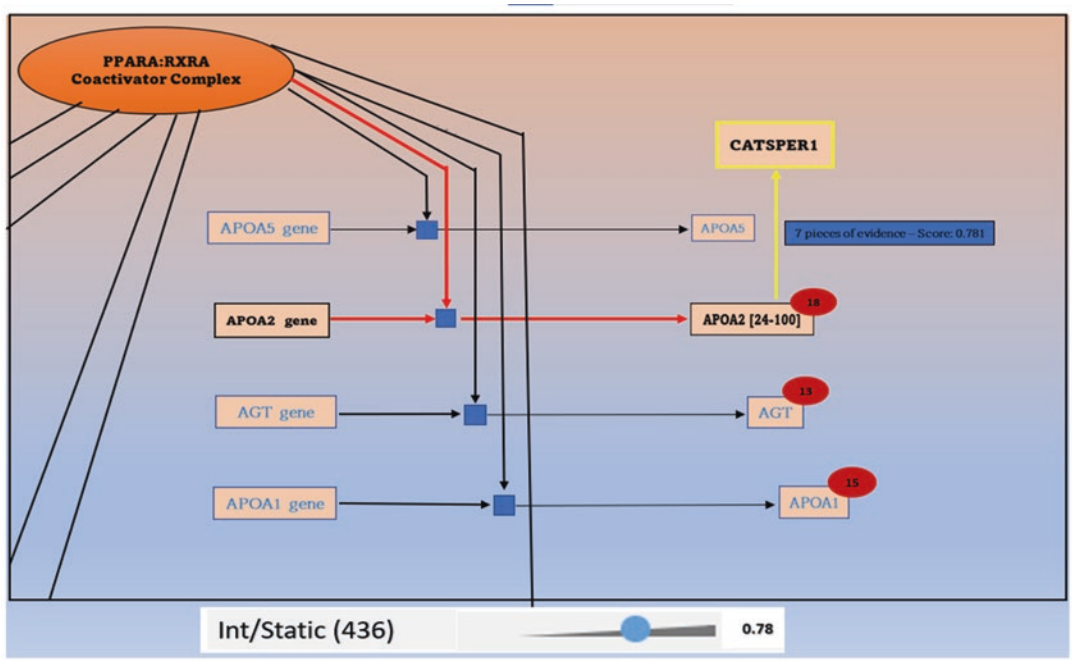


Fig. 18.4 Gene involved in lipid metabolism (APOA2) showed its interaction with CatSper1 gene with an interacting score 0.78

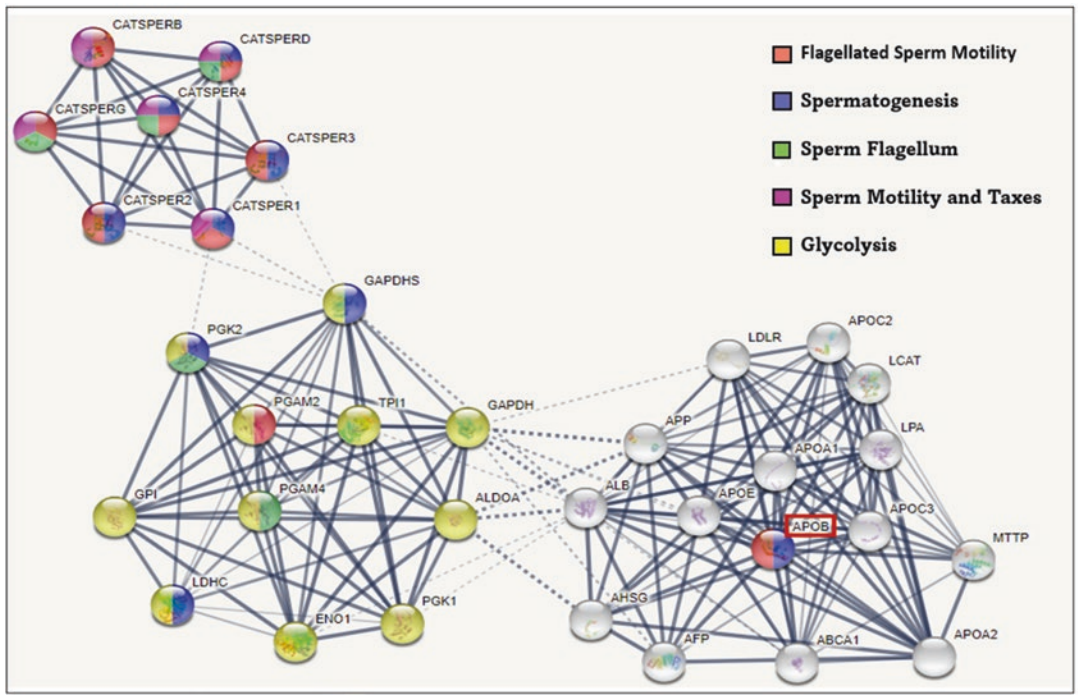


Fig. 18.5 PPI analysis that shows the association of APOB in sperm motility and spermatogenesis, in which red color indicates flagellated sperm motility, the blue color indicates spermatogenesis, green color indicates sperm flagellum, yellow color indicates glycolysis, and pink color indicates sperm motility and taxes

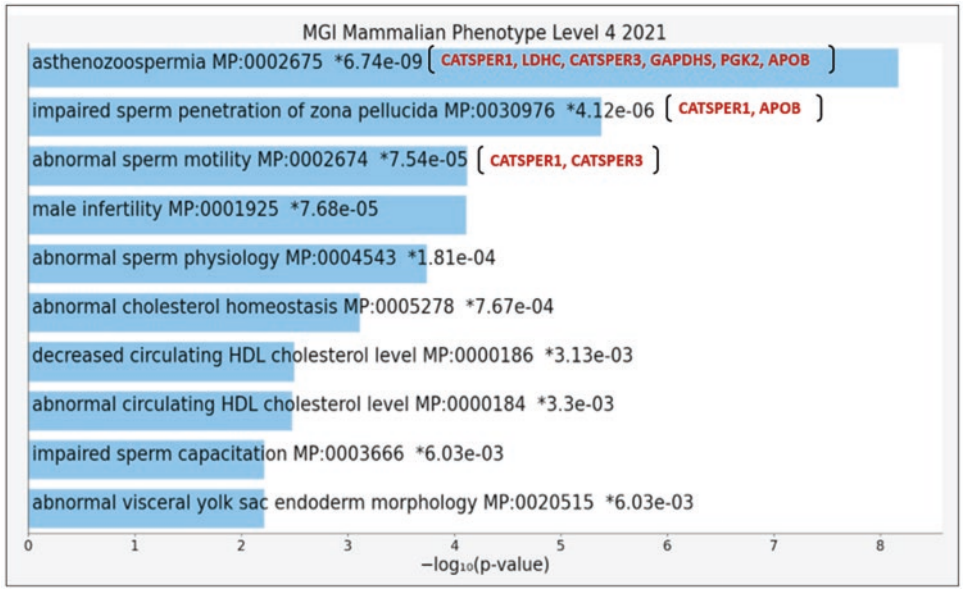


Fig. 18.6 Enrichr result showing the involvement of glycolytic genes and APOB in asthenozoospermia with *p*-value 6.74e-^{09}

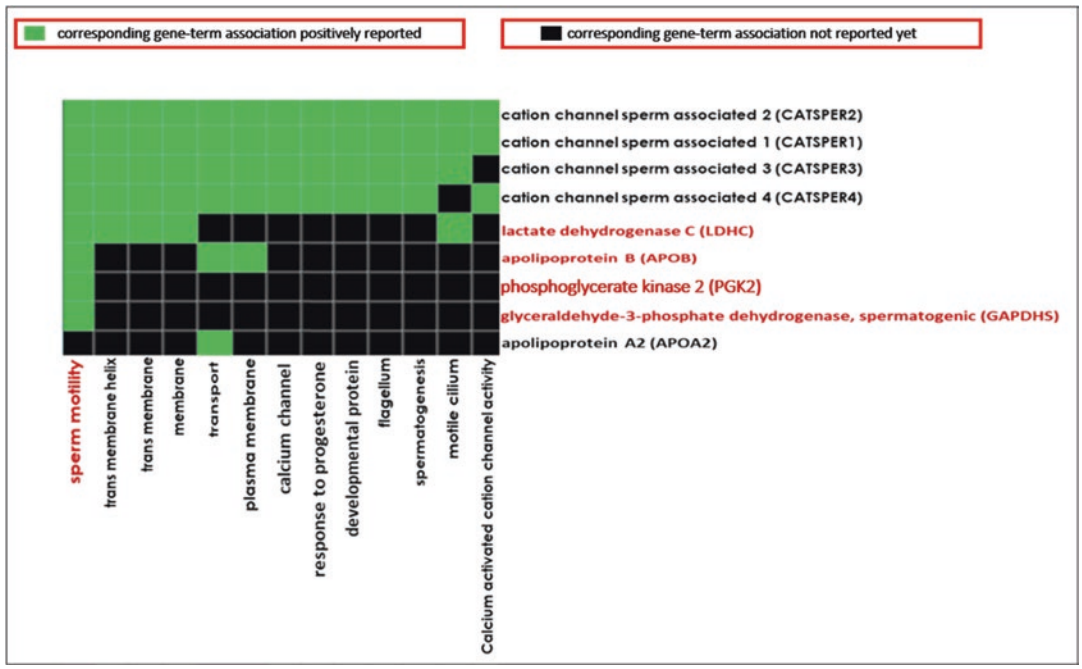
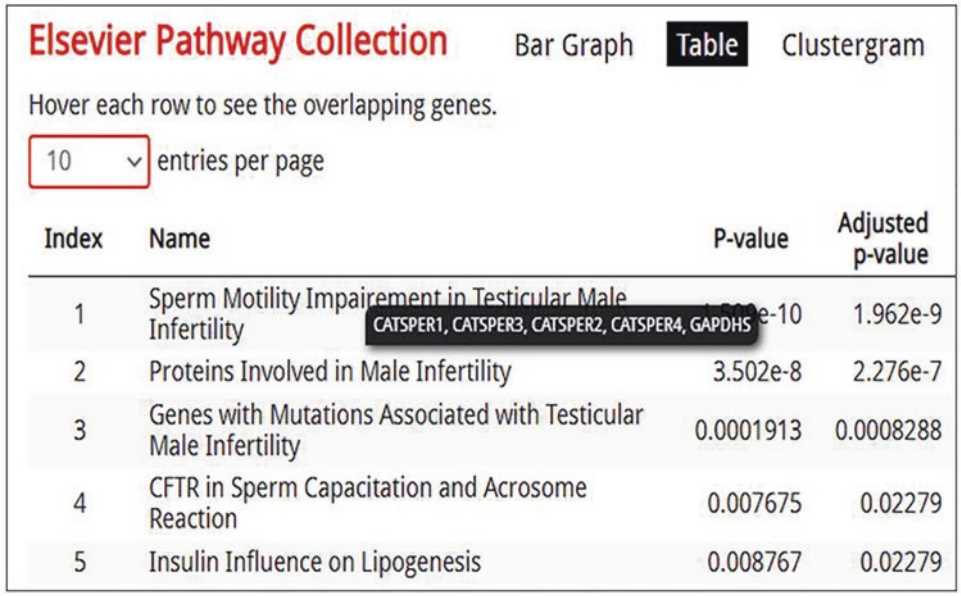


Fig. 18.7 Result of functional clustering from DAVID database showing involvement of glycolytic genes and APOB in sperm motility

of genes associated with asthenozoospermia and male infertility in humans provided a novel insight into the understanding of sperm motility. In the present study, we performed a systematic bioinformatics analysis by collecting genes associated with asthenozoospermia. Total nine genes were



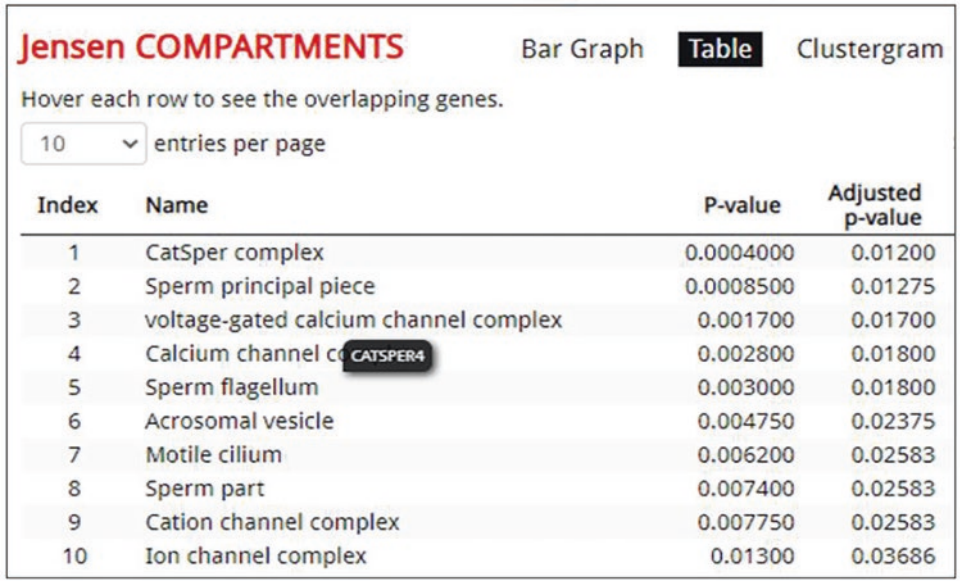
Elsevier Pathway Collection Bar Graph **Table** Clustergram

Hover each row to see the overlapping genes.

10 entries per page

Index	Name	P-value	Adjusted p-value
1	Sperm Motility Impairement in Testicular Male Infertility	e-10	1.962e-9
2	Proteins Involved in Male Infertility	3.502e-8	2.276e-7
3	Genes with Mutations Associated with Testicular Male Infertility	0.0001913	0.0008288
4	CFTR in Sperm Capacitation and Acrosome Reaction	0.007675	0.02279
5	Insulin Influence on Lipogenesis	0.008767	0.02279

Fig. 18.8 Enricher result showing CATSPER family proteins and GAPDHS involved in male infertility



Jensen COMPARTMENTS Bar Graph **Table** Clustergram

Hover each row to see the overlapping genes.

10 entries per page

Index	Name	P-value	Adjusted p-value
1	CatSper complex	0.0004000	0.01200
2	Sperm principal piece	0.0008500	0.01275
3	voltage-gated calcium channel complex	0.001700	0.01700
4	Calcium channel c	0.002800	0.01800
5	Sperm flagellum	0.003000	0.01800
6	Acrosomal vesicle	0.004750	0.02375
7	Motile cilium	0.006200	0.02583
8	Sperm part	0.007400	0.02583
9	Cation channel complex	0.007750	0.02583
10	Ion channel complex	0.01300	0.03686

Fig. 18.9 Enricher result showing CATSPER4 protein involved in sperm flagellum with *p*-value 0.003000

collected, i.e., *CatSper1*, *CatSper2*, *CatSper3*, *CatSper4*, GAPDHS, PGK2, LDHC, PGAM2, and PGM4. After this PPI network analysis was performed in STRING database, which showed the interaction between CATSPER1, CATSPER2, and CATSPER3 protein with glycolytic metabolic proteins, i.e., GAPDHS and PGK2, which clearly signifies the involvement of CATSPER protein in energy-driven pathway and downregulation of these genes will surely result in low ATP production which led to low sperm motility. To validate the PPI findings, we had gone for the pathway analysis by using the Reactome Pathway database. In that, we found the involvement of CATSPER1

protein in the lipid metabolic pathway and its interaction with the APOA2 gene with 0.78 interacting score, which clearly shows the strong association of the APOA2 gene in sperm motility. After finding the association of lipid metabolic gene in sperm motility, we again did the PPI network analysis between selected proteins (CATSPER1, CATSPER2, CATSPER3, CATSPER4, GAPDHS, PGK2, LDHC, PGAM2, and PGM4) with the APOA2 gene. In that, we find a novel protein APOB (lipid metabolic gene), showing its involvement in spermatogenesis and in sperm motility which confirms its involvement in male infertility by disrupting the metabolic pathway. Then for gene ontology, we selected Enricher and DAVID databases, in which we validated the role of the APOB gene in asthenozoospermia and in sperm motility which makes it clear that by targeting this gene in vitro, we will get a differential expression of this gene, and it can become a potential biomarker for the assessment of human sperm motility. Not only this, but we also find that except CATSPER family protein, glycolytic protein (i.e., GAPDHS) is also a contributor to male infertility, which signifies the association of metabolic genes in male infertility which need to be worked on to find stronger evidence. Lastly, CATSPER4 is the least studied protein among the other CATSPER protein, so its role is not clear yet. But in PPI results, CATSPER4 protein reported its role in sperm flagellum, and in gene ontology, the association of CATSPER4 in sperm flagellum was also found with p-value of 0.003000, which is statically strongly associated, and this tells us that this gene is needed to be studied in tail defeated spermatozoa and finding its protein structure will help us in drug development.

18.5 Conclusion

Recent findings of the mechanism(s) involved in the formation and motility of spermatozoa are still limited. Every stage of spermatogenesis involves the expression of particular gene patterns. These gene expressions are needed to study for understanding the molecular markers of germ cells at various stages of spermatogenesis. This study provided a piece of useful information and new ideas for further research on the genes involved in the formation of sperm tail and metabolic pathways involved in the regulation pattern of sperm motility and impact of ROS due to aging. This study also provided new ways and prospects for research in male contraception, diagnosis, and treatment of male infertility.

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19 Oxidative Stress and Toxicity in Reproductive Biology and Medicine: A Comprehensive Update on Male Infertility Volume II – Conclusion

Ralf Henkel

Abstract

Infertility is a globally under-recognized public health problem significantly impacting individual health and socioeconomics affecting millions of couples. The reasons for infertility are manifold and not only include many couples decision to postpone having children but also diseases (e.g., diabetes, infections, or varicocele), lifestyle (e.g., obesity), and environmental factors (e.g., bisphenol A, DTT or dioxin). In the pathology of many causes of infertility, oxidative stress plays a significant role as reactive oxygen species (ROS) exert significant detrimental effects. On the other hand, a small amount of ROS is essential to trigger physiological events such as capacitation. Therefore, a fine balance between oxidation and reduction has to be maintained. Apart from treating the underlying disease or correcting the cause of the infertility, oxidative stress can be treated by antioxidant supplementation. Since plants and their extracts contain numerous phytochemicals which exhibit antioxidant activity, many people tend to use herbal products. Alternatively, isolated antioxidants such as vitamin C or E are also used. However, when using purified antioxidants, it is essential that the redox balance is maintained to avoid a "reductive stress" situation, which is as harmful as oxidative stress.

Keywords

Infertility · Oxidative stress · Reductive stress · Environmental toxicants · Physiological balance

19.1 Introduction

Infertility is a globally under-recognized public health issue (McDonald Evens 2004) affecting almost 200 million people (Rutstein and Shah 2004; Inhorn and Patrizio 2015), i.e., about 15% of couples at reproductive age with an increasing trend as the population grows (Mascarenhas et al. 2012) and declining semen quality (Carlsen et al. 1992; Levine et al. 2017; Mishra et al. 2018). Yet, some authors question the latter because studies might suffer from potential selection and sociodemographic bias (Deonandan and Jaleel 2012; Merzenich et al. 2010; Pokhrel et al. 2019). On

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the other hand, many couples postpone having children to more advanced ages. In addition, environmental pollution and modern lifestyle is also negatively affecting fertility resulting in increased time to pregnancy (Giwercman and Bonde 1998; Bonde 2010; Buck Louis et al. 2014; Kumar et al. 2014; Wesselink et al. 2022). For all these causes of infertility, oxidative stress has been found to be involved or a major cause of the pathology in male and female (Gupta et al. 2013; Vaughan et al. 2020; Humaidan et al. 2022).

19.2 Oxidative Stress and Toxicity

Reactive oxygen species (ROS) are a group of oxygen derivatives that that exhibit an unpaired electron in the outer orbit, a chemical structure that makes these molecules highly reactive. Molecules showing this specific structure are called free radicals. Examples are superoxide or the hydroxyl radical. Due to their reactivity, ROS usually have half-life times in the nano- to millisecond range and react with other molecules at the site or close to the site of production. Hydrogen peroxide on the other hand is, although it is ranked among the ROS, an exception because it is not a radical. In contrast to other ROS, it is stable and can penetrate membranes just like water, thereby being able to cause damage inside cells. Considering that all aerobically living cells produce a certain amount of ROS, primarily as superoxide or hydrogen peroxide (Hayyan et al. 2016) as a result of leakage of about 1% to 5% of the consumed oxygen, is not converted into ATP in the mitochondria but released (Boveris and Chance 1973; Turrens 2003) and can thus damage cells. The damages to male germ cells include lipid peroxidation of the sperm plasma membranes (Aitken et al. 1989) and sperm DNA fragmentation (Lopes et al. 1998). On the other hand, a small, physiological amount of ROS is necessary to maintain and trigger essential cellular functions, which includes the regulation of apoptosis (Huang et al. 2000; Reed 2000; Castro et al. 2003) or the triggering of sperm capacitation (O'Flaherty et al. 2006; Aitken and Curry 2011). Hence, changes in the redox status of sperm and other cells are exposed which will have direct influence on the cellular functionality. Panner Selvam et al. (2020) showed that exposure of human sperm to too high ROS (oxidative stress) and too high antioxidant levels (reductive stress) significantly compromises sperm functions by changing mitochondrial protein expression. These changes are not only seen in the sperm cells but also reflect in proteomic changes in seminal plasma and the sperm cells.

On the other hand, excessive ROS levels can be produced as a result of numerous medical conditions including varicocele (Cho et al. 2016), diabetes (Amaral et al. 2008), infections (Vicari 1999; Henkel et al. 2020), or obesity (Barbagallo et al. 2021) leading to oxidative stress. Obesity is also associated with a systemic inflammatory response (Leisegang et al. 2019; Bhattacharya et al. 2020) with the release of pro-inflammatory cytokines such as interleukin (IL)-6, IL-8, or tumor necrosis factor-α, which in turn thereby acting as intermediary factor triggering to lipid peroxidation (Martinez et al. 2007; Fraczek et al. 2008). Several studies revealed specific differentially expressed proteins in patients with asthenozoospermia (Wang et al. 2009; Sharma et al. 2013), varicocele (Camargo et al. 2019; Panner Selvam and Agarwal 2021), or diabetes- and obesity-associated changes in sperm (Kriegel et al. 2009). Differences in sperm protein expression could even be observed between normally fertilizing normozoospermic patients in in vitro fertilization and normozoospermic patients after rescue-ICSI (Liu et al. 2018).

This question of too much (oxidative stress) and too little (reductive stress) ROS points out that the bodily redox equilibrium has to be in balance for normal physiological functions. Many studies have been conducted on the effects of oxidative stress in male (Aitken et al. 2003; Krajcir et al. 2008; Ritchie and Ko 2021; Mannucci et al. 2022) and female reproductive functions (Agarwal et al. 2005). In females, oxidative stress affects functions throughout the reproductive life, namely, oocyte development and maturation (Combelles et al. 2009), pregnancy (Myatt and Cui 2004), parturition (Fainaru et al. 2002), labor

(Mocatta et al. 2004), and menopause (Doshi and Agarwal 2013). In addition, since the oxygen tension in the fallopian tube is decreasing from the site of fertilization in the ampulla to the uterus, where the environment is almost anoxic until placentation is taking place (Leese 1995), this results in embryonic metabolic changes from oxidative phosphorylation to glycolysis and back to oxidative phosphorylation after placentation (Hansen et al. 2020). Hence, the redox levels in the female reproductive tract also affect embryo development and health (Hansen 2006; Hu et al. 2012; de Castro et al. 2016).

Since the detrimental impact of oxidative stress is abundantly clear, patients are taking antioxidants either to stay healthy or regain health in order to achieve pregnancy. A number of trials to investigate the efficacy of an antioxidant treatment to improve fertility have been conducted with different results. While some of the studies support the use of antioxidants to treat infertility (Arafa et al. 2020; Tremellen et al. 2020; Szymański et al. 2021), others could not find a beneficial effect (Smits et al. 2018; Steiner et al. 2020) or conclude that more studies are necessary to characterize the clinical value (Budani and Tiboni 2020; Humaidan et al. 2022). Recent Cochrane studies on the clinical value of antioxidants to treat male (Smits et al. 2019) and female infertility (Showell et al. 2017) conclude that there is low-quality to very low-quality evidence suggesting that antioxidant treatments are effective in treating infertility. Reasons for these conflicting outcomes of trials are, among others, inconsistencies in the formulation of the antioxidants used, the varying durations of the treatment, and different concentrations of the antioxidants used or the use of different outcome parameters (e.g., sperm DNA fragmentation, motility, fertilization or pregnancy). However, one has to accept that human infertility is a very complex medical condition with numerous male and female factors influencing the outcome. Therefore, one needs to understand that treating a man with antioxidants for infertility without proper examination and indication might not result in improved fertilization or pregnancy rates. In this context, one must also not forget that a limited physiological amount of ROS is essential for normal cellular function. Therefore, the common assumption that oxidative stress is generally harmful and antioxidants are generally healthy is not only misleading but also wrong as a fine redox balance between oxidants and reductants has to be maintained for normal physiological functions (Symeonidis et al. 2021). Patients and clinicians need to understand that antioxidants should only be taken if there is a real need due to illness or deficiency because reductive stress is as harmful as oxidative stress (Castagne et al. 1999; Henkel et al. 2019).

In vivo, the early embryo is facing changing redox conditions on its way from the site of fertilization, the ampulla, to the uterus where the environment is almost anoxic (Leese 1995) leading to a metabolic switch in the embryo from oxidative phosphorylation to glycolysis (Ufer et al. 2010; Hansen et al. 2020). With placentation taking place, the oxygen tension is increasing again and the embryonic metabolism is switching back to oxidative phosphorylation. In an effort to mimic this redox situation in the in vitro embryo culture, Truong and co-workers (2016) supplemented embryo culture medium with a combination of antioxidants including acetyl-L-carnitine and N-acetyl-L-cysteine and achieved significantly improved preimplantation embryo and fetal development of mouse embryos as compared to the standard culture system.

Like in any eukaryotic cell, including spermatozoa, ROS also play a significant role in the regulation of spermatogenesis and the maturation of the male germ cells. On the one hand, small amounts of ROS, especially hydrogen peroxide, are produced in the testes either via activation of NADPH oxidase or activation of xanthine oxidase and cytochrome P_{450} (Guerriero et al. 2014), have essential roles in the activation of signal transduction pathways regulating spermatogenesis (Morimoto et al. 2013, 2019). For the normal production of spermatogonia, small amounts of hydrogen peroxide are required to modulate phosphorylation and dephosphorylation of different MAP kinases (Fujii and Imai 2014; Morimoto et al. 2013). On the other hand, excessive ROS production as it is the case in patients with crypt-

orchidism (Ahotupa and Huhtaniemi 1992), varicocele (Agarwal et al. 2009), testicular torsion (Rodriguez et al. 2006), testicular infections/inflammations (Allen et al. 2004), or cancer treatments (Manda et al. 2007; O'Flaherty et al. 2008) leading to increased germ cell apoptosis and decreased spermatogenesis with infertility.

Due to increasing industrialization, another increasingly important cause of infertility is environmental pollution, which also triggers the production of excessive amount of ROS and thereby oxidative stress. Many endocrine disruptors such as polychlorinated biphenyls (PCBs), bisphenol A (BPA), dichlorodiphenyltrichloroethane (DDT), or 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin) are increasing testicular ROS production and thereby not only disrupt steroidogenesis but also spermatogenesis (Mathur and D'Cruz 2011). One example are PCBs causing a significant increase in testicular ROS production and modulation of inhibition of the steroidogenic enzymes 3β-HSD, 17β-HSD, and $P450_{(scc)}$ (Murugesan et al. 2007). Insecticides like lindane exert their detrimental actions on spermatogenesis also during critical stages of testicular development and have therefore been banned in many countries (Samanta et al. 1999). Occupational hazards such as scrotal heat exposure in foundries and steel workers, welders, or in long-distance drivers (Mieusset and Bujan 1995; Thonneau et al. 1998; Bujan et al. 2000) causes oxidative stress. Furthermore, heavy metals are also not only able to induce ROS production directly but also indirectly increase ROS levels resulting in cellular damage and apoptosis (Stohs and Bagchi 1995; Valko et al. 2005). It is also important to mention that environmental toxicants affect not only directly male fertility potential by acting on the testicles and the spermatozoa and the hypothalamus-pituitary-gonadal axis thereby disturbing the hormonal balance but also indirectly after prenatal exposure of the mother, neonatal, and pubertal exposure of boys (Henkel 2018). Consequently, exposure to environmental pollutants has to be reduced and stricter legal regulations have to be enforced.

According to Shein and Maehira (2005), up to 80% of the global population depend for their primary healthcare on traditional medicine. Plant extracts contain a multiple phytochemicals such as carotenoids and polyphenols like resveratrol and isoflavones, most of which have antioxidant activity. Thereby phytomedicines are not only able to treat disease but also provide powerful antioxidants to directly fight oxidative stress or are indirectly helpful in treating diseases by scavenging excessive ROS produced as a result of a disease. Among numerous other phytochemicals with antioxidant activity reported in the literature are vitamins C and E, resveratrol (a stilbenoid from grape seeds), and lycopene (a carotenoid from tomatoes) (Shi et al. 2003; Bhuvaneswari and Nagini 2005; Adewoyin et al. 2017). Extracts from plants like *Withania somnifera* (Ashwagandha, Indian ginger) and *Tribulus terrestris* (devil's thorn) scavenge ROS directly and indirectly via their antioxidant contents and their ability to enhance the activity of cellular antioxidant enzymes, respectively (Chhatre et al. 2014; Sengupta et al. 2018). In light of the huge variety of different phytochemicals and the fact that in the search for new pharmacologically active compounds medicine and pharmacy are going back to nature for the development of new drugs, phytomedicine is a rapidly developing area. Thereby, phytomedicine will not only open new avenues in the treatment of symptoms of diseases but also help reducing the detrimental effects of oxidative stress and its consequences.

19.3 Conclusion

In conclusion, in life under aerobic conditions, ROS play an essential role in the regulation of physiological processes and development including the production and maturation of germ cells (male and female), fertilization, and embryo development. On the other hand, the amount of these highly reactive chemical compounds has to be accurately controlled and balanced with a sufficient amount of antioxidants as elevated amounts of ROS would cause oxidative stress with cellular damage and cell death. Excessive ROS can derive either from diseases or iatrogenic by exposure to toxicants. In these cases, the sup-

plementation with antioxidants is helpful. However, an oversupply of the body with antioxidants can lead to reductive stress, a condition that is as harmful as oxidative stress.

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