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Oxidative Stress and Toxicity in Reproductive Biology and Medicine

A Comprehensive Update on Male Infertility- Volume One

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Foreword

The book by Dr. Kavindra Kumar Kesari and Dr. Shubhadeep Roychoudhury, ***Oxidative Stress and Toxicity in Reproductive Biology and Medicine: A Comprehensive Update on Male Infertility***, highlights the strong connection between oxidative stress and male infertility. This topic has gained prominence with new knowledge about the impact of lifestyle and mutagenic factors on male infertility. Exposure to several environmental toxicants, such as chemicals, radiations, and viral and microbial infections, represents risk factors for male infertility. The book's title itself defines the theme of the book, wherein the authors have considered relevant oxidative stress–induced toxicity in male fertility. This book contains 15 chapters which cover most of the recent progress in the field of free radical biology in cellular toxicology and clinical manifestations of various issues related to men's health and infertility along with the therapeutic use of herbal and natural medicines to control the oxidative stress. The introductory chapter by Prof. Ralf Henkel highlights the relationship between oxidative stress and male infertility with historical perspectives. Several chapters in this book focus on the physiological and pathological role of oxidative stress in male reproduction by providing appropriate pathways. The follow-up chapters mostly explore the role of oxidative stress at the molecular level, for example, Chapter 5 unravels the molecular impact of sperm DNA damage and repair mechanism along with certain other types of damage relevant to male reproduction.

The scientific value of this book is that most of the exiting factors which are associated with our daily life exposures are well covered. Several important chapters on uropathogenic factors (bacterial and viral infection) have been discussed to explore the role in male infertility. Especially Chapter 7 discusses the role of oxidative stress in bacteriospermia vis-a-vis male infertility. Chapters 9 and 10 on inflammation and varicocele describe how different types of lifestyle and environmental factors induce oxidative stress and in turn cause infertility. Last but not least, Chapter 15 presents an interesting study on sperm redox system equilibrium and its implication in fertilization and male fertility. Understanding the sperm redox system will allow us to explore the mechanism of free radical biology in reproductive toxicology. The book presents an update on the etiology of infertility, where a number

of chapters defining the known and probable causes of male infertility and therapeutic measures against oxidative stress are delineated.

The editors should be congratulated for providing chapters with concepts and mechanisms using illustrations, flow charts, and tables. The language is kept simple in this book so that an inquisitive reader with a scientific or nonscientific background may easily understand the subject matter. This book will be useful for general readers as it covers many important public issues. The editors of this book deserve special thanks for assembling a great team of contributors and for editing such an important book on a clinical topical issue.

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Preface and Acknowledgments

There are several factors in the indoor and outdoor environment which may affect reproductive health. Potential threats to reproduction and fertility that are encountered by men in everyday life emanate from chemical, physical, and biological sources. Most common etiology is oxidative stress which may induce cellular toxicity and enhance free radical production within the cells which often overwhelm the scavenging capacity by antioxidants due to excessive production of reactive oxygen species (ROS). Oxidative stress is a result of the imbalance between ROS and antioxidants in the body which may lead to sperm damage (DNA or count), deformity, and, eventually, male infertility. Environmental toxicants, such as radiations (ionizing and non-ionizing), radiations from radiotherapy, chemicals, toxic metals, fine particles, lifestyle factors, medications or drugs consumption, and microbial and viral contaminations, and other factors including climate change and pollution affect male fertility. This book presents various state-of-the-art chapters on the recent progress in the field of cellular toxicology and clinical manifestations of various issues related to men's health and fertility. This book also provides a way towards therapeutic use of natural antioxidants to control or manage oxidative stress for the prevention of male infertility outcome. This book has value-added collection of 15 chapters as Volume I, which discuss **an up-to-date review on the impact of oxidative stress factors in male reproduction with** multidisciplinary approaches with a focus on environmental toxicity. Chapter 1 by Prof. Ralf Henkel mainly introduces the role of oxidative stress and toxicity in reproductive biology and medicine. This chapter primarily points out the factors that are responsible for inducing oxidative stress, which may lead to male infertility, and these are presented with historical perspectives. Chapter 2 discusses the role of ROS and antioxidants in both male and female reproduction. This chapter largely highlights the origin of ROS in the female and male reproductive tracts with possible mechanisms of their generation. It also describes the specific physiological roles of the reactive molecules in upholding the structural integrity and functionality of both the reproductive systems. Chapter 3 discusses the pathological roles of ROS in male reproductive problems which are linked to infertility, erectile dysfunction, and prostate cancer. The chapter mainly emphasizes the role of lipid peroxidation, DNA damage, and

apoptosis induced by oxidative stress, with an insight into the probable mechanism through which ROS exerts its pathological impact. The effect of varicocele-induced ROS on infertility is value added discussion towards male in fertility in this chapter. In this continuation, Chapter 4 represents the molecular interactions which are directly or indirectly associated with oxidative stress–mediated male infertility. The chapter mainly highlights the association of proteins with dysregulated molecular pathways in sperm and seminal plasma due to oxidative stress. Chapter 4 also discusses the molecular interactions between proteins associated with oxidative stress and their potential role leading to male infertility with the help of advanced proteomic techniques and bioinformatic tools. Chapter 5 by Prof. Ashok Agarwal and his group, one of the leading scientists in the field of oxidative stress and male infertility research, focuses on the role of ROS in causing sperm DNA damage, along with certain other types of damages. This chapter elaborates the molecular mechanisms involved in DNA repair during spermatogenesis or after fertilization of the oocyte. The impact of sperm DNA damage on reproductive outcomes, such as fertilization and pregnancy rates, has been discussed with a focus on animal and human studies. Chapter 6 discusses the role of leukocytes and infection in male infertility. There are several types of infections that may affect human health, especially male urogenital infection, involving bacterial, viral, protozoal, and fungal infections. These infections are more or less asymptomatic in nature and pass on to sexual partner through intercourse which may lead to fertilization failure, pregnancy loss, and even development of illness in the offspring. Among these, leukocytospermia emerges as one of the most likely causes of ROS and oxidative stress-induced sperm dysfunction, poor fertilization, and male infertility. In this connection, Chapter 7 elaborates the role of oxidative stress towards bacteriospermia and male infertility. In most of the infection cases, bacteria attack the urological system, and these uropathogens are involved in infection and inflammation of the male urogenital tract. Bacteriospermia may induce infertility by interacting directly with sperm cells and start producing ROS and lead to impaired sperm parameters. Chapter 7 also discusses the protective measures by supplementing herbal medicines, antioxidants, and plant extracts. The role of herbal medicines against viral or microbial infections have been emphasized in recent years. Therefore, this book provides a collection of oxidative stress–induced infertility prevention and fertility protection measures. Chapter 8 discusses the oxidant-sensitive inflammatory pathways in male reproduction, where inflammation has been considered as a major signal of oxidative stress. Oxidative stress–induced male infertility and its mechanisms are extensively documented in this chapter along with several important fertility parameters. In this series, Chapter 9 discusses more about oxidative stress with emphasis on various factors of idiopathic male infertility and the mechanism by which several endogenous as well as exogenous factors may induce idiopathic male infertility. This chapter covers several important aspects such as inflammation, varicocele, obesity, and lifestyle factors which may induce oxidative stress and lead to infertility. It also elaborates the pathophysiology of male oxidative stress infertility with a focus on its diagnostic and therapeutic aspects. In this connection, Chapter 10 discusses an important clinical male infertility aspect – varicocele. This has been

reported as one of the most common causes of male subfertility, although the pathophysiology of varicocele remains largely unknown. This chapter mainly explores the correlation among oxidative stress, varicocele, and male infertility with focus on various fertility parameters. It also discusses several therapeutic aspects to explore the role of interventions and to reduce oxidative stress in men with varicocele-associated infertility. In a way, Chapters 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 pose oxidative stress to be the responsible factor behind elevated levels of ROS, and as a result may also be considered as a hallmark for diabetes-induced male infertility, as has been reported in Chapter 11. For better understanding of the underlying mechanisms, Chapter 11 discusses the central role of oxidative stress, which may play a major role as mediator of obesity, metabolic syndrome, and type 2 diabetes mellitus in men. This chapter mainly covers the pathophysiological role of oxidative stress in the management and mechanism of reproductive dysfunctions. Although, management of oxidative stress is not well defined, however, improved nutrition and exercise has been recommended for the improvement in male fertility parameters under the influence of oxidative stress. Chapter 12 discusses the key causes of decreased sperm motility and some of the muted genes and metabolic causes, wherein athenozoospermia or poor sperm motility has been reported as causes of infertility in the male. This chapter covers several important aspects related to the metabolic dysregulation with focus on signaling pathways and genes involved in sperm motility. This chapter explores the central role of oxidative stress and ROS production in association with sperm motility, molecular pathways, and lifestyle factors. Interestingly, a therapeutic approach for athenozoospermia has also been proposed. Chapter 13 discusses as to how viruses invade the male genital system thus in turn leading to detrimental consequences on male fertility. This chapter uncovers the facts associated with the tropism of various viruses in the male genital organs and explores their sexual transmissibility. It summarizes the functional and mechanistic approaches employed by the viruses in inducing oxidative stress inside spermatozoon thus leading to male infertility. Additionally, this chapter also highlights the various antiviral therapies that have been studied so far in order to ameliorate viral infections and to combat the harmful consequences leading to male infertility. This chapter would be highly interesting considering the current scenario of COVID-19 viral infections affecting human health. The male reproductive system is one of the attainable targets of many viruses including immunodeficiency virus, Zika virus, adenovirus, cytomegalovirus, and potentially severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and infection with such viruses may cause serious health issues which has been reported in Chapter 14. This chapter discusses the pathogenesis of viral infections and possible roles in male reproductive health. This is an evidence-based study where authors explore how the viruses affect the male reproductive system, including their distribution in tissues and body fluids, possible targets as well as the effects on the endocrine system. This chapter also covers several important therapeutic options to protect cells from the viruses. A last Chapter 15, the final chapter, mainly discusses the sperm redox system equilibrium and its implications for fertilization and male fertility. The role of ROS which has been responsible for cellular oxidative damage is discussed in this chapter. The

fundamental questions like how glutathione transferases, glutathione peroxidases, the mammalian testis thioredoxin system and their respective substrates and cofactors, specifically glutathione and selenium, facilitate the protection, successful development, and maturation of mammalian spermatozoa have been outlined in this chapter. This chapter shows high impact towards redox system and free radical research.

All the chapters presented in this book are interlinked and gradually lead to the conclusion that oxidative stress–induced ROS formation is a responsible factor behind male infertility. This book also focuses on the signaling pathways and molecular factors associated with environmental toxicity in understanding the mechanisms of toxicity associated with free radical generation and male infertility. One who could read all the chapters from 1 to 15 will inculcate a better understanding of environmental challenges for male reproduction and possible protective measures in the form of herbal medicine, bioactive compounds, therapeutic approaches, and antioxidant intakes. We hope this book will serve as both an excellent review and a valuable reference for formulating suitable measures against oxidative stress and for promoting the science involved in the male reproductive system.

Finally, we would like to dedicate this book to all the frontline workers globally, who are fighting 24/7 against COVID-19 to save our lives, and also to COVID-19 warriors who lost their lives saving humanity. We would like to thank all the authors who contributed to this book. Last but not least, our special 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. The editors are also thankful to the entire production team including Mr. Vishnu Prakash and Ms. A. Meenahkumary for their support.

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Contents

Chapter 1
Oxidative Stress and Toxicity in Reproductive Biology and Medicine: Historical Perspectives and Future Horizons in Male Fertility

Ralf Henkel

Abstract Since the discovery by John MacLeod in 1943 that spermatozoa produce small amounts of hydrogen peroxide, a member of the so-called reactive oxygen species (ROS), the importance and functions of these highly reactive oxygen derivatives in physiology and pathology are a subject of numerous studies. It has been shown that they play essential roles, not only in causing oxidative stress if their concentration is excessively high, but also in triggering crucial cellular functions if their concentration is low. On the other hand, antioxidants counterbalance the action of ROS to maintain a fine balance between oxidation and reduction as an excessive amount of antioxidants leads to a condition called reductive stress and is as harmful as oxidative stress. This book *"Oxidative Stress and Toxicity in Reproductive Biology and Medicine – A Comprehensive Update on Male Infertility"* authoritatively summarizes the current knowledge of various causes of oxidative stress including various andrological conditions and environmental pollution as well as the physiological effects of ROS. Moreover, this book expands into the treatment of oxidative stress with antioxidants and phytomedicine, a rapidly developing area. As a first of its kind, this book also sheds light on the effects of the redox potential during the fertilization process and thus highlights the importance of the correct balance of oxidants and antioxidants, even in the culture medium in assisted reproduction. The editors have brought together an impressive group of renowned

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experts to share their knowledge on the topic of oxidative stress and its clinical management in andrology and assisted reproduction.

Keywords Oxidative stress · Reductive stress · Antioxidants · Sperm functions · Fertilization

1.1 Introduction

It is almost 80 years ago that John MacLeod [45] made the pioneering discovery that the primary role of oxygen in spermatozoa is not for metabolism and motility as they cannot oxidize glucose, lactate, and pyruvate. MacLeod's observation went even further by indicating that high partial pressure of oxygen is not only inhibiting motility but also producing small amounts of hydrogen peroxide (H_2O_2), a highly oxidizing chemical compound that is now counted among the so-called reactive oxygen species (ROS). Since that time, scientists conducted a lot of research to understand not only the impact of extrinsic oxygen and highly reactive oxygen derivatives such as ROS but also cells' own intrinsic ROS production on cellular and organismal physiology and development and also their pathological impact on disease. By now, we have learnt much about the chemistry and biochemistry of oxygen [12] and its highly reactive derivatives with reaction times in the nano- to millisecond range [29], as well as the metabolic role of H_2O_2 in cellular signaling cascades and oxidative stress [53]. It is clear now that for normal physiologic function of cells, including reproduction in general and spermatozoa in specific, ROS at very low concentrations are essential because these molecules do not only trigger essential sperm functions such as capacitation, acrosome reaction, and spermatozoa-oocyte binding [10, 17, 49, 50] but also cause oxidative stress at higher concentrations [11].

Oxidative stress is a concept that was formulated by Helmut Sies in 1985 [52]. Groundbreaking work by John Aitken [9] and Ashok Agarwal [3] lead to the recognition of oxidative stress as major cause of male [2, 51] and female infertility [4, 5, 44] leading to miscarriage or birth defects [56]. Later it became clear that due to the dual effect of ROS on sperm function [18, 19] there must be a very fine balance between ROS essential for proper physiological activity and antioxidant protection from cellular oxidative damage. Hence, not only the ROS but also the antioxidants have to be regulated to counterbalance oxidative stress. Too high concentrations of antioxidants have also been found to be detrimental, as this condition can cause reductive stress [28, 34, 43] and is as harmful as oxidative stress [13]. In light of the recent, rapid developments in both the fields, redox-biology and human reproduction, it is time for a comprehensive update.

Oxidative Stress and Toxicity in Reproductive Biology and Medicine – A Comprehensive Update on Male Infertility provides such an update at the right time

highlighting the important topics. This book covers human fertility, including assisted reproduction, in general, the special emphasis is on male reproduction and provides a unique collection of chapters spanning from the description of various sources of ROS in the reproductive system and their physiological roles in the generation of ROS to their pathological influence on fertility in a variety of diseases. Since not only diseases or aging but also environmental pollution can cause increased oxidative stress, the book also describes a number of environmental toxicants as well as radiation and their effects on fertility. This is followed by a section dedicated to the treatment of oxidative stress with herbal medicine and antioxidants. The topics covered are then rounded off with chapters on the impact of male oxidative stress on recurrent pregnancy loss, the treatment of oxidative stress in embryo culture as well as the impact of reductive stress on male fertility.

The sources of ROS causing oxidative stress in the reproductive system are manifold and include physiological production of ROS in all aerobic living cells. These, small amounts of ROS have been shown to trigger essential physiological functions such as capacitation [17]. On the other hand, excessive production or exposure of cells to excessive amounts of ROS caused by adverse lifestyle [22, 26, 47] and related conditions such as obesity [42], diabetes mellitus [1, 24], or poor nutrition negatively [54, 62] affect fertility in men and women and modification of the lifestyle conditions can have positive effects on fertility [62]. In addition, genital tract infections/inflammations [33, 35], scrotal hyperthermia due to tight underwear [38, 48], sedentary position [25, 55], varicocele [37] or cancer [57] and its treatment with radiation [8, 15] and chemotherapy [20, 36] all have significant negative effects on fertility. On the other hand, infertility is also regarded as a surrogate marker for cancer development [40].

In addition to the aforementioned production of high ROS levels due to medical reasons, in the past century, environmental pollution has become a significant cause of infertility, of which parts of the causes are oxidative stress-driven where the pollution is directly or indirectly triggering high ROS production. The resultant oxidative stress does not only have a direct negative effect on the gametes [21, 60] but also detrimental effects on the testes [32, 46]. Furthermore, the detrimental effects are not only direct but also indirect if pregnant women are exposed to environmental toxicants. Among other problems, this can lead to disturbances in the sex determination.

About 80% of the global population is dependent on traditional and herbal medicine for their primary health care [27] including reproductive issues. This book addresses this globally important issue by including four chapters on the role of herbs and phytomedicine. Many plants are used not only due to their contents of pharmacologically active compounds such as atropine, triterpene saponins, and lactones, or flavonoids and tannins, but also due to the high concentrations of antioxidants such as lycopene and various vitamins. Interestingly, about 25% of modern prescription medicines contain bioactive plant compounds. In Western medicine, still little is known about herbal remedies to treat diseases [14]. Therefore, it is important to bring these aspects of the usage of herbal medicine as primary or supplementary therapy option to the attention of the readers [16].

Since ROS play such important physiological roles in all bodily functions, shedding light on redox imbalances in reproductive events as a whole is important, including embryo development [31, 59]. The oxygen concentration in the embryo surrounding environment is only about 8% in the fallopian tube, about 5% or less in the uterus [58] and is becoming hypoxic or anoxic during early implantation [41]. This situation makes a metabolic shift from oxidative phosphorylation to glycolysis necessary [31, 59]. Hence, keeping the redox balance between oxidants and antioxidants is of utmost importance for normal embryonic development as too high concentrations of either ROS or antioxidants detrimental because essential transcription factors such as Nrf2, NFκB, or AP1 are redox-sensitive and if not properly triggered can interfere in signaling pathways [23, 59] and lead to embryonic death and teratogenesis [30, 61]. Therefore, this book, as the first of its kind in the field of human reproduction, throws more light not only on the pathology of male factors leading to recurrent pregnancy loss but also on the essential functions of the finely balanced redox systems needed for successful fertilization with its implications even in embryo culture systems.

Last, but not least, this book on oxidative stress and its toxicity in reproductive biology also addresses the use of antioxidants, which are not only freely available over the counter but also often prescribed by clinicians to treat oxidative stress-related conditions of infertility. In most cases, this is done without having properly diagnosed the patient for oxidative stress. Oral antioxidants provide an excellent safety and are cost-effective [6, 7, 39]. On the other hand, in respect to their effects, the bivalent actions of both ROS and antioxidants to trigger essential physiological reactions and to cause harm are widely unknown. Whereas for ROS the detrimental effects are generally known, the detrimental effects of antioxidants are either not known or are ignored. However, as ROS have beneficial effects in triggering e.g. sperm capacitation, antioxidants have detrimental effects, particularly if they are available at too high concentrations when they are causing reductive stress [34], a condition which is as harmful as oxidative stress [13].

Since the discovery of ROS and the recognition of oxidative stress as major contributing factor to numerous diseases including infertility, we have learnt a lot about oxidative stress and generally associate negative events with it. Opposed to this, antioxidants are thought to be ‘good’ and ‘healthy’ and neutralize the “bad” effects of oxidative stress. With the increase of knowledge, however, we began to realize that such “black and white painting” is incorrect. It is now clear that ROS are also crucial triggers for essential physiological functions. On the other hand, for antioxidants, we are just at the beginning of understanding that they can also have serious detrimental effects. This includes not only their functions but also the realization of reductive stress as cause of disease and infertility. The lesson that we have to learn is that only a finely regulated balance between oxidation and reduction provides a healthy environment for cellular function. Therefore, it is essential to properly diagnose the redox status and the causes of a patient’s infertility before starting with a treatment which could worsen the fertility status. Hence, a personalized diagnosis with subsequent adjusted treatment is of utmost importance. The problem that we are facing at this stage is that we do not know what is “normal,” not only for male

infertility but also for female infertility. For embryo development, there are even no data available yet.

To come to this point has been a long journey, and with this limited information available for human infertility treatment, this book provides the reader with an authoritative overview of redox stress in human reproduction. The comprehensive knowledge provided in this book will assist not only andrologists and urologists in better comprehension of male infertility but is also a source of information for gynecologists, embryologists, and basic scientists to further their understanding of fertilization as a highly complex process. With all the up-to-date evidence provided, this book will be an invaluable source of information to improve patient management and laboratory procedures.

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Chapter 2
Reactive Oxygen Species in the Reproductive System: Sources and Physiological Roles

Anandan Das and Shubhadeep Roychoudhury

Abstract Reactive oxygen species (ROS) are oxygen-containing molecules which are reactive in nature and are capable of independent existence in the body. ROS comprise mostly of free radicals that contain at least one unpaired electron. Endogenous sources are the foremost birthplaces of ROS, which include mitochondrial electron transport chain, endoplasmic reticulum and peroxisome. Conversely, numerous enzymatic pathways such as xanthine oxidase and cyclooxygenase systems are among the prominent generators of cellular ROS. Major sources of ROS in the female reproductive tract include Graafian follicles, follicular fluid, fallopian tube, peritoneal cavity and endometrium. On the contrary, leukocytes, immature spermatozoa and varicocele are the key originators of ROS in the male reproductive system. For the sake of maintaining a proper oxidative balance, cells have evolved a variety of antioxidative molecules. From the physiological perspective, ROS and antioxidants are actively involved in the regulation of myriad female reproductive processes, such as cyclic luteal and endometrial changes, follicular development, ovulation, fertilization, embryonic implantation, maintenance of pregnancy and parturition. Similarly, physiological amounts of ROS are crucial in the accomplishment of various male reproductive functions as well, which include spermatozoa maturation, capacitation, hyperactivation and acrosome reaction. This chapter highlights the birthplaces of ROS in the female and male reproductive tract along with mechanisms of their production. This chapter will also put forward specific physiological roles of these reactive molecules in upholding the structural integrity and functionality of both the reproductive systems.

Keywords Oxidative stress · Free radicals · Female · Endometrium · Ovulation · Implantation · Male · Spermatozoa · Hyperactivation · Acrosome reaction

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2.1 Introduction

Reactive oxygen species (ROS) are extremely reactive oxidizing molecules which are endogenously produced during mitochondrial oxidative phosphorylation of the cellular aerobic metabolism [1]. Among the endogenous sources, mitochondria are the most prominent sources of ROS [2]. Apart from the mitochondrial electron transport chain (ETC), the major intracellular sources of ROS production include endoplasmic reticulum and peroxisomes [3]. Moreover, enzymatic pathways such as reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, lipoxygenase, cyclooxygenase and the cytochrome P450 systems serve as prominent sources of ROS generators in the cell [4]. ROS may also arise exogenously from interactions with external sources like environmental pollutants, ionizing radiations, ultraviolet light, chemotherapeutic agents, inflammatory cytokines, xenobiotic compounds and bacterial invasion [1, 5]. ROS are mostly free radicals in nature, most significant of which are superoxide ($O_2^{\bullet -}$) and hydroxyl anions ($OH^{\bullet}$) [6]. Apart from free radicals, non-radicals such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are also considered as important forms of ROS [7].

ROS, irrespective of its class, possess the fundamental chemical properties necessary for conferring reactivity to different biological targets. ROS also play the role of signalling molecules which help in regulating biological and physiological processes [8, 9]. From evolutionary biology studies, it is quite evident that nature had selected ROS as signal transducers for the allowance of adaptation to changes in nutrient availability and the oxidative environment [10]. When present in reasonable strength, ROS play vital roles in maintaining a large number of physiological activities in both female and male reproductive systems [11]. In the female, ROS are known to modulate several physiological processes ranging from ovulation to fertilization. They also play a critical role during gestation and normal parturition as well as initiation of pre-term labour [3]. In men, ROS are actively involved in the normal physiology of the spermatozoa, including facilitation of the maturational stages, thereby, maximizing their fertilizing ability [12].

As already mentioned, ROS serve as key signalling molecules in physiological processes when present in optimum concentration, but when the balance is interrupted towards a surplus of ROS, it gives rise to various pathological conditions. Due to their ability to play both physiological and pathological roles, ROS are often regarded as a double-edged sword [3]. In battling the excess ROS there exists an antioxidant defence mechanism in the body, comprising enzymes or non-enzymes which are capable of neutralizing the reactive molecules. Antioxidant defence is surpassed when ROS concentration rise beyond its normal capacity, thereby disrupting the balance, which leads to a condition called oxidative stress. Oxidative stress can result in disruption of cellular functioning or even cell death through several mechanisms including lipid peroxidation, nucleic acid, and protein damages [13].

This chapter highlights some of the important free radicals and non-radicals that exert biological effects on the reproductive system. Along with this, some of the

major endogenous sources responsible for production of ROS in the female and the male reproductive tracts have been delineated. Finally, it sheds light on various ROS-mediated reproductive physiologies and their complicated mechanisms of action.

2.2 Reactive Oxygen Species (ROS)

ROS is a recurrently utilized term in biological sciences and medicine. Aerobic organisms utilize molecular oxygen (O_2) which results in the generation of a large number of oxygen-containing reactive molecules that are collectively known as ROS [14]. ROS is a cumulative term to include $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$,$^1O_2$, peroxyl radical ($LOO^{\bullet}$), alkoxyl radical ($LO^{\bullet}$), lipid hydroperoxide (LOOH), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl) and ozone (O_3), among others [15]. The oxides of nitrogen such as nitric oxide ($NO^{\bullet}$), $ONOO^-$ and nitrogen dioxide ($NO_2^{\bullet}$) are generally included under the category of reactive nitrogen species (RNS). RNS are a family of nitrogen moieties associated with O_2, which are produced during the reaction of NO with $O_2^{\bullet-}$ and H_2O_2. Similarly, chlorine-containing reactive species, such as HOCl, that are capable of chlorinating and oxidizing other molecules are called reactive chlorine species (RCS). However, these are not a frequent matter of concern in biology and medicine, as compared to ROS and RNS [14].

Some of the O_2-containing reactive molecules have unpaired electrons and are thus called O_2-free radicals. On the other hand, those ROS which are devoid of unpaired electrons are called non-radical ROS [14]. Some of the major free radicals and non-radicals are discussed below.

2.2.1 Free Radicals

Free radicals are defined as atoms or molecules containing one or more unpaired electrons in the outer electronic orbital that are capable of existing independently in the body. Their high reactive nature renders them the ability to abstract electrons from other compounds to attain stability. The molecule which is attacked eventually loses its electrons and becomes a free radical itself, which then initiates a chain of reaction cascade [16].

2.2.2 Superoxide Anion ($O_2^{\bullet-}$)

Superoxide ($O_2^{\bullet-}$) is an anionic radical which is generated during the one-electron reduction of O_2 [17]. This reaction can occur non-enzymatically in the mitochondrial ETC or by the activity of NADPH oxidase and xanthine oxidase enzymes [18].

The concentration of $O_2^{\bullet-}$ are kept in check by effective compartmentalization of the sequential decrease of O_2 and by widespread expressions of an enzyme called superoxide dismutase (SOD), which converts these anions into non-radical species such as $H_2O_2$and $^1O_2$ [19, 20].

Superoxide ($O_2^{\bullet-}$) is fairly non-reactive to most of the biological molecules, even though the low level of $O_2^{\bullet-}$ tolerable in cells and tissues indicate that restraining cell exposures to $O_2^{\bullet-}$ is a significant selector for survival [21].

2.2.3 Hydroxyl Radical (OH•)

Hydroxyl radicals ($OH^{\bullet}$) are generated in situ by either oxidation of water or hydroxide ions. The interaction between H_2O_2 and reduced transition metals such as copper and iron may also lead to the production of $OH^{\bullet}$. Moreover, water molecules when exposed to ionizing radiation can give rise to $OH^{\bullet}$ [17, 18, 22].

Hydroxyl radicals ($OH^{\bullet}$) are highly reactive species and most of the organic molecules are vulnerable to its attack. The oxidation potential of these molecules renders them highly oxidizing in nature [23]. Furthermore, due to the non-selective nature of $OH^{\bullet}$, many susceptible organic molecules in the body such as acids, aromatics and ketones get degraded by its action [24].

2.2.4 Nitric Oxide (NO•)

Nitric oxide ($NO^{\bullet}$) is a gas and a diatomic free radical which is synthesized in vivo from L-arginine and O_2 by nitric oxide synthase (NOS). Heme-containing proteins are accountable for biological synthesis of $NO^{\bullet}$, which is generated by two successive monooxygenase reactions at the NOS heme active site [25].

Nitric oxide ($NO^{\bullet}$) mediates myriad biological functions through stimulation of its primary cellular receptor haemoprotein-soluble guanylate cyclase, which in turn, produces the second messenger molecule cyclic-guanosine monophosphate (cGMP) [25]. The bioavailability and actions of $NO^{\bullet}$ are mediated by its reaction with $O_2^{\bullet-}$, which yields reactive peroxides and $ONOO^-$, thereby merging O_2 radicals and $NO^{\bullet}$ pathways [26]. $NO^{\bullet}$ regulates endothelium-dependent regulation of blood flow and pressure as well as inhibits the activation of blood platelets, which holds particular importance in achieving a penile erection. Moreover, $NO^{\bullet}$ is recognized as a neurotransmitter in certain types of nerves [27].

2.3 Non-radicals

Non-radicals are the atoms or molecules which, unlike the free radicals, are devoid of unpaired electrons [14]. The non-radical species mediates their biological effects by initiating free radical reactions in the living organisms [28].

2.3.1 Hydrogen Peroxide (H_2O_2)

It is primarily produced as a metabolic by-product of aerobic respiration taking place in the mitochondria, where partial reduction of O_2 results in the generation of $O_2^{\bullet-}$. In addition, enzymes such as SOD helps in the production of H_2O_2 from $O_2^{\bullet-}$ [29]. Moreover, H_2O_2 may also be generated endogenously by $p66^{Shc}$ enzyme activity, a member of the Src homology/collagen protein family [30]. H_2O_2 may get decomposed to water in cytosol and mitochondria by the activity of scavenger enzyme glutathione peroxidase (GPx) or in the peroxisome by the action of catalase [31].

It has been considered a toxic molecule for living systems for a long time [32]. However, recent findings have elucidated the physiological roles of H_2O_2 and has recognized it as a ubiquitous endogenous molecule. H_2O_2 has been suspected to play the role of second messenger system with a pro-survival activity in several physiological processes [33].

2.3.2 Singlet Oxygen ($^1O_2$)

Singlet oxygen ($^1O_2$) is a non-radical that represents an excited state of O_2. In humans, $^1O_2$ is produced by enzymatic activation of O_2 and during phagocytosis by macrophages [34, 35]. $^1O_2$ is not as stable as the ground state O_2 and tends to change back fairly quickly at the cost of surrounding molecule, thereby inducing a chemical effect [34].

Being a strong oxidant, $^1O_2$ possesses the capability of readily oxidizing lipids, proteins and nucleic acids present in the cell [36]. Though, unlike radical O_2 species, $^1O_2$ is rather mild and non-toxic to mammalian tissues [37]. $^1O_2$ exerts its biological effects by acting as a cell signal messenger through the activation of Ca^{2+}-regulated K^+ channels [38].

Table 2.1 Major ROS in human body along with their biosynthetic pathways

	ROS	Mechanism of biosynthesis	References
Free radicals	Superoxide ($O_2^{\bullet -}$)	Enzymatic or non-enzymatic reduction of molecular oxygen (O_2)	[17,18]
	Hydroxyl ($OH^{\bullet}$)	Oxidation or ionization of water molecules	[17,18]
	Nitric oxide ($NO^{\bullet}$)	Reaction of L-arginine and molecular oxygen (O_2) in presence of NOS	[25]
Non-radicals	Hydrogen peroxide (H_2O_2)	Mostly by the partial reduction of molecular oxygen (O_2) and subsequent action of SOD during aerobic metabolism	[29]
	Singlet oxygen ($^1O_2$)	Enzymatic activation of oxygen (O_2) and during phagocytosis	[35]
	Ozone (O_3)	Decomposition of dihydrogen trioxide (H_2O_3) during antibody-catalyzed water oxidation pathway	[39]

2.3.3 Ozone (O_3)

Ozone (O_3) is best known as a gaseous compound occurring in the stratosphere where it shields the entry of harmful solar radiations. However, it has been suspected that O_3 is also produced endogenously in biological systems through the process of antibody-catalyzed water oxidation pathway [39]. This reaction occurs in a hydrophobic site in the antibody molecule, where dihydrogen trioxide (H_2O_3) is formed during the initial reaction of water with $^1O_2$, which subsequently decomposes to H_2O_2 and O_3 [40].

Ozone (O_3) is a highly reactive gaseous allotrope of O_2 and it can have pathogenetic consequences by cleaving the unsaturated lipids and oxidizing the sulphur and nitrogen atoms of proteins [41]. However, judicious concentration of O_3 can reduce oxidative stress by upregulating intracellular antioxidant enzymes and it can also provoke the generation of ROS acting as natural physiological activators of various biological functions [42]. The major ROS produced in the human body along with their respective biosynthetic pathways are summarized in Table 2.1.

2.4 Sources of ROS in the Female Reproductive System

ROS have been linked with various physiological and pathological functions of the female reproductive system [3]. The female reproductive tract is thought to be a potent site of ROS production. ROS are mostly generated in the ovarian cells such as the Graafian follicles and in other non-ovarian tissues like the fallopian tube, the endometrium and the peritoneum [7].

2.4.1 Graafian Follicle

Graafian follicles have been claimed to be a possible source of ROS because of the oocyte and various immune cells such as macrophages and neutrophils. The oocyte produces ROS by various mechanisms – the most prominent being oxidative phosphorylation that takes place in the oocyte for production of ATP by utilizing O_2. Another pathway is the xanthine oxidase system of the follicle, which generates ROS during the elimination of nitrogenous wastes mainly in the form of uric acid. Furthermore, immune cells produce ROS in the form of HOCl by the NADPH oxidase system to protect Graafian follicles against microbial attack [43].

The role of NADPH oxidase along with SOD type 3 (SOD3) has been studied extensively in the follicular cells of *Drosophila*. An NADPH oxidase and an extracellular SOD3 functions in follicular cells of *Drosophila* egg chamber to produce H_2O_2, which is critical for follicular rupture and ovulation. Human follicles also express these two enzymes and thus could perform similar roles in women [44]. The mammalian NADPH oxidase family comprises of seven members, of which, the expression of NADPH oxidase 4 and 5 (NOX4 and NOX5) are found in human granulosa cells [45]. NOX4 and NOX5 allocate an electron across the granulosa cell membrane from NADPH in the cytosol to O_2 in the luminal or extracellular space. This intercellular transportation of electrons eventually generates $O_2^{\bullet -}$, which in turn, is rapidly converted to H_2O_2 by SODs [44]. ROS can also generate from the cumulous oophorus, which originates from undifferentiated granulosa cells [46].

Generation of native ROS is essential for cellular physiology while widespread ROS may be harmful [44]. Oxidative stress initiating from excessive production of ROS in the granulosa cells and cumulous oophorus is detrimental to oocyte functioning and viability and can even lead to DNA damage [47].

2.4.2 Follicular Fluid

The production of follicular fluid is undertaken by granulosa cells and theca interna cells, which are the likely producers of ROS [48]. Follicular fluid contains various steroid hormones, growth factors, leukocytes and cytokines, all of which are the potent generators of ROS [49]. During the ovulatory process, ROS are produced in the follicular fluid physiologically and serve as key signalling molecules in regulating various reproductive functions such as maturation of oocyte, steroid production by ovary, and functions of the corpus luteum, as well as the development of fertilization and embryo [50].

Follicular fluid creates a unique microenvironment necessary for maintaining the quality of oocyte, implantation, and the development of early embryo. Overproduction of ROS in follicular fluid may alter the fine balance of distinctive composition which are critical for female fertility [51]. For this reason, follicular fluid also houses various antioxidant enzymes, which under physiological conditions, avoid

ROS production and forage prevailing free radicals, thereby protecting the ovarian somatic cells, and maintaining a healthy oocyte physiology [52].

2.4.3 Fallopian Tube

Some animal and human studies have confirmed the presence of ROS in the fallopian tubes – the most noteworthy is $NO^{\bullet}$ [53–55]. An endogenous ROS generation system exists in the fallopian tube which has been demonstrated by positive NADPH diaphorase staining [53]. The formation of $NO^{\bullet}$ is catalyzed by NOS which exists in the human tubal cells. It has already been mentioned that $NO^{\bullet}$ arises from O_2 and L-arginine by the action of NOS. $NO^{\bullet}$ further reacts with $O_2^{\bullet-}$ to give rise to $ONOO^-$, which is necessary for the process of signal transduction [25].

There are three isoforms of NOS: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) [3]. The three isoforms of NOS are produced differently in various tissues of the human fallopian tube [56]. However, only tubal epithelial cells produce all the isoforms of NOS, signifying that the tubal epithelial cells are potentially significant determinant of the effect of $NO^{\bullet}$ in the fallopian tube [57]. eNOS and nNOS are accountable for incessant basal release of $NO^{\bullet}$ and both require calcium/calmodulin system for activation [58]. The third isoform iNOS is an inducible form independent of calcium and is released in reply to inflammatory cytokines and lipopolysaccharides [59]. A study conducted on knockout mouse model has indicated that the activity of $NO^{\bullet}$ is sustained at appropriate levels by the action of NOS isoforms [60].

Studies have shown that $NO^{\bullet}$ could affect the contraction of fallopian tube smooth muscle and ciliary beat frequency present in epithelial cells of airway via cGMP pathway, which is necessary for smooth passage of ovum from the ovary [55, 61]. However, at high concentrations, it can lead to immune reactions and tissue damage [57]. Thus, to limit ROS toxicity, antioxidant enzymes are supplied in abundance to the fallopian tubes [3]. It is noteworthy that decreased level of $NO^{\bullet}$ can have negative effects on the ovum [47].

2.4.4 Peritoneal Cavity

The space between the parietal peritoneum and visceral peritoneum is called peritoneal cavity. It largely houses the visceral organs such as liver, stomach, small and large intestines, and associated smaller organs. The female reproductive organs such as uterus, fallopian tubes and ovaries also project into the peritoneal cavity [62, 63].

The main origin points of ROS in the peritoneal cavity include macrophages, apoptotic endometrial cells, red blood cells (RBC) and menstrual-reflux debris [64, 65]. Various pro-oxidant and pro-inflammatory factors are released by RBCs, such as haemoglobin and its by-products heme and Fe^{2+}, into the peritoneal conditions.

Heme and Fe^{2+} are indispensable for human cells but play a crucial role in the generation of lethal ROS, unless appropriately chelated [66].

Disruption in the balance of ROS and antioxidant enzymes in the peritoneal cavity is suspected to cause endometriosis, a major causal factor of female infertility [67]. Increased level of ROS may also regulate the expression of genes encoding immunoregulators, cytokines and cell-adhesion molecules implicated in the induction of endometriosis [64].

2.4.5 Endometrium

The endometrium is the innermost epithelial lining of the uterus, which is highly organized in nature and is under cyclical control of ovarian steroids and pituitary hormones [68]. Various physiological processes related to the endometrium are controlled by ROS present in physiological concentrations. Endometrial stromal cells are speculated to be the potential source of ROS. The endoplasmic reticulum and the electron transport system both in mitochondria and in the nucleus of these stromal cells are the intracellular sources of ROS [69]. The production of ROS increases during the late secretory phase of the endometrium, which is believed to carry out a vital role in endometrial shedding, prior to menstruation [70].

A fine balance exists between ROS and the various defence systems against oxidative stress in the endometrium. SODs present in cytosol and mitochondria metabolize $O_2^{\bullet -}$, whereas GPx metabolize H_2O_2. Disruption of oxidative balance in the endometrium results in abnormalities of normal reproductive physiological functions such as menstruation and decidualization [69].

Various endogenous sources of ROS in the female reproductive tract have been elucidated in Fig. 2.1.

2.5 Sources of ROS in the Male Reproductive System

Human spermatozoa are the active production sites of ROS and $O_2^{\bullet -}$ is considered the most abundant ROS [71, 72]. In fact, $O_2^{\bullet -}$ is the precursor of other ROS types in spermatozoa. The reduced product of $O_2^{\bullet -}$ reacts with itself via dismutation to generate H_2O_2. In presence of Fe^{2+} and Cu^{2+}, H_2O_2 further reacts with $O_2^{\bullet -}$ to undergo the Haber-Weiss reaction which generates the highly reactive $OH^{\bullet}$ [73, 74]. The generation of ROS in spermatozoa occur via two methods: (a) the NADPH oxidase system at the level of spermatozoa plasma membrane, and (b) the reduced nicotinamide adenine dinucleotide (NADH)-dependent oxidoreductase system at the level of mitochondria [75].

NADPH oxidases are a set of enzymes located in the plasma membrane of spermatozoa, which catalyze the conversion of bivalent O_2 to $O_2^{\bullet -}$. NADPH molecules produced by the hexose monophosphate shunt act as the substrate for this reaction

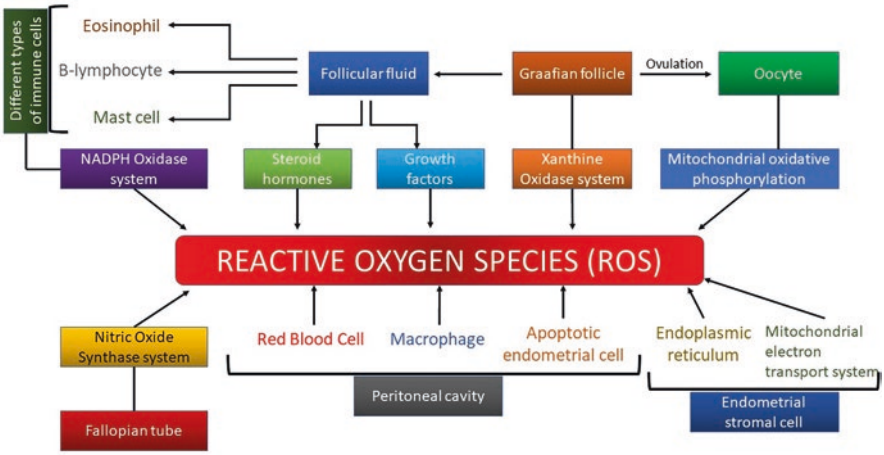

Fig. 2.1 Endogenous sources of ROS in the female reproductive system. ROS may be produced in the Graafian follicle via the xanthine oxidase system or by the oxidative phosphorylation that takes place in the mitochondria of the oocyte. The follicular fluid present inside the Graafian follicle harbours various steroid hormones and growth factors which may also give rise to ROS. Immune cells such as eosinophils, mast cells and B-cells present in the follicular fluid also produce ROS via the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system. The nitric oxide synthase system operating in the tubal cells of fallopian tube contributes to the generation of ROS. Red blood cells, macrophages and apoptotic endometrial cells present in the peritoneal cavity also release ROS. Endoplasmic reticulum and the mitochondrial electron transport chain present in the endometrial stromal cells are also a potent generation site of ROS.

[76]. NOX5 is a member of the NADPH oxidase family, the presence of which has been confirmed in the acrosome and the midpiece region of spermatozoa [77]. NOX5 is stimulated when Ca^{2+} attaches to its cytosolic N-terminal domain. Eventually, several conformational changes in the cell initiate the production of $O_2^{\bullet-}$ [72].

The mitochondrial oxidoreductase system contributes to the bulk of ROS in the human spermatozoa. This is due to the abundance of mitochondria, which is required to provide relentless supply of energy for the sake of spermatozoa motility [75]. Respiring spermatozoa are an active foundation of ROS, in which the NADH oxidoreductase catalyzes the transfer of electrons from NADH to coenzyme Q10 in the mitochondrial ETC. During this process of inverse transfer of electron, electron leakage occurs, which reduces O_2 to $O_2^{\bullet-}$ [78]. The upsurge of the respiratory rate of spermatozoa increases the concentration of O_2 in the mitochondria, which in turn results in escalated release of $O_2^{\bullet-}$ [79].

ROS are mainly detected in the seminal plasma, which are endogenously originated from different sources. The human ejaculate contains diverse types of round cells from different developmental stages of spermatozoa, leukocytes and epithelial cells. Leukocytes mainly neutrophils and macrophages, and immature spermatozoa are considered the chief endogenous generators of ROS [80, 81].