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Oxidative stress: Major executioner in disease pathology, role in sperm DNA damage and preventive strategies

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1. ABSTRACT

Oxidative stress (OS) has been implicated in a wide array of diseases such as neurodegenerative disorders, autoimmune diseases, complex lifestyle diseases and cancer. OS is caused by an imbalance in production of Reactive Oxygen Species (ROS) and antioxidant defenses in the cell which results in the damage of cellular components, inactivate essential metabolic enzymes and disrupt signal transduction pathways. OS induces peroxidative damage to the sperm plasma membrane, DNA fragmentation in sperm nuclear/mitochondrial genome and causes dysregulation in levels of mRNAs/transcripts. OS induced sperm DNA damage is associated with male infertility, recurrent pregnancy loss (RPL), congenital malformations and high frequency of childhood disorders. OS induced pathologies are caused by endogenous and exogenous factors, majority of which, are modifiable. Antioxidant supplementation could help in relieving OS, however, its long-term usage may disrupt the intricate oxidation-reduction balance and can lead to “Reductive Stress”. Adoption of simple lifestyle interventions may relieve OS and can also aid in its management. This may improve

Table 1. Classification of reactive oxygen species and reactive nitrogen species

Reactive Oxygen Species
Radicals
Superoxide (O_2^-)
Hydroxyl (OH^-)
Peroxyl (RO_2^-)
Alkoxyl (RO^-)
Hydroperoxyl (HO_2^-)
Non-radicals
Hydrogen peroxide (H_2O_2)
Hypochlorous acid (HOCl)
Hypobromous acid (HOBr)
Ozone (O_3)
Singlet oxygen (Δ g)
Reactive Nitrogen Species
Radicals
Nitric oxide (NO^-)
Nitrous acid (HNO_2)
Non-radicals
Nitrogen dioxide (NO_2)
Nitrosyl cation (NO)
Nitrosyl anion (NO^-)
Dinitrogen tetroxide (N_2O_4)
Dinitrogen trioxide (N_2O_3)
Peroxynitrite ($ONOO^-$)
Peroxynitrous acid (ONOOH)
Alkylperoxynitrites (ROONO)

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overall quality of life (QOL) and can reduce prevalence of OS induced diseases.

2. INTRODUCTION

Oxygen is an imperative element that has sustained life on Earth. It is utilized by aerobic living organisms "aerobes" for energy production via the mitochondrial electron transport chain (ETC). During this energy deriving i.e., adenosine triphosphate (ATP) synthesizing chemical reaction, free radicals are generated as by-products which chiefly includes ROS and Reactive Nitrogen Species (RNS). ROS are highly reactive oxidizing agents belonging to the class of free radicals or reactive species and includes hydrogen peroxide (H_2O_2), hydroxyl or superoxide radicals, superoxide anion, peroxyl or hydroxyl radicals and singlet oxygen whereas RNS includes nitric oxide, nitrous acid, peroxynitrite and dinitrogen trioxide (Table 1). Free radicals (ROS and RNS) are constantly produced during physiological conditions and serves beneficial as well as detrimental impact on the biological system depending upon their relative concentrations. The aerobic system is also well-endowed with antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione reductase (GR), ascorbate, α-tocopherol etc. which protects the aerobic system from free radical induced toxicity and methodize total ROS/RNS levels to maintain physiological homeostasis. OS is a condition originated as a result of imbalance between pro-oxidant and antioxidant systems which thereby disrupts cellular metabolism and its regulation and ultimately damages essential cellular components (DNA, proteins and lipids) (1). At lower, moderate or physiological levels, ROS and RNS serves detrimental role in various biological processes and serves as inter and intra-cellular signaling intermediates, messengers of information to nucleolus, messengers of metabolism and essential

immune functions whereas at higher levels ROS/RNS generates OS. OS is associated with chronic and regressive ailments such as accelerated ageing, cancer, autoimmune dysfunction, cardiovascular disease, neurological disorders, pulmonary diseases, rheumatoid arthritis, nephropathy, ocular diseases, disease of the reproductive system (male and female infertility) etc. (2–4). Sperm plasma membrane is most accessible to OS induced injury because of the high content of polyunsaturated fatty acids (PUFAs) which provides exuberant sites for free radical induced damage (5). It also has a limited cytosolic space with minimal antioxidants post spermiogenesis. Sperm harbors a truncated DNA damage detection and repair system which makes sperm further vulnerable to accumulate oxidatively induced damage to both mitochondrial and nuclear DNA (6). Sperm with higher levels of DNA damage and DNA fragmentation is associated with clinical pathologies such as male factor infertility, RPL, and paternally mediated increased risk of congenital malformations, neuropsychiatric disorders and childhood cancers (7). Sperm with higher levels of oxidative DNA damage and associated single/double strand breaks, telomere shortening, GC to TA transversions and dysregulated sperm transcripts is associated with impaired and slower cleavage, poor blastocyst morphology, pre- and post-implantation losses, higher risk of embryonic death and poor fertilization outcomes in Assisted Reproductive Techniques (ARTs) such as *In vitro* fertilization (IVF) and Intra cytoplasmic sperm injection (ICSI). Therefore, integrity of paternal sperm DNA/RNA is a critical factor and cannot be ignored while discussing the role of paternal factors in fertilization outcomes, pregnancy rates and risk of genetic/epigenetic disorders in the offspring (8). Impact of advanced paternal age/delayed parenthood (after 30 years) is associated with increased mitochondrial and nuclear DNA damage, increased ROS levels, genomic fatigue in oocyte with suboptimal repair of sperm DNA damage and its persistence in the subsequent generations, which predisposed to several childhood diseases (9). Administration of various antioxidants or their combinations (in food or in supplement form) by increasing dietary intake of fruits and vegetables highly enriched in polyphenolic antioxidants or by using synthetic form of antioxidants may exert beneficiary effect on the human health and may help in reducing OS burden. However, indiscriminate and sustained usage of antioxidants doesn't have prolonged effects and its sustained usage is harmful ultimately leading to the generation of "Reductive Stress" impairing numerous redox sensitive metabolic processes. This enigma is called as "antioxidant paradox" (10). Till date the exact therapeutic doses of antioxidants at which there is a positive impact on sperm DNA integrity is still not clearly documented and therefore, regular monitoring is warranted as moderate level of OS are beneficial in maintaining genomic integrity by maintaining telomere length (11). During reductive stress, ROS production overwhelms the ROS scavenging capability employed by antioxidants and thereby generates net mitochondrial ROS spillover causing further oxidative damage and injury (12). OS induced pathologies are caused by factors that could be easily modified by maintaining healthy habits like moderate exercise, cessation of smoking and reducing intake of alcoholic beverages, avoiding non-vegetarian food, avoiding pesticide exposure and avoiding excessive usage of mobile phones and adopting simple lifestyle interventions like yoga, meditation and breathing practices may improve the QOL and help in cessation of OS as well (13, 14). This review started with an aim to investigate the role of OS in disease initiation and progression. Subsequently, we focused on OS induced sperm DNA damage and its clinical manifestations. We also discussed the role of antioxidant therapy for reducing cellular and seminal OS followed by reviewing the role of lifestyle modifications and interventions to mitigate the impact of OS induced cell injury.

3. OXIDATIVE STRESS: ORIGIN AND HISTORY

3.1 Evolution of the concept of cellular oxidative stress

Gershman's (1954) "Free radical theory" which suggested that the toxic effect of oxygen is due to partially reduced forms of oxygen has laid the foundation for the origin of the field of free radical biochemistry. After the second world war (1939–1945) and Hiroshima-Nagasaki (1945) massive casualty, Gershman and Gilbert tried to explore the factors responsible for huge loss of life during the Hiroshima-Nagasaki catastrophe and came to the conclusion that ionizing radiations has the lethal impact on biological systems which is mediated by the action of ROS on cellular machinery. The usage of the term "Stress" dated back to 1658 when Robert Hooke, a British Physicist used this term to explain Hooke's law to illustrate the theory of elasticity (15). The term stress was first used in the context of biological science by Sir Hans Selye in his short letter to *Nature* in the year 1936 to explain the "general adaptation syndrome" (which he later named as stress) in rats as represented by the triad of enlarged adrenal glands, lymph node and thymic atrophy, and gastric erosions/ulcers (16). Denham Harman in 1956 paraphrase the role of free radical in ageing process. Stress is now defined as a condition originated due to perturbation in biochemical homeostasis originated due to sociological, philosophical, psychological or environmental stressor which can be any stimulus or agent anticipated by the body as challenging and hence, the body's response is generated by activating and coordinating various organ systems (especially the pulmonary, cardiovascular

and renal systems) to protect against the damaging legacy employed by the stressor (10). Thus, previous studies done in the subject of free radical biology has prompted many scientists to explore the ruinous effects of free radicals and its negative impact on the biological systems and has opened up unlimited opportunities for further research in this emerging field. Free radical mediated OS generation is now considered as the basic mechanism underlying the origin of many human diseases. Various mechanisms and pathways converge together in the OS generation has opened new frontiers for further research in this never-ending field of free radical biology.

3.2 Evolution of the concept of seminal oxidative stress

Human spermatozoa are profoundly vulnerable to OS induced damage because of the presence of bountiful PUFAs on their plasma membrane, low amounts of antioxidants due to constrained volume, restricted distribution of cytoplasmic space and an inefficient DNA damage detection and repair system unable to completely remove oxidative DNA lesions (17).OS damages the sperm plasma membrane deteriorating its fluidity and also damages the integrity of sperm nuclear and mitochondrial genome and initiates pathologies such as male infertility, RPL, paternally mediated increased risk to childhood disease such as neuropsychiatric disorders, developmental anomalies, congenital malformations and childhood cancer. ROS play a central role in mediating the pathogenesis of many reproductive processes and OS-induced sperm DNA damage hasten up the process of germ cell apoptosis leading to decline in sperm count associated with male factor infertility. The first observation of OS induced defective sperm function was made by Dr. John MacLeod in 1943 when he reported a remarkable observation that human spermatozoa when incubated in high oxygen tension lose their motility by mechanism which could be recovered by concomitant presence of catalase-a specific scavenger of H_2O_2. The clinical implication of this finding divulges that human spermatozoa have the capacity to generate ROS (mainly H_2O_2) which have negative impact on its motility. In 1946, a paper published in *Nature* by Tosic and Walton confirmed the notion that spermatozoa could generate ROS; specifically H_2O_2 (18). In 1950, Tosic and Walton wonderfully demonstrated that bovine spermatozoa possess an enzyme system which give rise to H_2O_2 in concentrations which, although relatively low, are toxic to the respiration and motility of the spermatozoa. In this paper, the authors described that L-amino acid oxidase acts on aromatic amino acids specifically L-phenylalanine in the presence of molecular O_2 and produce H_2O_2 by dehydrogenation and deamination (19). In 1982, Shannon and Curson confirmed the presence of L-amino acid oxidase in bovine spermatozoa which is active only in dead spermatozoa and actively generates H_2O_2 as a toxic by-product of phenylalanine oxidation under conditions of high oxygen tension. The rate of production of H_2O_2 depends upon number of dead spermatozoa, substrate concentration, oxygen tension and temperature (20). In 1976, Yanagimachi *et al.*, introduced a novel technique the 'zona-free hamster oocyte penetration assay' to selectively assess the fertilizing potential of human spermatozoa using zona pellucida free (hamster) ova as a substitute for human ova (21). This assay further laid down the foundation of an important observation which was later on confirmed by Aitken *et al.,* (1993) that sperm show reduced fertilizing potential in infertile men as compared to the fertile individuals even when their spermatozoa were treated with A23187 (cation ionophore which induces calcium influx in the sperm plasma membrane to induce acrosome reaction and sperm-oocyte fusion) (22). This important observation evoked scientists to find out the causes and mechanisms of OS induced decline in sperm function and its impact on fertilizing potential of the spermatozoa. In 1987, Aitken and Clarkson and Alvarez independently described that human spermatozoa have the capacity to generate ROS in the male germ line (23). The above-mentioned breakthroughs in the era of modern andrology prompted many Andrologists to further investigate the cause and consequences of OS induced damage to human spermatozoa and its association with the rising burden of defective sperm associated pathologies and increased disease burden.

4. OXIDATIVE STRESS: ROLE IN CELL PHYSIOLOGY, DISEASE PATHOGENESIS AND INCREASED RISK OF DISEASE

Free radicals are the chemical species having an unpaired electron, hence are very unstable and react quickly with other molecules to acquire the needed electron in order to become stable. (24). Free radicals possess very small size and molecular weight lesser than that of proteins and some of the signaling molecules which makes some of the free radicals able to penetrate through the cell membranes (25). The sources of free radicals are endogenous and exogenous both (Figure 1). Free radicals are generated as by-product of cellular metabolism during aerobic respiration in the mitochondria (during ETC) which constitutes the major proportion of endogenous free radical production. The other endogenous sources of free radicals include peroxisome, lipoxygenases, neutrophils, eosinophils, NADPH (Nicotinamide adenine dinucleotide phosphate) oxidase, xanthine oxidase etc. The exogenous sources of free radical production include ionizing radiation, UV- light, chemotherapeutic agents, environmental factors such as infectious agents, food, smoking, alcohol, pollution, chemicals, etc. (26). Elevated levels of free radicals generate OS which is defined as a state resulting due to disproportionate pro-oxidant/antioxidant levels in favor of the pro-oxidants leading to cellular and tissue

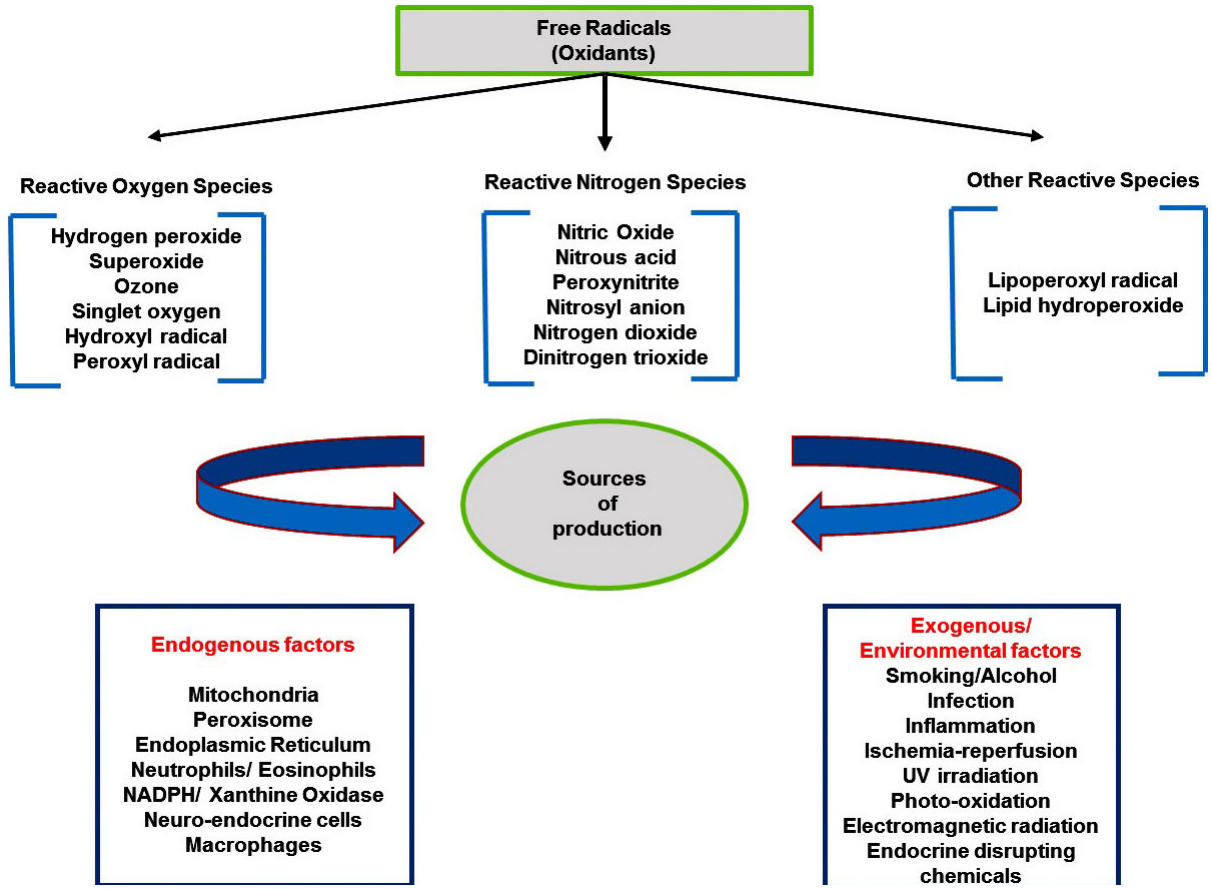

Figure 1. Types of free radicals/oxidants and their endogenous and exogenous sources of generation.

damage. Elevated oxidant levels in the biological system activates specific signaling pathways leading to negative impact on cellular processes which ultimately ends up in accelerated ageing and age-associated disorders (27). According to the "free radical theory of ageing" given by Denham Harman in 1956, the process of ageing and age-associated degenerative disorders which involves damage to cellular components and connective tissues are caused by the free radicals generated by biological reactions involving molecular oxygen and arise primarily as a result of aerobic metabolism (28).

4.1. Reactive oxygen species: Are they essential?

ROS also known as Reactive oxygen intermediates (ROI) are the chemically reactive species having a single unpaired electron in their outermost shell of electrons and originated as by-products of cellular metabolism. Low or moderate ROS levels in the body serve some very vital functions such as host defense mechanism, wound healing, physiological regulation of cell signaling, cellular growth, regulation of cytokines, neuromodulation, immune modulation and immune defense, inflammatory response, apoptosis, ion transport, T-cell regulation, fibroblast adhesion and spreading, regulation of gene expression etc. (29–31). In aerobic cells, electron transport and oxidative phosphorylation are the vital activities of the protein complexes in the inner mitochondrial membrane, which serves as the major source of cellular energy/ATP synthesis. This process of cellular energy metabolism generates ROS as by-products. The mitochondrial ETC harbors complexes I-IV and complex V (ATP synthase). Pairs of electrons enter ETC from NADH (the reduced form of Nicotinamide adenine dinucleotide) (produced during glycolysis and citric acid cycle) in complex I (NADH-CoQ Reductase) which are then transferred to Coenzyme Q, which carries electrons through the membrane to complex III ($CoQH_2$-cytochrome c reductase). During this electron transfer process, superoxide (O_2^-) radical is generated and about 80% of the generated superoxide is released into the mitochondrial intermembrane space and rest 20% is released into the mitochondrial matrix (32). Superoxide anion is then released into the cytoplasm mediated by the mitochondrial permeability transition pore which is present at contact sites between the mitochondrial outer and inner membranes (33). Superoxide further dismutates into H_2O_2 by either mitochondrial antioxidant manganese superoxide dismutase (MnSOD) or by cytoplasmic Copper and

zinc-containing superoxide dismutase (Cu/ZnSOD). Reduction of H_2O_2 to water is mediated by CAT and GPx. However, in the presence of transition metals, such as iron and copper, H_2O_2 is reduced to hydroxyl radical **(•OH)** (34). The reactivity of hydroxyl radical is so great that it does not diffuse more than one or two molecular diameters before reacting with its cellular target and must be generated immediately adjacent to DNA to oxidize it. Therefore, it is likely that H_2O_2 serves as a diffusible, latent form of hydroxyl radical that reacts with a metal ion in the vicinity of a DNA molecule to generate hydroxyl radicals (25, 35). **Fenton's reaction** (as described below) is an important biochemical reaction which is mainly dependent upon H_2O_2 concentrations and causes localized damage close to its site of formation.

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

The highly reactive hydroxyl radical then can readily oxidize proteins, lipids, carbohydrates, DNA, and RNA (Richter *et al.*, 1995). Peroxisomes are the second major sites of ROS generation and scavenging where superoxide and H_2O_2 are generated by xanthine oxidase during peroxisomal β-oxidation pathway (36). In the Endoplasmic Reticulum (ER), ROS is generated by redox-signaling mediators under ER-associated stress conditions which holds an important step of the ER mediated unfolded protein response (UPR), involved in several pathophysiological processes (37). Primary cellular antioxidants such as glutathione and thioredoxin are also maintained in their functional redox state by the ROS levels and any changes or perturbations in the balance between ROS production and the capacity to rapidly detoxify it, generates OS (38). Being a major intracellular signaling molecules, ROS also plays a crucial role in tumor development and progression. Novel curative strategies for cancer treatment involving chemotherapeutic agents and radiotherapies rely on regulating intracellular ROS signaling, depriving the malignant cells from ROS-induced tumor promotion and ROS generation to destroy malignant cells by inducing apoptosis (39).

4.2. Oxidative stress induced damage to biomolecules

4.2.1. Effect of oxidative stress on DNA

Oxidative DNA damage can be induced by oxygen radicals, various oxidizing agents, photo-oxidation and ionizing radiations. ROS induced DNA damage results in generation of apurinic/apyrimidinic (AP) sites, single/double strand breaks, thymine glycol and oxidatively generated clustered DNA lesions (40). Oxidatively induced lesions in DNA (possibly due to ionizing radiations) results in chromosome breakage leading to mutation and genomic instability and cancer (41). Single strand breaks and specifically the double strand DNA breaks are the most genotoxic and unrepaired lesions which leads to neurodegeneration and neurodegenerative diseases (Alzheimer, Parkinson, Amyotrophic lateral sclerosis, etc.) (42). 8-hydroxy-2'-deoxyguanosine (8-OHdG) is the most common oxidative DNA adduct and serve as the most potential indicator of OS mediated DNA damage (43).

4.2.2 Effect of oxidative stress on lipids

Excessive ROS levels in the cell reacts with the PUFAs present on the cell membrane and initiates lipid peroxidation reaction leading to the formation of lipoperoxyl radical (LOO•) which further reacts with a lipid to yield a lipid radical and a lipid hydroperoxide (LOOH). This lipid peroxidation cascade eventually terminates in the generation of highly reactive lipid peroxidation products such as malondialdehyde (MDA), 4-Hydroxynonenal (4-HNE), acrolein and isoprostanes (44). Lipid peroxidation by ROS leads to loss of membrane properties such as membrane permeability and membrane potential and also affects the cellular integrity. 4-HNE and MDA lipid peroxidation products are the most thoroughly investigated, they further react with DNA and proteins due to their electrophilic nature (26). 4-HNE is considered as the secondary messenger of OS execution and considered as highly toxic because of its ability to rapidly react with thiols and amino groups of proteins resulting in the formation of protein carbonyls and also reacts with DNA to form ethano DNA adducts. 4-HNE is a dynamic modulator of various cellular process such as signal transduction, cell proliferation and apoptosis and have been shown to be associated with pathologies such as neurodegeneration, cancer and accelerated ageing. Because of the ability of 4-HNE to execute apoptosis in cancer cells, its therapeutic potential to specifically target and to kill cancer cells has been extensively explored since last few decades (45). MDA is considered as the most mutagenic product of lipid peroxidation and due to its highly stable nature, plasma concentrations of MDA are a predictive indicator of lipid peroxidation and OS (46).

4.2.3 Effect of oxidative stress on proteins

OS due to UV irradiation, photo-oxidation, inflammation, ischemia or reperfusion injury induces reversible and irreversible modifications in the protein which includes protein oxidation where the excessive ROS reacts with amino acid side chain and peptide backbone (47). The reversible oxidation-reduction involves that of thiol groups and cysteine and methionine oxidation but these amino acids are brought back to their reduced state by the action of protein repair enzymes for instance the oxidation of methionine to methionine sulfoxide is reversed by the action of methionine sulfoxide reductase present in eukaryotes and prokaryotes both (48). The irreversible

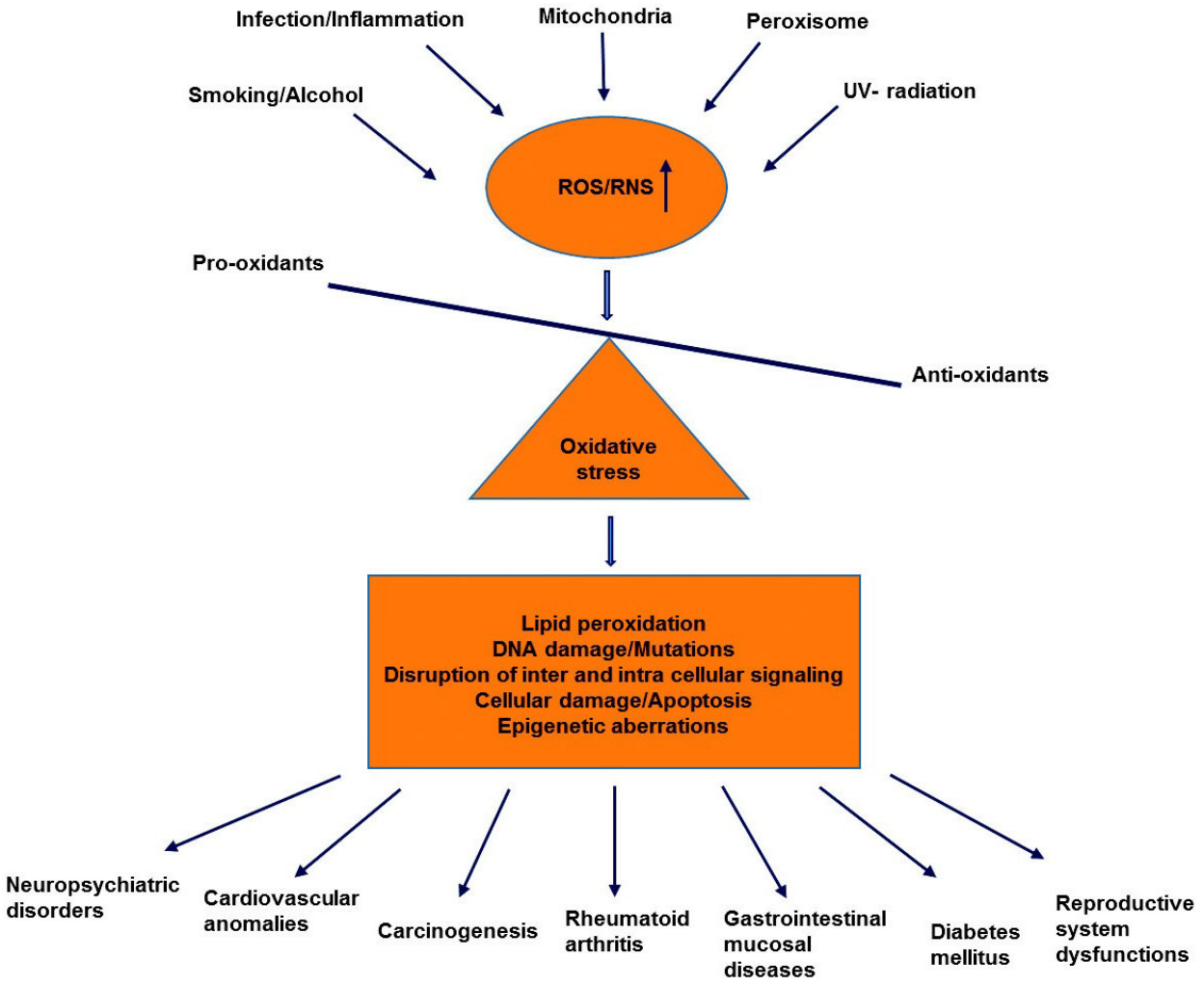

Figure 2. Pathway elucidating free-radical mediated oxidative stress generation and its role in the execution of various diseases.

oxidative damage includes ring cleavage in amino acid histidine or tryptophan, carbonylation and tyrosine nitration which are used as potent biomarkers for evaluation of OS induced diseases (49).

4.3. Oxidative stress and role in various diseases

OS plays an important role in the pathogenesis of various diseases. OS induced execution of various human diseases is described briefly in the following section and also with the help of Figure 2. A detailed pathway showing ROS/RNS generation, subsequent chain reactions and their consequences on cellular system has been explained via Figure 3.

4.3.1 Oxidative stress and Neurodegenerative disorders

Neurodegenerative disorders are characterized by the progressive loss or dysfunction of specific neurons and protein aggregation and includes Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS), depression, dementia and Multiple sclerosis (MS). The pathology of neurodegenerative disorders is OS induced neuronal death, apoptosis and excitotoxicity which are the main attributes of neuronal diseases (50).

AD is characterized by neuronal degeneration and cognitive decline affecting a large proportion of the ageing population. Brain tissue damage and accumulation of amyloid β-plaques in AD patients is caused due to protein/lipid/DNA oxidation, advanced glycation end products, lipid peroxidation products such as 4-HNE, and glycoxidation which is a result of OS which accumulates with the advancing age (51).

PD is characterized by the selective loss of dopaminergic neurons in the substantia nigra pars compacta of the brain and accumulation of intracellular inclusion bodies known as Lewy bodies of α-synuclein. The exact cause for PD is still

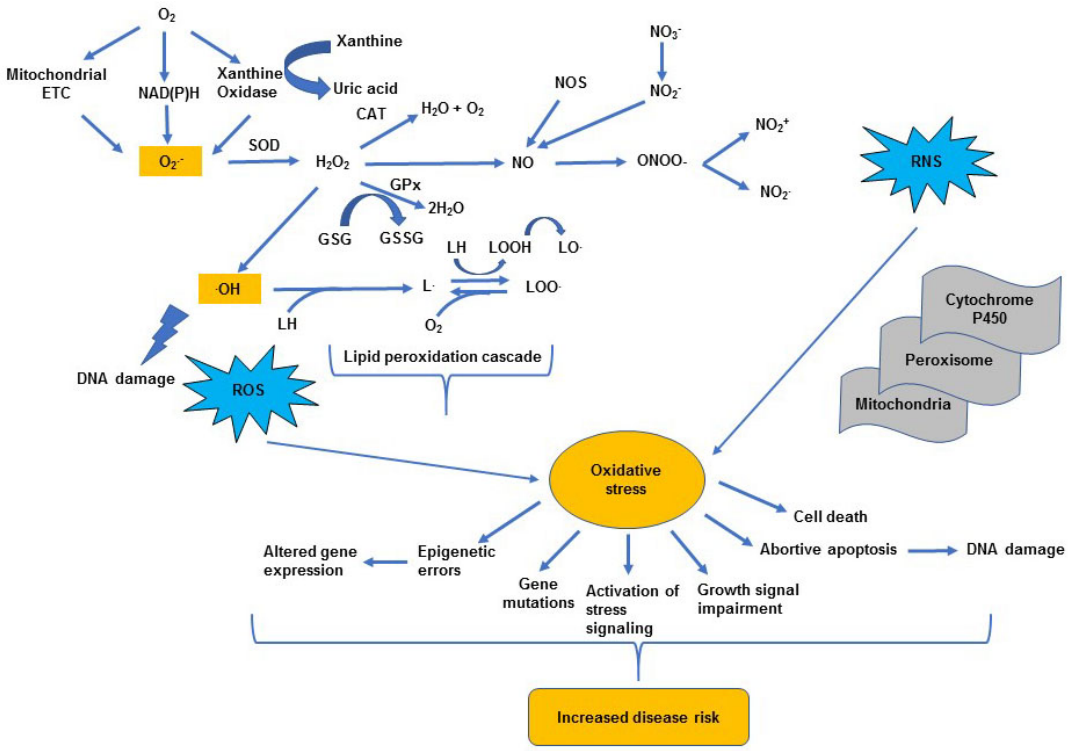

Figure 3. Pathway showing ROS/RNS generation, subsequent chain reactions and their consequences on the cellular system.

questionable but idiopathic and genetic cases of PD are mainly attributed due to excessive ROS which leads to OS induced dopaminergic neurotoxicity (52). The metabolic process of dopamine which involves its oxidation and quinone modification also contributes to OS generation leading to cellular dysfunction and cell death (53).

ALS or Motor neuron disease (MND) is one of the most common adult-onset neurodegenerative disorder characterized by progressive degeneration and death of lower motor neurons located in the spinal cord and brain stem and upper motor neurons located in the motor cortex. The mechanism behind ALS pathology is still elusive however, 20% of familial MND cases are caused by the mutations in Copper or Zinc SOD (Cu/Zn *SOD1*), the gene encoding for copper–zinc superoxide dismutase (54). More than 100 mutations have been already reported in Cu/Zn *SOD1* gene leading to the toxic gain-of-function by SOD causing motor neurons death as evident in ALS patients. Two theories have been given to explain this gain-of-function by SOD. The oligomerization hypothesis suggests that mutant SOD proteins become misfolded and subsequently oligomerize into high molecular weight species that ultimately lead to the death of motor neurons whereas the oxidative damage hypothesis suggests that mutant SOD proteins harbors loss of the active sites required for Cu binding which result in conversion of SOD protein itself into a pro-oxidant which participates in ROS generation and thus, leading to OS (55).

4.3.2 Oxidative stress and Cardiovascular anomalies

At physiological levels, ROS serves essential role as cell signaling mediators, maintains cardiovascular homeostasis and regulate vascular functions such as vascular inflammation in atherogenesis and vascular smooth muscle cell relaxation and contraction. In the recent years, *in vitro* studies and studies on various animal models showed the role of OS in pathogenesis of cardiovascular diseases (CVDs) such as coronary heart disease, atherosclerosis, ischemic heart disease, ischemia-reperfusion injury, cardiomyopathy, arrhythmias, cardiomyopathy and hypertension (56). The vascular risk factors for the development of CVDs are many and includes smoking, alcohol, diabetes, hypertension, age, physical idleness, overweight etc. All of these factors together markedly increases vascular ROS production and cause oxidative damage by the common underlying mechanism involving mitochondrial dysfunction, enzyme degradation, cellular damage and apoptosis (57).

4.3.3 Oxidative stress and cancer

Cancer is a multistage, multistep process caused by amalgamation of various exogenous

and endogenous factors which include physical and chemical agents that mediates molecular and cellular changes transforming a normal cell into a malignant neoplastic cell. During neoplastic transformation of a normal cell, various events such as modification of redox homeostasis, modification of energy metabolism, oncogene activation, mitochondrial dysfunction lead to OS generation (58). Elevated ROS levels causes oxidative DNA damage resulting in single or double strand DNA breaks, replication errors, base modification, base oxidation, DNA cross-linking which leads to cell dysfunction, cell death, DNA mutation which if not repaired prior to replication will lead to genomic instability and thus, cancer (59). Elevated ROS can affect various signaling pathways and activates key transcription factors such as Nuclear factor erythroid 2-related factor 2 (Nrf2) and Nuclear factor-kappaB (NF-kB) resulting in altered gene expression patterns which mediate the cancer progression (60). 8-OHdG is the most common oxidative base adduct DNA which is formed due to elevated OS and has been widely used for the estimation of endogenous oxidative DNA damage and a risk factor for many diseases including cancer (43). Chronic inflammation as induced by biochemical or physical factors also increases risk of developing tumors probably by mechanism underlying genetic or epigenetic instability, altered gene expression, resistance to cell death and metastasis. Hence, inflammation is considered as "silent killer" as it poses risk for developing cancers in later stages (61).

4.3.4 Oxidative stress and Reproductive system dysfunctions

Balance in the pro-oxidant and antioxidant levels are very essential for regulating various reproductive processes such as spermatogenesis, testis function, sperm maturation, folliculogenesis, oocyte maturation, embryogenesis, fetal growth, etc. Imbalance in pro-oxidant/antioxidant levels and OS has been associated with initiation and development of pathological processes affecting male and female fertility status.

Male factor infertility accounts for nearly 50% cases of all the infertility cases. OS is considered as a significant contributor for male factor infertility (62). The primary sources of ROS generation in sperm include activated leukocytes in the seminal plasma and mitochondria in the spermatozoa. Men with elevated levels of ROS show sperm DNA damage and sperm DNA fragmentation leading to infertility (63). ROS are required by the spermatozoa at normal/physiological levels for its maturation and function, motility, sperm-oocyte fusion, acrosome reactions and sperm capacitation via activation of tyrosine phosphorylation whereas pathophysiological ROS levels in spermatozoa causes lipid peroxidation which damages sperm plasma membrane and at much higher levels ROS can also induce sperm apoptosis (64).

A plethora of studies have already documented the role of OS in pregnancy complications and outcome. Imbalance in pro-oxidant and antioxidant levels and a reduction in antioxidants is associated with clinical implications such as polycystic ovary syndrome (PCOS), endometriosis, RPL, spontaneous abortion, preeclampsia and unexplained female infertility, intrauterine growth restriction. Maternal lifestyle habits such as cigarette smoking, alcohol use, obesity, malnutrition, recreational drug use, environmental and occupational exposures lead to oxidative convulsion and thus, alter the reproductive stability (65).

4.4. Oxidative stress and ageing

Commoner *et al.*, (1954) with the help of electron spin resonance technique were the first to explain the generation of free radicals in living system and also explained that free radicals are produced more in those tissues that are highly metabolically active as compared to the tissue that are less metabolically active (66). Followed by Commoner, Denham Harman in 1956 articulated the "Free Radical/Oxidative Stress Theory of Ageing" which is the first theory to explain the molecular basis of age-associated accumulation of free-radicals which is the major determinant of lifespan. Free radicals are accumulated with advancing age causing cellular OS leading to lipid peroxidation of cell membranes, homeostatic imbalance, dysfunction of cell macromolecules, enzyme inactivation, increased mutation rate and cell death/injury. This could be prevented by adoption of healthy lifestyle habits such as calorie restriction (CR), physical exercise, use of food items rich in antioxidants which may reduce levels of free-radicals, decrease cellular senescence, increases longevity and declines risk of age-associated diseases (67). However, several *in vitro* and *in vivo* experimental models of antioxidant administration failed to explain the cause behind lifespan reduction which has led to the basis of mitochondrial theory of ageing. This theory more specifically explains the ROS induced damage and cause of lifespan reduction because mitochondria are not only the primary endogenous source of ROS generation within the cell but also the most potent targets of ROS induced damage (68). Summative damage caused by ROS to mitochondria and mitochondrial DNA is one of the major causes of ageing as mitochondrial damage further boost the ROS generation causing age related diseases such as cardiac ageing, muscular ageing, age associated neurodegenerative disorders and age-related predisposition to certain cancers (69). Several researchers have given their views regarding the mechanism underlying the process of ageing but none of them proven as a landmark which could explain this above- mentioned mechanism precisely therefore,

further research is required to be carried out in the field of ageing biology which could decipher the associated novel pathways which may further help in finding a cure for age-associated pathologies and disease risk.

The emanating evidences from previous studies done in the field of ageing biology showed that ageing is a multifactorial phenomenon regulated by genetic make-up of the individual and persuaded by the epigenetic factors (70). Mitochondria are considered as the chief sources of endogenous free radical generation via the ETC (71). Electron leakage during ETC directly goes to O_2 and generates unstable, short-lived free radicals such as superoxide anion, hydroxyl radical whereas the non-radical so produced such as H_2O_2 is comparatively long lived and can freely diffuse across cell membrane (2). H_2O_2 has both reducing and oxidizing properties, acts as a signaling molecule, involved in several redox signaling pathways and mediates several physiological processes such as cell proliferation, differentiation, and migration. Due to its linear or trans-planar conformation, H_2O_2 cannot pass easily across cell membranes to mediate its functions and hence, diffusion of H_2O_2 across cell membranes is mediated via channel proteins such as aquaporins. The free radicals and non-radicals generated during mitochondrial respiration causes oxidative devaluation in DNA, lipids, carbohydrates and lipids and play a crucial role in ageing. Mitochondria also play a crucial role in regulating apoptosis via modulating the apoptotic machinery (72). Further evidence for mitochondrial ROS associated ageing came from studies on mice having targeted mutation of *p66*^{Shc} gene. *p66*^{Shc} gene encodes for P66^{Shc} which is a mitochondrial redox enzyme located in the mitochondrial intermembrane and generates H_2O_2 during the ETC. P66^{Shc} regulates the levels of ROS, induction of apoptosis and lifespan in mammals and mice with *p66*$^{Shc-/-}$ genotype showed increased resistance to ROS and age-associated disease pathologies and thus, showed a 30% increase in their lifespan (73, 74). Age-associated accumulation of mitochondrial OS activates apoptosis which contributes to the ageing process (75). In addition to the endogenous source of ROS generated by the mitochondria, the NADPH oxidases which are a family of plasma membrane-associated enzymes found in a variety of cell types and first described in neutrophils, also generates superoxide anion from oxygen using electrons from NADPH there by trigger cellular transformation or replicative senescence (76, 77). The three cardinal markers of ageing namely OS, DNA damage and shortening of telomeres is seen in infertile men with normozoospermia and oligozoospermia. Thus, we believe that infertility could be accelerated testicular ageing (78). Though in any system we may not be able to reverse ageing but by adoption of simple lifestyle modifications like yoga and meditation based lifestyle intervention (YMLI), having plant based whole food, minimal intake of nutritiously depleted food can significantly reduce the OS levels and OS induced damage to both nuclear and mitochondrial genomes. YMLI can also upregulate the levels and activity of telomerase, a reverse transcriptase and assists in telomere length maintenance and maintenance of genomic integrity and chromosomal stability (14, 79).

Sirtuins

Sirtuins are a group of conserved nicotinamide adenine dinucleotide (NAD)-dependent histone/protein deacetylases, homologous to the Sir2 (silent information regulator 2) of *Sacchromyces cerevisiae*. Sirtuins are shown to regulate lifespan in many model organisms including yeast and mice. Sirtuins regulates metabolic and genotoxic stress in an organism by regulating and coordinating diverse array of cellular functions such as cell survival pathways, cell-cycle control, DNA damage response, autophagy and metabolic homeostasis. Sirtuins are ubiquitously expressed and found in the cytoplasm, mitochondria or nucleus of the cell (80). OS and oxidative DNA damage accumulates with advancing age and a major cause for cellular senescence. Dietary regimen called as CR declines the steady-state levels of OS and its associated damage and increase longevity and maximum life-span in mammals as depicted by studies done by increasing the enzymatic antioxidant defenses in transgenic *Drosophila melanogaster* (81). *SIRT1* located in the nucleus and *SIRT3* located in the mitochondria are the mammalian homolog of yeast *Sir2* and forms an auto-regulatory feedback loop to regulate the ROS levels, OS and cellular longevity. Under CR conditions, *SIRT1* deacetylases several transcription factors that regulate antioxidant genes. For example, *SIRT1* activates Forkhead box *protein* O1 (FOXO) family of transcription factors that further activates *SOD1* (stress response gene). It also controls the gluconeogenic/glycolytic pathways in liver by activating the transcriptional coactivator peroxisome proliferator-activated receptor coactivator 1-α (PGC-1α) which induces mitochondrial biogenesis and expression of antioxidant genes such as CAT and GPx. *SIRT1* inhibits the cytokine-mediated cytotoxicity, Nitric Oxide (NO) production, and inducible nitric oxide synthase (iNOS) expression by deacetylating the p65 subunit of the nuclear factor NF-κB and hence decreases the OS levels. Mitochondrial *SIRT3* acts by deacetylating the enzymes required for maintaining the ROS levels. *SIRT3* deacetylases the mitochondrial antioxidant enzyme *SOD2* at two critical lysine residues and enhances its antioxidant property and thereby reduces the total cellular ROS. *SIRT3* modulates the activity of mitochondrial isocitrate dehydrogenase 2 (*IDH2*) by deacetylation and thereby increasing the NADPH levels. An increased ratio of reduced to oxidized glutathione in mitochondria protects from OS induced cell death/injury. *SIRT3* reduces tumor growth/progression by inhibiting the activity of hypoxia

inducible factor-1α (*HIF-1α*) probably by reducing the ROS levels which mediates *HIF-1α* activity in hypoxic environment. Thus, *SIRT1* and *SIRT3* together mediate the antioxidant protective role which intensify the cellular longevity (82). YMLI can significantly upregulate levels of *SIRT1* independent of CR (83).

5. MITOCHONDRIAL METABOLISM AND OXIDATIVE STRESS

The first evidence regarding mitochondrial ROS generation was given by Jensen in 1966 but this discovery remained obscure until 1971 when Loschen *et al.* first demonstrated that intact pigeon heart mitochondria showed succinate dependent H_2O_2 production during the mitochondrial respiratory chain reaction which could be completely prevented by the uncoupling agent pentachlorophenol (84, 85).

Mitochondria "the powerhouses of the cell" carried two major functions in aerobic organisms. The first function is the energy production and metabolism and the second is the regulation of programmed cell-death or apoptosis. Mitochondria are the major sources of intracellular ROS generation which are generated mainly at complex I (NADH-CoQ Reductase) and complex III ($CoQH_2$-cytochrome c reductase) of the ETC. Electron leakage during electron transfer from one complex to the other of the ETC leads to electron leakage which ultimately reaches O_2 and generates free-radical such as superoxide anion which is a predecessor of ROS and further disseminates to produce H_2O_2 that can further react to form hydroxyl radical. About 1% of the total O_2 consumption by mitochondria is utilized to generate superoxide with the help of eight known sites present in the mitochondria that are filthy engaged in this process (86). Elevated ROS levels due to mitochondrial metabolism forms a "vicious cycle" and executes more ROS production thereby causing cellular damage (71). Mitochondria are also the executioners of intrinsic apoptotic cascade and mitochondrial ROS play crucial role in the release of cytochrome *c* from the mitochondrial intermembrane space, the activation of caspase-9 and the subsequent cell death (87). To perform its highly-specialized functions such as energy production, regulation of calcium and redox homeostasis and apoptosis, mitochondria continuously change their structure and morphology and engaged themselves in a process called as mitochondrial dynamics which includes repeated cycles of fission and fusion that intermixes the lipids and contents of a mitochondrial population in order to maintain mitochondrial homeostasis and quality (88). High mitochondrial ROS levels and OS affects mitochondrial dynamics and alterations in mitochondrial dynamics has implications in many human diseases including cancer (89). Dysregulation in mitochondrial dynamics due to OS increases Dynamin-1-like protein (Drp-1) mediated mitochondrial fission thereby increasing metastasis and chemoresistance in cancer cell (90). Mitochondrial ROS mitigates various cellular signaling pathways which has important roles in several biological and biochemical processes such as stress response, cell survival and function and neoplastic transformation of a normal cell. Hypoxia or low oxygen tension induces expression of *HIF-1α* which is a key regulator of a broad range of cellular and systemic responses to hypoxia in mammalian cells (91). Hypoxia increases ROS generation from complex III of mitochondria via the Q-cycle which creates a cytosolic signal to facilitate the *HIF-1α* levels by stabilizing the *HIF-α* subunit which is normally degraded and hence the expression of genes regulating the cell cycle and regulation, glycolysis and angiogenesis is enhanced (92). A key target of the secondary messenger Phosphatidylinositol 3,4,5-triphosphate (PIP_3) which is critical for cell proliferation and survival is a protein-serine/threonine kinase known as Akt. PIP_3 binding with Akt recruites Akt to the plasma membrane and its subsequent activation. Once activated Akt phosphorylates a number of target proteins that are direct regulators of cell survival, transcription factors and other protein kinases. Akt activation is also influenced by the mitochondrial ROS which is a result of increased mitochondrial metabolism (93). The other cell signaling pathways mediated by mitochondrial ROS includes NF-κB and TNF-α mediated cell death, stress response pathway mediated by *SIRT-1* leading to mitochondrial biogenesis, regulation of phosphatase activity, etc. (94, 95). In the male germ cells, mitochondria are not only the primary source of ROS production but are also the potential targets of ROS induced free radical damage. When there is an inadvertent ROS generation, the mitochondrial-nuclear cross talk in the male germ cells which is required to be maintained in harmony so as to drive essential sperm functions such as sperm capacitation, sperm-oocyte fusion has been disrupted. As a result of inadvertent ROS generation, mitochondrial stress may occur which may lead to mitochondrial DNA copy number variations, mitochondrial DNA mutations and disruption/disintegration of the electron transport chain. This initiates the mitochondrial-nuclear retrograde signaling which may induce global changes in sperm nuclear gene expression mediated by hnRNPA2 (novel histone acetyltransferase)-dependent epigenetic regulation by mitochondrial stress (96). Therefore, imbalance in the mitochondrial-nuclear cross talk in the male germ cells and mitochondrial-nuclear retrograde signaling may cause the transcriptional reprogramming of the genes essential for regulating spermatogenesis, developmentally essential genes and genes for sperm maturation and function. This may increase risk for testicular cancers and other associated pathologies such as male infertility, recurrent pregnancy losses, increased risk of childhood cancers, etc. Recent studies published in the context of mitochondrial

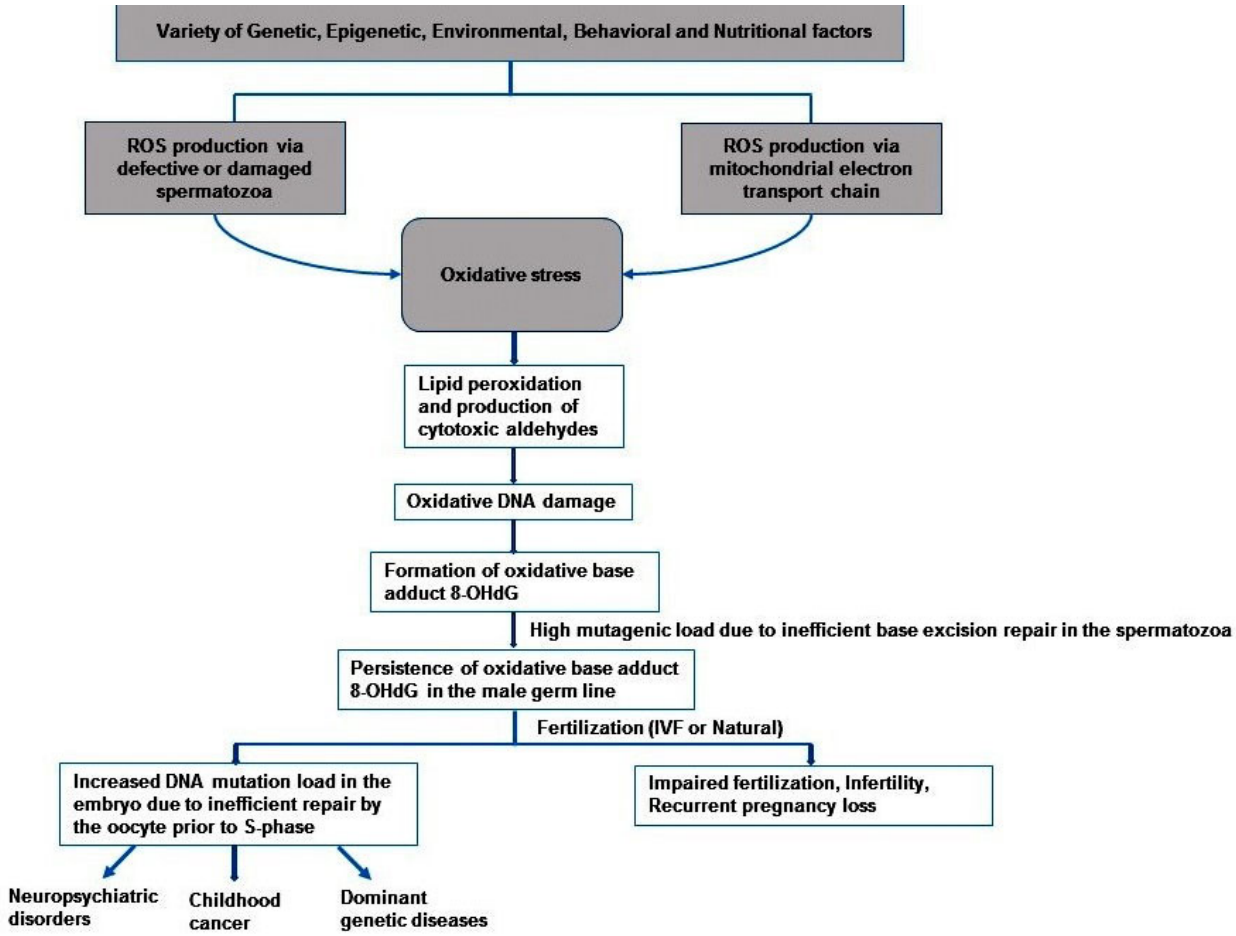

Figure 4. Seminal oxidative stress, oxidative DNA damage and its consequences.

stress biology and mitochondrial redox regulation has highlighted the role of the mitochondrial ROS as signal mediators and their requirement for cellular processes such as proliferation and differentiation and their supra-physiological levels causing cell damage or apoptosis. Therefore, further research on this field may open up new frontiers and may serve as therapeutic targets for many diseases where mitochondria plays a critical role.

6. OXIDATIVE STRESS: ROLE IN SPERM DNA DAMAGE AND CLINICAL MANIFESTATIONS

ROS is associated with the pathology of many reproductive phenomenon and hence, OS is found to be associated with the pathophysiology of male and female reproductive disorders such as male factor infertility, endometriosis, unexplained female infertility, poor outcomes in IVF, embryotoxicity and poor *in vitro* embryonic development (17). Human spermatozoa are highly specialized and polarized cells that fertilize the oocyte and convey the paternal contribution of the embryonic genome to the MII oocytes. During fertilization, the spermatozoa also delivers the mRNAs, microRNAs and other genetic and epigenetic factors that regulate embryogenesis and embryonic gene expression (97). Human spermatozoa are highly vulnerable to OS because they infest abundant PUFAs mainly docosahexaenoic acid (DHA) which provides plentiful sites for free radical induced lipid peroxidation, incomplete DNA damage detection and repair system with only one enzyme i.e., 8-Oxoguanine glycosylase (*OGG1*) of the base excision repair (BER) pathway (98).

Infertility affects nearly 8–12% couples globally in their reproductive age group and the cause is idiopathic/unexplained in nearly half of the cases and mainly attributed to male factor infertility (99). Male infertility is a serious complication of the developing world affecting nearly 1 in 20 men in their prime reproductive age and is further compounded by unhealthy lifestyle and delayed parenthood (100). Human male infertility is a multifactorial disorder encompassing genetic, epigenetic, environmental, behavioral and nutritional factors which altogether affect spermatogenesis and infertility phenotype (101). OS abolish sperm motility via induction of lipid peroxidation, damages sperm nuclear and

Table 2. Tests for sperm DNA damage detection and evaluation

Test	Detected parameter of sperm DNA damage	Characteristic feature of the test
Comet	Single and double stranded DNA breaks	Based on single cell gel electrophoresis, highly sensitive, direct assay
SCSA	Detect chromatin decondensation	Based on flow cytometer, low variability, indirect assay
TUNEL	Quantify both single and double stranded DNA breaks	Based on flow cytometer, direct assay
SCD	Detect sperm DNA fragmentation mainly single stranded DNA fragments	Based on characteristic halo produced by sperm, indirect assay
ISNT	Detect single stranded breaks via incorporation of probes catalyzed by DNA polymerase I	Less sensitive, direct assay
DNA oxidation	Detect oxidative base adduct 8-OHdG	Based on ELISA, direct assay

Reproduced with permission from (117–119). SCSA= Sperm chromatin structure assay; TUNEL= Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling; SCD= Sperm chromatin dispersion; ISNT= *In situ* nick translation; 8-OHdG= 8-hydroxy-2'-deoxyguanosine; ELISA= Enzyme linked immunosorbent assay

mitochondrial DNA and alter the sperm epigenetic profile and thus, negatively affects the sperm fertilizing potential (102). ROS are produced by the immature sperm cells or by the activated leukocytes in the seminal plasma. Spermatozoa harbors a wide range of antioxidants to maintain the optimum levels of ROS and exert beneficial effects on sperm function, pregnancy rate and pregnancy outcomes. Seminal plasma is also well-endowed with enzymatic antioxidants (CAT, SOD, and GPx) and non-enzymatic antioxidants (pyruvate, ascorbic acid, α-tocopherol, glutathione, taurine, hypotaurine, L-carnitine, coenzyme Q10, vitamins A, E, C, and B complex) and micronutrients such as zinc, selenium, copper and iron. Antioxidants provides the optimum protection to the spermatozoa against the ROS in two ways. Firstly, via the inactivation of ROS produced during sperm mitochondrial metabolism and rendering them inactive for the lipid peroxidation and secondly, by decreasing down the catalytic ROS generation (103). Total antioxidant capacity (TAC) as well as total reactive antioxidant potential (TRAP) was found to be relatively low in seminal plasma of sub-fertile male subjects as compared to the fertile controls (104, 105). Variations such as polymorphisms or genetic disruptions in antioxidant genes is associated with multifactorial phenotype of human male infertility which includes poor semen parameters (motility, morphology and sperm count), oligoasthenoteratozoospermia and oligozoospermia (106). In male infertility subjects, single nucleotide polymorphisms (SNPs) in antioxidant genes such as *Nrf2*, *SOD*, *NOS*, *GPx*, *CAT* may occur in consort with the environmental ROS. Therefore, assessment of antioxidant genes serve as potential biomarkers for male infertility assessment and also help in deciphering novel ROS mediated antioxidant signaling pathways and networks that work in consortium to cause male infertility (107). The lipid peroxidation is another major cascade driven by OS in human spermatozoa. The lipid peroxidation in spermatozoa is executed via two phases i.e., initiation phase and the propagation phase. The sperm plasma membrane is highly enriched in PUFAs (mainly DHA having six double bonds per molecule) which have unconjugated double bonds separated by methylene groups (bis-allylic position, -CH=CH-). The carbon-hydrogen dissociation energies are lowest at the bis-allylic position which provides numerous sites for free radical attack. The lipid peroxidation is initiated by hydroxyl radical which leads to rearrangement of the double bond and formation of diene that can be further oxidized (108).The lipid peroxidation cascade which is initiated due to hydrogen abstraction event is prompted and generates carbon-centered lipid radicals which combine with oxygen to produce peroxyl radical ($ROO^{\cdot}$) or alkoxyl radical ($RO^{\cdot}$) which in order to stabilize- sequesters hydrogen from the adjacent PUFAs to stabilize themselves and generate additional lipid radicals and thus, promotes the propagation of the lipid peroxidation chain reaction. The lipid peroxides so generated via this mechanism now became the potent targets of phospholipase A2 which cleave out the lipid peroxides to generate lysophospholipids which destabilizes the sperm plasma membrane, affects the functions of integral membrane proteins such as ATP-dependent ion pumps and voltage-regulated ion channels that are crucial for the maintenance of sperm motility (109). The lipid peroxidation chain reaction culminates together in the formation of small molecular mass electrophilic aldehydes such as acrolein, 4-HNE, and MDA (102). 4-HNE, an alkenal is a product of lipid peroxidation and highly toxic because of its ability to react rapidly with thiols and amino group. 4-HNE binds with mitochondrial proteins and trigger electron leakage and generation of ROS by sperm mitochondria. This ROS which propagates itself by the lipid peroxidation chain reaction and generates OS leads to oxidative damage, sequesters the spermatozoa to enter the intrinsic apoptotic cascade, DNA single and double strand breaks and ultimately cell death (110). MDA is an alkanal, formed during omega-6 fatty acids lipid peroxidation and it exists in electrophilic and nucleophilic both forms and form MDA-MDA dimers which are pro-mutagenic but also form MDA-DNA adducts known to induce mutations in

Table 3. Factors causing seminal oxidative stress in the male germline, their associated damage and the resulting pathologies

Factors (Genetic/ Lifestyle/ Environmental)	Characteristic of the damage to the spermatozoa	Resulting pathology
Smoking	Associated with sperm DNA fragmentation, leukocytospermia, carcinogenic	Increased risk of gonadal/extra-gonadal cancers, sperm DNA damage and increased disease burden/cancer in children.
Advanced age	Associated with oxidative DNA damage to the sperm	Increased risk of miscarriages, increased rate of *de novo* germline mutations in the offspring or other diseases such as schizophrenia, ASD, BPD, congenital malformations, huntington's disease, childhood cancer, etc.
Environmental toxicants (Lead, BPA, PCB, pesticides, herbicides, insecticides, air pollution, heavy metals)	Associated with increased sperm DNA fragmentation	Alteration in hormonal milieu, increased risk of children with congenital malformations.
Mobile phone	Associated with damage to the male reproductive system, hormonal imbalances and DNA fragmentation *in vitro*	Increased risk of developing male subfertility.
Dietary deficiencies (Nutritionally depleted processed food)	Associated with high levels of sperm DNA damage	Accelerated ageing and early onset of complex lifestyle diseases.

Reproduced with permission from (111, 116, 121, 123, 157). ASD= Autism spectrum disorder, BPD= Bipolar disorder; BPA= Bisphenol A; PCB= polychlorinated biphenyl

tumor suppressor genes and oncogenes (46). Increase in MDA levels in seminal plasma of infertile men is associated with impairment of sperm motility, sperm DNA fragmentation, hyperviscosity of seminal plasma and decline in sperm-oocyte fusion (111, 112). OS stress impedes sperm functions rendering it inactive to fertilize and the products of plasma membrane peroxidation such as 4-HNE and MDA increases the risk of developing testicular cancers in later stages. Therefore, OS can be designated as a forbearer of testicular cancers (113). Acrolein is a powerful electrophile that binds with mitochondrial proteins such as axoneme and dynein and form adducts, alter their confirmation, causes electron leakage and thus, trigger mitochondrial ROS generation which affects sperm movement (98). Figure 4 describes seminal OS, oxidative DNA damage and its consequences.

OS induces fragmentation in both nuclear and mitochondrial DNA and affects sperm motility by peroxidative damage to axoneme and depletion in intracellular ATP and also results in impairment in development and differentiation of microtubule apparatus. Spermatozoa with tremendously higher levels of DNA damaged (due to chromatin fragmentation as a result of elevated OS) have very low potential for natural fertility (114). It is essential to explore the factors that cause decline in sperm function which disrupt genomic integrity and factors which aid in its maintenance. The elevated levels of OS marker 8-OHdG as measured by Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) or sperm chromatin dispersion assays in spermatozoa of infertile subjects as compare to fertile controls also represent that most of the DNA damage in the spermatozoa is oxidatively induced (120). Due to highly truncated and inefficient repair mechanism with only one functional enzyme i.e*, OGG1* and absence of the downstream enzymes i.e., apurinic/apyrimidinic endonuclease 1 (*APE1*) and X-ray repair cross complementing 1 (*XRCC1*), spermatozoa depend upon the oocyte to correct any unrepaired lesion which has downstream enzymes of BER pathway i.e., *APE1* and *XRCC1*. High mutation load in the spermatozoa will bypass the post-fertilization repair process done by the oocyte prior to the S-phase will lead to the persistence of mutagenic lesion in the germline which has potential for developing *de novo* mutations in the offspring and may be the underlying cause of many childhood diseases (6, 115, 116). Sperm with DNA damage have adverse effects on reproductive outcomes and ARTs which circumvent the natural selection mechanism employed by the spermatozoa to prevent fertilization via the spermatozoa with higher levels of DNA damage and thus, end up in increased risk of birth defects and genetic and epigenetic abnormalities in the child (117). Therefore, tests for assessment of sperm DNA damage have clinical value for predicting or determining the reproductive outcome. These tests are mentioned briefly in Table 2. These are direct tests (DNA fragmentation/oxidation is measured directly by incorporating probes at the site of DNA damage) and indirect tests (DNA fragmentation is measured indirectly by measuring chromatin compaction or susceptibility to DNA denaturation) (117–119).

6.1. Factors causing oxidative stress in the male germline

Defective sperm function is encompassed by multi-faceted causal factors including genetic, epigenetic, age, environment and lifestyle which work individually or in consortium to exert their detrimental

impact on sperm function (120). The genetic factor includes sperm DNA fragmentation which is not included in conventional semen parameters (motility, morphology and sperm count) and is the most critical factor. Other possible pathologies associated with OS generation in male germline includes genital tract infections, varicocele, diabetes, hyper-homocysteinaemia, etc. The unhealthy lifestyle habits such as cigarette smoking or consumption of tobacco in any form, excessive alcohol intake, excessive use of mobile phones, late parenthood, obesity, sedentary lifestyle, physical inactivity, use of recreational drugs, intake of fast food or non-vegetarian food items, psychological stress, exercise intolerance, dietary deficiencies, indiscriminate usage of the antioxidants and environmental factors including exposure to pesticides such as lindane or methoxychlor, herbicides such as dioxin-2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), air pollutants, heat, heavy metals (cadmium, lead, iron, and copper), environmental toxicants (acrylamide, endosulfan, bisphenol A (BPA), phthalates), electromagnetic radiation, etc. (111, 121–123). All of the above-mentioned factors culminate together in the generation of seminal OS and thus affect the reproductive outcomes. The factors associated with oxidative damage to the germline and its consequences were listed briefly in Table 3.

6.2. Oxidative stress and impact on sperm epigenome

OS induces epigenetic alteration (or epimutations) in the sperm genome which are reversible and affect the phenotype without inducing any change in the genetic make-up of the individual. Epigenetic alterations which includes DNA methylation, histone modifications, chromatin remodeling and so on are easily inducible even via the environmental agents and affects the process of spermatogenesis. Epigenetic alterations in the sperm genome affects or sometimes alters a number of biological processes and thus, play an important role in several disorders including male infertility, RPL and can also affect the outcome of ARTs. Because of their heritable nature, epigenetic modifications in the genome can also have impact on early embryogenesis or can affect the spermatogenesis in the subsequent generation also (124). Epigenetic alterations which are most commonly mediated by DNA methyl transferases (DNMTs) and act via methylation of Cytosine residues in the CpG island is considered to be the most important epigenetic mechanisms regulating gene expression and imprinting (125). OS induces oxidative DNA damage which in turn impairs the process of global sperm DNA methylation and epigenetic reprogramming as seen in male infertility patients (aberrant global sperm DNA methylation patterns mainly hypomethylation) as compare to the fertile controls (126). Age-associated sperm DNA methylation alteration as seen in children of older men increases risk of developing complex neuropsychiatric disorders (schizophrenia, autism, bipolar disorder), trinucleotide expansion associated diseases (myotonic dystrophy, Huntington's, etc.), cleft lip and palate and even childhood cancers. This can be mediated either by affecting the epigenetically induced changes in the sperm transcriptionor by compromising the epigenetic reprogramming by the embryo (127). Methylation status of the paternal genome play an important role in regulating the fertility outcome and early embryonic development. Experiments on murine model of spermatogenesis having wild-type *DNMT1* expression, were incorporated with cytidine analogue 5-aza-2'-deoxycytidine into DNA showed decrease in DNA methylation, reduced pregnancy rates and elevated preimplantation loss (128). Children conceived via ART (IVF) have a higher incidence of developing genomic imprinting pathologies such as Angelman syndrome, Beckwith–Wiedeman syndrome, Russell-Silver syndrome, and Retinoblastoma (RB) (129–131). Epigenetic changes are reversible and thus, could be reverted by use of antioxidant supplementation and modification of lifestyle habits.

6.3. Oxidative stress and testicular cancers

Male infertility is believed to be an early biomarker of cancer. Infertile men with abnormal semen parameters have higher incidence of developing gonadal and extra-gonadal tumors and increased tumor susceptibility in their offspring (132). OS induces DNA damage which further induces mutations, accumulation of mutagenic DNA adducts, generation of electrophilic aldehydes, telomere shortening, disruption of cell-cycle regulation and intracellular signal transduction pathways and inactivation of transcription factors. Testicular cancer is the cancer of the testicles and the most common malignancy in men aged between 15–35 years and one of the multiple life threatening diseases in men. Infertile male with elevated seminal OS, high levels of DNA damage and fragmentation and abnormal semen parameters (sperm motility, morphology and count) not only show reduced fecundity but are also at 20 times higher risk of developing testicular tumors and a 4–5 times higher risk of developing extragonadal tumors as compared to the general population (113). Impaired sperm production/function, testicular cancers, cryptorchidism, undescended testis and hypospadias are the common features of an underlying entity known as testicular dysgenesis (TDS). TDS is influenced primarily by lifestyle or environmental factors and to some extent by genetic aberrations or polymorphisms in the genes regulating various phases of spermatogenesis (133). Male sub-infertility and testicular cancer patients harbor mutations in genes involved in DNA damage and repair mechanisms which ends up either by forming unstable compounds or expansions of DNA repeat sequences of genes involved in meiotic recombination

Table 4. Antioxidants used for the treatment of male infertility, their characteristic features and impact on seminal parameters

Antioxidant	Characteristic features	Impact on seminal parameters
Vitamin E	Non-enzymatic, localized mainly in cell membrane, major chain breaking antioxidant	Decreases ROS production and hence, protects the sperm plasma membrane from lipid peroxidation, preserves sperm motility
Vitamin C	High antioxidant potential, chain breaking antioxidant	Protects sperm DNA from harmful effects of ROS, reduces DNA damage, preserves sperm motility
N–acetyl–cysteine	Antioxidant and mucolytic agent, glutathione precursor, increases cellular pools of free radical scavengers	Prevents reduction of sperm motility, increases sperm motility, count, morphology and viscosity, prevents oxidative DNA damage, reduces ROS levels
Glutathione	Most abundant intracellular thiol, regulates generation of some important antioxidants such as Vitamin C and E	Protects sperm plasma membrane from lipid peroxidation, reduces ROS induced damage, increases sperm motility and count
Coenzyme Q10	Antioxidant and energy promoting agent	Significantly improves sperm motility and function with no effect on motility, morphology and count, prevents lipid peroxidation

Reproduced with permission from (15, 146–149).

such as human mutL homologue 1 (*hMLH1*) and human mutS homologue 2 (*hMSH2*), which further enhances the mutational load in the male germ line (134). *p53* plays an inevitable role in regulating normal spermatogenesis by regulating spermatogonial cell proliferation by regulating apoptosis and by removing damaged spermatogonia formed after irradiation. Loss-of-function mutation in *p53* tumor suppressor gene also has important role in the genesis of testicular cancer and male infertility (135, 136). Telomere attrition and age-associated accumulation of mitochondrial DNA mutations and OS induced genome wide epimutations such as hypomethylation contribute to genome hypermutability and genetic instability and may predispose to cancer (137, 138). Therefore, oxidative DNA lesions, mitochondrial mutations, mitochondrial DNA fragmentation and its insertion into nuclear genome may aid the process of carcinogenesis and explains for the link between male infertility and its association with gonadal and extragonadal tumors. OS is also associated with accelerated shortening of telomeres and chromosomal instability which may predispose to microsatellite instability and may also lead to aberrant recombination events which may lead to loss of Y chromosome. Loss of Y chromosome is observed in older men and also in men with hematological malignancies and solid tumors and associated with shorter survival rate (139, 140).

7. ANTIOXIDANTS AND LIFE STYLE MODIFICATIONS: IMPACT ON OXIDATIVE STRESS

7.1. Antioxidants: Role in the treatment of oxidative stress related disorders

The reduced and oxidized state of the cell are required to be maintained in harmony so as to drive essential cellular functions of cell proliferation, cell cycle regulation, cell signaling, apoptosis, etc. Cells employ natural antioxidant system which includes enzymatic and non-enzymatic antioxidants to regulate the negative impact of potentially harmful ROS. During pathological conditions, the antioxidant system of the cell is overwhelmed by the deleterious synthesis of ROS thus, disrupting the redox balanced state of the cell and leading to OS. (141). Antioxidants function by reducing ROS levels and thus, nullify the impact of OS in cell and prevents OS induced damage to DNA, proteins and lipids. Antioxidants are classified as enzymatic and non-enzymatic. The enzymatic antioxidants include SOD, CAT, GPx, GR, etc. and the non-enzymatic antioxidants include Vitamin E and C, carotenoids, omega-3 and -6 fatty acids, reduced glutathione (GSH), coenzyme Q10, L-arginine, etc. Antioxidants can also be classified as endogenous and exogenous or dietary antioxidants. The endogenous antioxidants include lipoid acid, NADPH, NADH, ubiquinone (coenzyme Q10), glutathione, SOD, CAT, GPx, GR, etc. whereas the exogenous or dietary antioxidants include vitamin C and E, β-carotene, lycopene, lutein, polyphenols, etc. (15).

Male infertility is a complex lifestyle disease resulting due to OS which cause oxidative DNA damage and formation of oxidative base adduct 8-OHdG. Therefore, early diagnosis of male infertility, its underlying causes and timely initiation of treatment can aid in reducing disease severity, preventing its sequelae and reduce disease burden of genetic and epigenetic disorder in the future generations. Oral antioxidant supplementation is in usage since long and considered as the standard practice for the treatment of male infertility to reduce ROS levels and to improve the fertility in these patients (142). But there are conflicting reports and cumbersome evidences which suggests that indiscriminate or long-term usage of antioxidants for the treatment of male infertility can result in a state of "Reductive Stress" which is harmful and interferes with the physiological ROS levels causing enhanced ROS generation by mitochondria leading to oxidative injury to the cell (12). Prolonged usage of antioxidant

regimen consisting of vitamins C and E (400 mg each), β-carotene (18 mg), zinc (500 μmol) and selenium (1 μmol) which is generally given to male infertility patients with higher levels of DNA fragmentation index (DFI) (greater than 15%) showed reduction in DFI but increase in sperm chromatin decondensation (143, 144). This has negative impact as sperm with higher levels of chromatin decondensation (greater than 28%) can result in asynchronous chromosome decondensation and may lead to cytoplasmic fragmentation in the developing embryo as seen in ICSI or IVF outcomes (145). Antioxidant scavenging system plays an important role in inactivating ROS therefore multiple studies were already conducted to find out the efficacy of antioxidant supplementation in the treatment of male infertility so as to improve the fertilization rates and the pregnancy outcomes (15, 146–149). Some of the antioxidants used for the treatment of male infertility, their characteristics and impact on semen parameters is briefly outlined in Table 4.

7.2. Lifestyle modifications/interventions: Role in oxidative stress management

OS is the most common cause of male infertility and the chief cause of other reproductive system disorders resulting in poor fertility rate and poor outcomes in natural and assisted conceptions. Sperm DNA integrity is the most essential and the crucial parameter for reproduction and serve as a predictor of the reproductive outcome (117). DFI as assessed via Sperm Chromatin Structure Assay (SCSA) showed that DFI above 30 leads to failure to conceive spontaneously and DFI greater than 24 leads to conception but may result in RPL (150). Majority of factors leading to OS induced sperm DNA fragmentation are modifiable by simple lifestyle modifications, physical activity, and adoption of interventions like yoga and meditation (14). These mentioned factors can improve fertility potential and outcomes by improving sperm DNA integrity, regulating OS, maintaining sperm telomere length and reducing the rate of cellular ageing and may also help in reducing the burden of paternally mediated disease risk to the offspring and reduces pathologies associated with sperm DNA damage (13, 79, 151).

Smoking causes testicular inflammation, decrease in mitochondrial activity, leukocytospermia (seminal leukocytes>$1x10^6$ leukocytes/ml), increase in DFI, telomere shortening and increase incidence of childhood cancer (116). Cessation of smoking may help in improving sperm chromatin integrity and therefore, advised to men with unexplained infertility (116, 152). Leukocytospermia which occurs due to infection or inflammation and represented by elevated leukocytes in the seminal plasma of infertile men could be reduced by antibiotic supplementation such as doxycycline and can result in positive benefits such as spontaneous conception (153). Xenobiotic compounds (organochlorides such as plastics and pesticides, polychlorinated biphenyls, naturally occurring polyaromatic hydrocarbons) and toxic metals particularly iron and copper increase seminal ROS levels as well as DNA fragmentation. Therefore, the exposure to xenobiotics and heavy metals should be minimized as far as possible (154, 155). BPA is environmental endocrine disruptor used mainly for the production of polycarbonate plastics and epoxy resins. Increasing urinary concentrations of BPA are associated with increased DNA damage. Therefore, plastic packaging or heating food in plastic containers should be avoided as much as possible (156). Excessive usage of mobile phones (>4 hours daily) and their storage in the trouser pockets is associated with high sperm DNA fragmentation, decline in sperm motility, hormonal imbalances and decreased activity of antioxidant enzymes (157). The other lifestyle factors that can be modified includes avoiding excessive exposure to pesticides, insecticides or herbicides, reducing excessive alcohol intake, avoiding cycling with tight pants, avoiding excessive laptop usage especially on closed legs, CR in obese men via exercise and increased physical activity, avoid increased intake of non-vegetarian food items, avoiding exposures to chemotherapeutic agents and other environmental toxicants, minimizing psychological stress, increasing intake of food items rich in dietary antioxidants such as vitamin C (papaya, strawberries, kiwi, broccoli, oranges), vitamin E (spinach, sunflower seeds, almonds, papaya), selenium (mushroom, mustard seeds), zinc (spinach, sesame seeds, pumpkin seeds, oats), etc. (158–160).

Yoga is basically a psycho-somatic-spiritual discipline, aimed at achieving union and harmony between mind, body, and soul and brings balance to all the aspects of one's being from physical, mental, emotional to spiritual spectrum. This ancient Indian discipline includes all aspects of an individual from health to self-realization. YMLI practices which is an adjunct to modern therapy includes regulation of diet, mental attitude and the practice of specific techniques such as asanas (postures), breathing practices (pranayamas) and meditation, to attain the highest level of consciousness and thus, leading to individual's well-being. YMLI reduces psychological stress, anxiety, depression, decreases levels of salivary cortisol, slows down cellular ageing and enhances QOL, cognitive abilities and increases long term cardio-vagal tone (161, 162). YMLI practices help in the management of stress and stress induces disorders via down-regulation of the hypothalamic–pituitary–adrenal (HPA) axis response to stress and regulation of the sympathetic nervous system (163). YMLI practices are now becoming increasingly popular worldwide to treat a number of medical conditions

such as bronchial asthma, cardiovascular disorders, depression, etc. (164–166). YMLI is increasingly used as an alternative and complementary medicine to treat reproductive health problems such as male infertility and to produce successful pregnancy outcomes (167). Yoga based techniques especially *kundalini yoga* and *moola bandha* yoga acts on endocrine axes to enhance male reproductive functions and helps in improving reproductive health by improving reproductive behavior, mood, and also by reducing anxiety and stress (167). Studies from our lab have reported that YMLI based lifestyle intervention can reduce OS, improves sperm DNA integrity and can reduce the incidences of childhood cancer by reducing OS induced damage to paternal genome (168). This intervention also increases expression of DNA repair genes, decreases the levels of inflammatory cytokines such as Interleukin-6 (IL-6), Mitogen activated protein kinase 10 (MAPK10), MAPK15 and upregulates the levels of anti-inflammatory cytokines such as IL-2 and IL-4 (169, 170). YMLI based lifestyle intervention can reduce rate of cellular ageing, enhances telomerase levels and its activity and aids in maintenance of telomere length, genomic stability and chromosomal integrity (79). This may further aid in maintenance of spermatogonial proliferation and production of sperm with low levels of DNA damage and thereby, enhance the male fertility. It may also reduce the number of couples opting for assisted conception due to male factor infertility and thereby reduced both psychological and financial stress associated with such treatment.

8. CONCLUSION

In the last decade, OS has become the paramount research interest of many scientists working in the field of free-radical biology because of the havoc it creates in the biological system and its role in initiation of various diseases. The underlying molecular mechanism by which OS induces disease initiation and execution are many ranging from disruption of cell cycle regulation and cellular homeostasis, activation of oncogenes and inflammatory cytokines, inactivation of tumor suppressor genes, transcription factors and anti-inflammatory cytokines, alteration of epigenome, accelerated cellular ageing leading to cell damage/apoptosis, etc. OS is the chief cause of defective sperm function, sperm DNA damage and its high levels were detected in 30–80% cases with male factor infertility. Antioxidant therapy which is currently being used for the treatment of male infertility and OS induced other diseases are beneficial but cannot be used indiscriminately and does not provide prolonged health benefits and may result in "hypo-oxidative" stress and disrupt several redox sensitive processes. Therefore, deciphering the signaling networks which were initiated by free radicals during OS and how the cell respond to such condition will help in better understanding of the disease pathogenesis and may help to develop new therapies to manage a variety of disease conditions for which current therapies are not available. The dire need of the hour is to identify lifestyle factors which are modifiable and to develop simple lifestyle modifications or interventions which can help in the management of OS and its associated disease pathologies.

9. ACKNOWLEDGEMENTS

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