

Free Radical Theory of Aging: Implications in Male Infertility

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This review examines the effect of mitochondrial generation of reactive oxygen species (ROS) and aging on human spermatozoa and seminal antioxidants. We discuss the effect of continuous ROS production on biomarkers of aging, such as germ cell telomeres and telomerase, lipofuscin, and amyloid. These markers may be responsible for telomere shortening and subsequent decrease in sperm count, decline in testosterone concentration, and decline in motility with aging. Excessive ROS can also damage mitochondrial deoxyribonucleic acid and sperm nuclear DNA, contributing to paternally transmitted diseases. ROS generation has a central role in the pathophysiology of age-related decrease in male fertility. UROLOGY xx: xxx, xxxx. © 2009 Elsevier Inc.

The recent trend toward delayed parenthood raises major safety concerns because of the adverse effects of aging on fertility.^{1,2} Although it is well known that female fertility declines with aging as a result of diminution of oocyte reserve and quality, recent studies have demonstrated the effects of aging on the male reproductive system.^{1,3,4} However, the mechanism of this observed age-related decline in male fertility remains to be established.

Mechanisms of cell senescence have been well studied in the last decade, and the free radical theory, conceived by Denham Harman in 1956, has attracted considerable attention.⁵ Continuous production of reactive oxygen species (ROS), mainly from mitochondria and subsequent mitochondrial deoxyribonucleic acid (mtDNA) damage, as well as the secondary build-up of macromolecular damage, leads to a decline in cellular functional capacity, commonly associated with cell senescence and aging.⁶ The physiological and pathologic role of free radicals in the male reproductive system is well defined. ROS level $> 0.0185 \times 10^6$ counted photons per minute or 20×10^6 sperm in neat semen is associated with male infertility.⁷ ROS levels are significantly higher in men aged > 40 years compared with levels found in semen of younger men.^{7,8}

However, the correlation between male infertility as a result of aging and the free radical theory of aging is not well studied. Despite the established effects of free radicals on biomarkers of aging, such as telomere dysfunction, telomerase (often found in the germ line to ensure

genomic integrity), lipofuscin, and amyloid, their correlation with male infertility is not clear.^{9,10}

The aim of this review is to discuss the molecular mechanism of male infertility related to aging. We examine the implications of the free radical theory of aging and biomarkers of aging (telomere dysfunction, lipofuscin, amyloid) on the observed age-related declines in male infertility.

DISCUSSION

ROS Generation From Mitochondria

Mitochondria play an important role in cellular energy generation, apoptosis regulation, and calcium homeostasis.¹¹ Coupled to the tricarboxylic acid cycle, the electron transport chain (ETC), and adenosine triphosphate (ATP) synthase in the mitochondria generate ATP, a source of most cellular energy.¹¹ Mitochondria are the major source of ROS in the cell. Superoxide is continually produced as a byproduct of normal cellular respiration.¹² As electrons are passed from complexes I to IV in the mitochondrial ETC, continuous leakage of electrons occurs, forming superoxide (1% of total rate of electron transport).¹³ This superoxide is converted to hydrogen peroxide by manganese superoxide dismutase in the mitochondrial matrix under physiological conditions.¹³ Superoxide oxidizes iron-sulphur-containing enzymes such as aconitase that produces hydrogen peroxide and ferrous iron.¹⁴

The formation of hydrogen peroxide from superoxide and its transformation to hydroxyl radical is apparent, especially when the mitochondrial ETC is abnormal or compromised.¹² Among 5 mitochondrial ETC complexes, complex I (nicotinamide-adenine dinucleotide [NADH] ubiquinone oxidoreductase) and complex III (ubiquinol-cytochrome c oxidoreductase) are responsible for most of the superoxide production.¹² Numerous studies have used mitochondrial ETC inhibitors such as ro-

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tenone and antimycin to disrupt the flow of electrons through complexes I and III, respectively.^{12,15,16} Mitochondrial superoxide production generally is greater in antimycin-inhibited complex III than in rotenone-inhibited complex I.^{12,16} However, superoxide production by complex III is minimal in the absence of antimycin.¹⁶ Therefore, it can be suggested that *in vivo* complex I is responsible for much of mitochondrial ROS production through reversed and forward electron transport.¹⁶

Mitochondria are more susceptible to oxidative damage because of the active production of ROS, as mtDNA is not protected by histones.¹⁷ Consequently, mitochondrial ROS production damages the mitochondria themselves.^{6,17} Mutations or deletions in mtDNA lead to defects in oxidative phosphorylations, defective cellular calcium dyshomeostasis, and other related mtDNA diseases.⁶ A dysfunctional mitochondrial respiratory chain would lead to more ROS production. Oxidative damage is more prevalent in mtDNA and protein *in vivo* than in other cell components.⁵

ROS and Human Spermatozoa

Small amounts of ROS are continuously produced in the spermatozoa and play a crucial role in capacitation, hyperactivation, motility, acrosome reaction, and ultimately, normal fertilization.⁷ Located at the midpiece of the spermatozoa, mitochondria function to produce ATP necessary for the physiological functions of the spermatozoa. Electrons leaking out of the mitochondrial respiratory chain contribute to superoxide production in the spermatozoa, such as in somatic cells.¹⁵ Koppers et al¹⁵ recently were the first to demonstrate that mitochondria in human spermatozoa can produce ROS. They also suggested that rotenone-inhibited complex I, not antimycin, induces peroxidative damage in the midpiece of the spermatozoa. This continuous ROS generation induces oxidative damage to somatic cells as well as the spermatozoa, although spermatozoa are particularly susceptible to ROS damage because of the high content of polyunsaturated fatty acid in the cell membrane. ROS also reduces sperm motility by decreasing axonemal protein phosphorylation, as well as by lipid peroxidation.⁷

Antioxidants and Aging

Antioxidants play a crucial role in minimizing oxidative damage in the spermatozoa.¹⁸ Studies have shown that along with an increase in ROS with aging, the antioxidant defense activity decreases with aging.^{17,19} In a study using Brown Norway rats, Weir et al²⁰ compared the antioxidant enzymatic activity in epididymal spermatozoa from young (4 months old) and old (21 months old) rats. Their study showed that antioxidants such as glutathione peroxidase (Gpx1, Gpx4) and superoxide dismutase had decreased activity in aging spermatozoa, whereas both ROS production and lipid peroxidation were significantly increased. Luo et al²¹ examined activities of copper–zinc superoxide dismutase, manganese su-

peroxide dismutase, and glutathione peroxidase in Leydig cells isolated from 4- and 20-month-old rats, noting age-related decreases in all antioxidant enzymatic activities. They concluded that age-related reductions in testosterone levels of Leydig cells may be associated with the impairment of the antioxidant defense system of these cells.

Telomere and Telomerase

Telomeres are noncoding, repetitive DNA sequences (TTAGGG) at the ends of eukaryotic chromosomes that function to stabilize and protect chromosome ends. Progressive reduction of telomere length to a critically short size is related to the cessation of cell division and the onset of senescence. The enzyme telomerase adds specific TTAGGG sequence at the chromosome ends and maintains the length of the telomere. Telomerase is found in the male germline cell,²² activated lymphocytes, and certain stem cell populations.²³

In 1998, Fujisawa et al²⁴ studied telomerase activity in human testes. Telomerase activity is highly critical in the germline cells because of its task of preserving the full-length chromosome of the cells and to maintain spermatogenesis. Yashima et al²⁵ found variations in the location of telomerase expression in testis between the end of the prepubertal period and adulthood. Before puberty, telomerase is highly expressed in immature Sertoli cells, whereas in advanced age groups, the telomerase expression is highest in the germ cells of the seminiferous tubules.²⁵

Effects of ROS on Telomere and Telomerase

ROS-induced telomere shortening may be due to direct injury to guanine repeat telomere DNA by ROS. Using a culture of human WI-38 fibroblasts, Zglinicki and co-workers²⁶ demonstrated that telomere shortening is significantly increased under mild oxidative stress compared with that observed under normal conditions. The addition of an antioxidant suppresses the rate of telomere shortening in somatic cells. Furumoto et al²⁷ showed that the telomere shortening rate slowed after enrichment by ascorbic acid, a strong antioxidant. Overexpression of the extracellular superoxide dismutase gene in human fibroblasts decreased the peroxide content, further decreasing the rate of telomere shortening.²⁶ The rate of telomere shortening in sheep and humans is directly related to the cellular oxidative stress levels.⁹

Deleterious conditions including dysfunctional telomeres, genomic instability, and apoptosis may result from telomere shortening in both somatic and germline cells. A shortened telomere may limit the potential for replication and could result in genomic instability. This instability may play a role in about 70% of conceptions lost before birth, and also in 50% of spontaneous abortions with detectable chromosomal abnormalities.²⁸ Hence, maintaining telomere length may be essential for healthy human spermatozoa. Despite a lack of studies correlating

ROS and telomere length in spermatozoa, we hypothesize that continuous ROS production by sperm may have deleterious effects on telomere function.

Effect of ROS on Telomerase Activity

ROS also can affect telomere function indirectly by their interaction with telomerase. Intracellular ROS leads to a loss of activity of telomerase reverse transcriptase (TERT), whereas antioxidants such as *N*-acetylcysteine delay the onset of cell senescence.²⁹ Zhu et al³⁰ suggested that decreased activity of catalytic subunits of telomerase (mTERT) increases neuronal cell susceptibility to oxidative stress. In embryonic mouse neurons, the decrease in the level of mTERT is directly related to the increase in apoptosis induced by oxidative stress. Therefore, the catalytic subunits of telomerase may play a role in protection from oxidative stress.

Pathogenesis of Lipofuscin and Amyloid Accumulation in Male Reproductive Tract. Lipofuscin, also known as “age pigment,” is a marker of cell senescence.³¹ It is an intralysosomal polymeric material that cannot be degraded or exocytosed, and continuously accumulates during the lifespan of a cell. Terman et al³¹ suggested that lipofuscin formation is governed by ROS, mainly hydrogen peroxide, which is a byproduct of normal oxygen metabolism that is produced continuously by mitochondria, peroxisomes, and cytosolic oxidases. It is largely eliminated by catalase and glutathione peroxidase. However, it partially diffuses through lysosomal membranes. The Fenton reaction takes place in the presence of iron; the reducing substance (such as cysteine) is converted into a hydroxyl radical that induces peroxidation of various degraded macromolecules in the lysosome. This results in the formation of a cross-linked, nondegradable material called lipofuscin. Thus, ROS are involved in the accumulation of lipofuscin inside lysosomes.

An age-related increase in lipofuscin formation is also seen in male reproductive tissue. Studies have demonstrated that lipofuscin accumulation increases with aging in epididymal cells and Leydig cells.¹⁰ Significantly large amounts of lipofuscin were found in the Leydig cells of elderly men compared to younger men.³² Lipofuscin content matches the apoptosis indices of Leydig and epididymal cells.⁴ ROS are produced by Leydig cells during steroid genesis (testosterone synthesis), as well as by spermatozoal mitochondria.³² This ROS production might be responsible for Leydig cell aging, considering that inhibition of steroid genesis has been shown to prevent Leydig cell aging.³² Aging-associated increases in lipofuscin accumulation in Leydig cells¹⁰ and decreased testosterone³³ support this theory (Fig. 1).

Amyloid deposition affords additional evidence that ROS play a significant role in the loss of testicular function with aging. An age-dependent increase in oxidative stress and oxidative damage to cellular proteins contribute to amyloid (an insoluble protein aggregate) formation.³⁴ Amyloid formation is associated with age-related

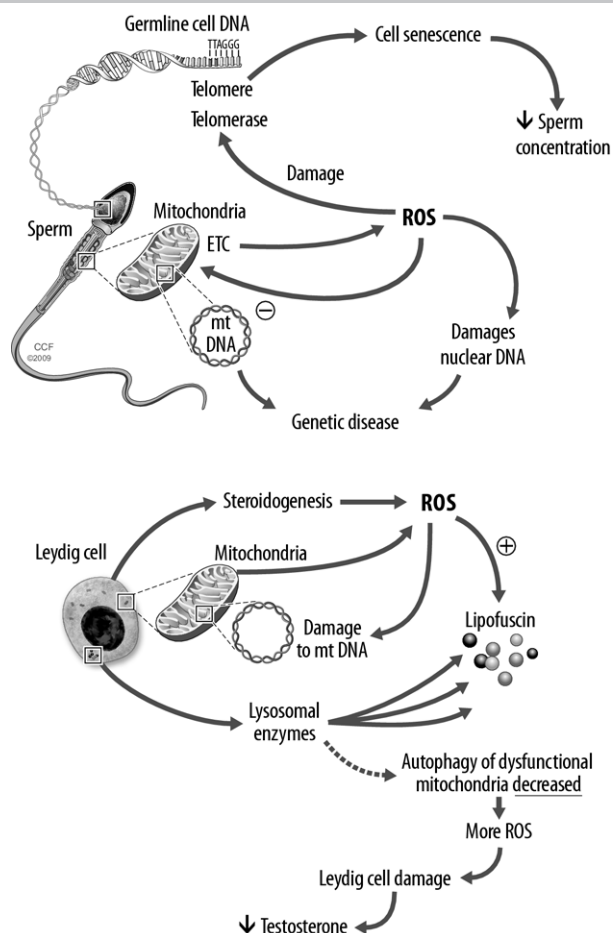


Figure 1. Showing continuous production of reactive oxygen species (ROS) from sperm and Leydig cell mitochondrial electron transport chain as well as Leydig cell steroidogenesis. ROS will damage germline cell telomere and telomerase, sperm and Leydig cell mitochondrial deoxyribonucleic acid, nuclear DNA, and mitochondria. This may result in decreased sperm concentration with aging, possible risk of genetic diseases transmission, and increase in ROS production because of damage to the mitochondria. ROS production also stimulates lipofuscin formation. Lysosomal enzymes will be used up for degradation of newly formed lipofuscin. Lesser amount of lysosomal enzymes will be available for degradation of dysfunctional mitochondria. These dysfunctional mitochondria will produce more ROS, which will be damaging to Leydig cells. Damage to Leydig cell eventually leads to decrease in testosterone production with age.

increases in the fraction of abnormally folded proteins and decreases in the functions of proteases that degrade newly synthesized proteins.³⁴ The protein aggregates, in turn, further promote formation of protein aggregates by serving as nucleation sites. Amyloid fibrils with protease-resistant structures are not reversible when formed, and promote further formation of protein aggregates. The age-related increase in amyloid in male reproductive tissue was first studied by Herriot and Walker³⁵ They studied the distribution of amyloid in normal human testes from fetal life to old age, noting amyloid deposits in most

men aged > 20 years, but never in males aged < 18. Age-related accumulation of intracellular amyloid fibrils in Sertoli cells of atrophic testes has also been noted.³⁶

Correlation Between Mitochondria Production of ROS, Telomere Shortening, and Lipofuscin Accumulation. Mitochondrial production of ROS, lipofuscin accumulation, and telomere damage are correlated, and each event promotes other events, leading to cell aging (Fig. 1).³¹ Mitochondrial ROS production can damage the mitochondria themselves, and subsequent mtDNA damage can lead to a higher production of ROS.

In the lipofuscin-loaded aging cell, many lysosomal enzymes degrade lipofuscin-loaded lysosomes as well as the damaged mitochondria. These lysosomal enzymes degrade within lipofuscin-loaded lysosomes, and enzyme activity available for autophagy (of damaged mitochondria) is diminished, leading to accumulation of damaged mitochondria. Dysfunctional mitochondria produce more ROS and, therefore, lipofuscin accumulation sensitizes cells to ROS-induced damage.³¹ ROS production leads to increased accumulation of lipofuscin, which in turn promotes more mitochondrial damage, which can become a self-amplifying, vicious cycle (Fig. 1).

A similar self-amplifying cycle (Fig. 1) between telomere sequence and mitochondrial ROS also has been suggested. It is now clear that mitochondrial dysfunction leads to oxidative stress and subsequent shortening of telomeres. Mitochondrial localization of telomerase increases mitochondrial susceptibility to oxidative stress. Telomerase expression in mitochondria makes mtDNA more susceptible to oxidative stress. So, mitochondrial telomerase expression increases mtDNA damage because of ROS.³⁷ Damaged mitochondria produce more ROS that damages telomeres, causing cell senescence.

Decrease in Testosterone Level and Decline in Sperm Parameters With Aging: Role of ROS. Testosterone levels decline by as much as 50% between ages 20 and 80.³³ The level of biologically available testosterone is reduced below the normal limit in 7% of men aged between 40 and 60 years, in 20% of men aged between 60– and 80 years, and 35% of men aged > 80 years.³⁸ The mechanism responsible for this decline in male hormone remains unclear. Accumulations of lipofuscin and amyloid contribute significantly to a decline in numerous physiological functions such as bone density, muscle strength, and libido.³⁹ Decreases in testosterone levels with aging³³ may reflect ROS elevations, with resulting accumulation of amyloid and lipofuscin around Leydig cells, and ultimately affecting reproduction and male fertility.

Aging is also associated with a decrease in semen quality. Jung et al⁴⁰ showed a significant decrease in semen quality of older men (≥ 50 years; $n = 66$) in comparison with that of younger men (21–25 years; $n = 134$). They demonstrated a 27% decline in progressive motility in older men (after adjustment for duration of

sexual abstinence).⁴⁰ Total sperm count, a variable dependent on semen volume and sperm concentration, peaks between age 30 and 35 while suffering a 24.3% decline in the age group ≥ 55 years.⁴¹ Although the etiology behind decreasing sperm parameters is not completely clear, increased ROS production has been suggested as a possible cause.⁸ Decline in sperm concentration may be suggested by effect of ROS on germ cell telomere and telomerase, whereas decline in motility with aging can be explained by effect of ROS on sperm axonemal phosphorylation and lipid peroxidation.

Oxidative stress may result in unfavorable, physiological changes in the reproductive organs, including the epididymis and accessory glands.⁴² Damage localized to the epididymis may affect normal sperm maturation processes. Reduced semen volume caused by damaged accessory glands is another physical manifestation of oxidative stress.^{1,41} Therefore, oxidative stress coupled with aging correlates with decreased semen quality.

Aging and Sperm DNA Damage: Genomic Defects in Offspring

The association between aging and sperm DNA damage has been extensively studied during the last decade. Singh et al⁴³ showed higher levels of double-stranded DNA breaks in older men (by comet assay). Wyrobek et al⁴⁴ found an inverse relationship between DNA fragmentation index and male age by sperm chromatin structure assay. Increasing oxidative stress levels associated with aging might be responsible for this increase in DNA damage with age. Oxidative stress-mediated DNA damage may be an etiology for repeated assisted reproductive technology failures in older men.⁴⁵ Increasing male age may have an influence on DNA fragmentation in the form of single-strand breaks.⁴⁶ This may not have any effect on fertilization because the oocyte can repair single-strand breaks. However, if the oocyte repair mechanisms are dysfunctional, this may result in poor, if not failed, blastocyst formation. Thus, oxidative stress-induced DNA damage can lead to various genomic defects.^{2,47} Plas et al² showed a positive correlation between structural chromosomal abnormalities and paternal age, with a 4-fold increase in abnormalities in the 45+ age group compared with men aged 20–24 years. They also suggested that children of men in advanced age groups have 20% higher risk of carrying autosomal dominant diseases potentially due to increasing germ cell mitoses and meioses.²

It is interesting to note that a direct relationship exists between paternal aging and offspring development. Considerable evidence shows a connection between aging and offspring learning and cognition. One study conducted by Reichenberg et al⁴⁸ examining Israeli births over a consecutive 6-year period, concluded that offspring of men 40 years or older were 5.75 times more likely to develop autism spectrum disorder than those of men younger than 30 years. Purported etiology consisted

of genetic imprinting alterations and de novo mutations. An older study showed an increased risk of trisomy 21 in offspring of older men, with a profound age effect in men 41 years and older.⁴⁹ Because the spontaneous mutation rate closely relates to paternal age, it can be suggested that the progeny of advanced male age groups experience a higher frequency of imprinting disorders such as Beckwith–Wiedemann syndrome, which involves insulin-like growth factor 2, a paternally expressed gene. This syndrome is characterized by abdominal wall defects, macroglossia, and susceptibility to embryonic tumor.⁵⁰

CONCLUSIONS

In conclusion, ROS are continually produced in the mitochondria of spermatozoa and play an important role in age-related male reproductive pathophysiology. The increased ROS level in semen observed with aging is associated with a possible decrease in antioxidant enzyme activity. This imbalance between pro-oxidants and antioxidants induces oxidative damage, resulting in abnormalities in telomeres and telomerase in male germline cells. This sequence of events may explain the decrease in sperm concentration seen with aging. Oxidative stress in aging male reproductive system may inhibit sperm axonemal phosphorylation and increase lipid peroxidation, which can decrease sperm motility. This oxidative stress can also lead to lipofuscin and amyloid accumulation in the male reproductive tract, potentially the cause of decreased Leydig cell function and a subsequent decrease in blood testosterone levels. A higher rate of lipofuscin accumulation in turn may increase the amount of dysfunctional mitochondria in spermatozoa, thus increasing ROS formation. Along with its negative effect on the fertilizing potential of spermatozoa, ROS also leads to offspring malformation (if fertilization is successful). Oxidative stress-induced mtDNA damage and nuclear DNA damage in aging men may put them at a higher risk for transmitting multiple genetic and chromosomal defects. Thus, this review suggests that ROS might play a central role in decreased male fertility with aging. This hypothesis provides guidance for future study and experiments, focusing on specific biomarkers of aging in men (telomere function, lipofuscin, amyloid) and their comparison with semen parameters and male fertility.

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