Human sperm DNA damage: Impact of *in vitro* oxidative stress and *in vivo* vitamins on conventional and advanced sperm function parameters

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III. Abstract

Introduction: Reactive oxygen species (ROS) and oxidative stress (OS) play a major role in sperm DNA damage which may adversely affect fertilisation and embryo development. ROS induces sperm DNA damage and impairs sperm motility via multiple mechanisms (lipid peroxidation process, initiation of apoptosis, and DNA oxidation). Oral antioxidants may have a favorable effect in decreasing ROS and protecting the sperm against DNA damage. However, a physiologic balance between oxidation and reduction is vital for sperm function.

Material & methods: For in vitro OS study, we performed a prospective study of 10 fertile donors at McGill University Health Center in Montreal. Semen samples were collected and sperm parameters (progressive motility and viability) were obtained. For the *in vivo* vitamin study, we conducted a prospective study of 24 men presenting with idiopathic infertility at the OVO fertility clinic in Montreal between January 2016 and January 2018. We included 6 healthy fertile sperm donors as a control. All patients received 6 months of oral antioxidant treatment. We assessed sperm parameters (concentration, progressive motility) and aniline blue (AB) staining. For both studies, we also evaluated DNA fragmentation index (DFI), high DNA stainability (HDS), iodoacetamide fluorescein (IAF), at baseline and at increasing doses of hydrogen peroxide (H_2O_2) of 100, 250, and 500 μM H_2O_2 for *in vitro* study, and before and 6 months after antioxidant treatment for *in vivo* study.

Results: In the *in vitro* study, the mean viability, mean % progressive motility, and mean % DFI showed a statistically significant differences among the groups ($P < 0.05$). H_2O_2 induced a statistically significant increase in %DFI as the dose of H_2O_2 increased ($P < 0.05$). % positive IAF fluorescence increased with increasing dose of H_2O_2 but without statistical significance ($P > 0.05$). In term of the effect of H_2O_2 on % HDS, there was an improvement with increasing H_2O_2

concentrations, but it was not of statistical significance ($P > 0.05$). In the *in vivo* study, the fertile donor group had a higher mean sperm concentration and mean % progressive motility than the group of infertile men ($P < 0.05$). The mean % DFI, % positive IAF fluorescence, % positive AB staining of infertile group were significantly higher than that of the fertile control group ($P < 0.05$). Conversely, the mean % HDS in the infertile group was not significantly different than the control group. There was an improvement in sperm parameters after antioxidant treatment, but the difference was not statistically significant ($P > 0.05$). Unexpectedly, the chromatin integrity measures (% HDS, % positive IAF fluorescence, % positive AB) worsened after 6 months of antioxidant treatment, but without statistical significance ($P > 0.05$). There was a trend toward improvement in % DFI after antioxidants supplementation with borderline statistical significance (mean: 23.4 vs. 19.1; $P = 0.06$).

Conclusions: In vitro oxidative stress (OS) resulted in a significant increase in DFI with impaired motility at higher concentration of H_2O_2 with no impact on chromatin compaction. In our study, 6 months of antioxidant supplementation had no significant impact on sperm parameters, DFI, and chromatin integrity measures.

IV. RÉSUMÉ

Introduction: Les espèces réactives de l'oxygène (ROS) et le stress oxydatif (SO) jouent un rôle majeur dans les dommages à l'ADN des spermatozoïdes, susceptibles de nuire à la fécondation et au développement de l'embryon. Les ROS induisent des dommages à l'ADN des spermatozoïdes et altèrent leur motilité par l'intermédiaire de multiples mécanismes (processus de peroxydation lipidique, amorce de l'apoptose et modification de l'ADN). Les antioxydants oraux peuvent avoir un effet favorable en diminuant les ROS et en protégeant le sperme contre les dommages de l'ADN. Cependant, un équilibre physiologique entre oxydation et réduction est vital pour la fonction du sperme.

Matériel et méthodes: Pour une étude in vitro sur la SO, nous avons mené une étude prospective sur 10 donneurs fertiles au Centre universitaire de santé McGill à Montréal. Des échantillons de sperme ont été prélevés et les paramètres du sperme (motilité et viabilité progressives) ont été obtenues. Pour l'étude in vivo sur les vitamines, nous avons mené une étude prospective sur 24 hommes présentant une stérilité idiopathique à la clinique de fertilité OVO de Montréal entre janvier 2016 et janvier 2018. Nous avons inclus 6 donneurs de sperme fertiles comme témoins. Tous les patients ont reçu un traitement anti-oxydant pendant 6 mois. Nous avons évalué les paramètres du sperme (concentration, motilité progressive) et la coloration au bleu d'aniline (AB). Pour les deux études, nous avons évalué l'indice de fragmentation de l'ADN (DFI), la haute aptitude à la coloration de l'ADN (HDS) et l'iodoacétamide fluorescéine (IAF) au départ et à des doses croissantes de peroxyde d'hydrogène (H2O2) de 100, 250 et 500 μM H2O2 pour l'étude in vitro et avant et 6 mois après le traitement anti-oxydant pour l'étude in vivo.

Résultats: Pour les études in vitro, la viabilité moyenne, le pourcentage moyen de motilité progressive et le pourcentage moyen de DFI ont montré des différences statistiquement significatives entre les groupes ($p < 0,05$). H_2O_2 a induit une augmentation statistiquement significative du% de DFI lorsque la dose de H_2O_2 a augmenté ($P < 0,05$). Le pourcentage de IAF positif a augmenté avec l'augmentation de la dose de H_2O_2 mais sans signification statistique ($P > 0,05$). En termes d'effet de H_2O_2 sur le % de HDS, il y avait une amélioration avec l'augmentation des concentrations de H_2O_2, mais ce n'était pas statistiquement significatif ($P > 0,05$). Pour l'étude in vivo, le groupe de donneurs fertiles présentait une concentration moyenne de spermatozoïdes moyenne et une motilité progressive des hommes% par rapport au groupe des antioxydants ($p < 0,05$). Le pourcentage moyen DFI, le pourcentage de fluorescence IAF positive, le pourcentage de coloration AB positif du groupe antioxydant étaient significativement plus élevés que ceux du groupe témoin fertile ($p < 0,05$). À l'inverse, le pourcentage moyen de HDS dans le groupe des antioxydants n'était pas significativement différent de celui du groupe témoin. Il y avait une amélioration des paramètres du sperme après un traitement antioxydant, mais la différence n'était pas statistiquement significative ($P > 0,05$). De manière inattendue, les mesures d'intégrité de la chromatine (% HDS, % fluorescence IAF positive, % AB positive) s'aggravent après 6 mois de traitement antioxydant, mais sans signification statistique ($P > 0,05$). Il y avait une tendance à l'amélioration du pourcentage de DFI après la supplémentation en antioxydants avec une signification statistique limite (moyenne: 23,4 vs 19,1; $p = 0,06$).

Conclusions: Le stress oxydatif (SO) in vitro a entraîné une augmentation significative du DFI avec une motilité altérée sans aucun impact sur la compaction de la chromatine. De plus, une supplémentation en antioxydants pendant 6 mois n'a eu aucun impact significatif sur les paramètres du sperme, le DFI et les mesures d'intégrité de la chromatine.

V. Acknowledgement

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A. Review of literature

A-1: Overview of oxidative stress

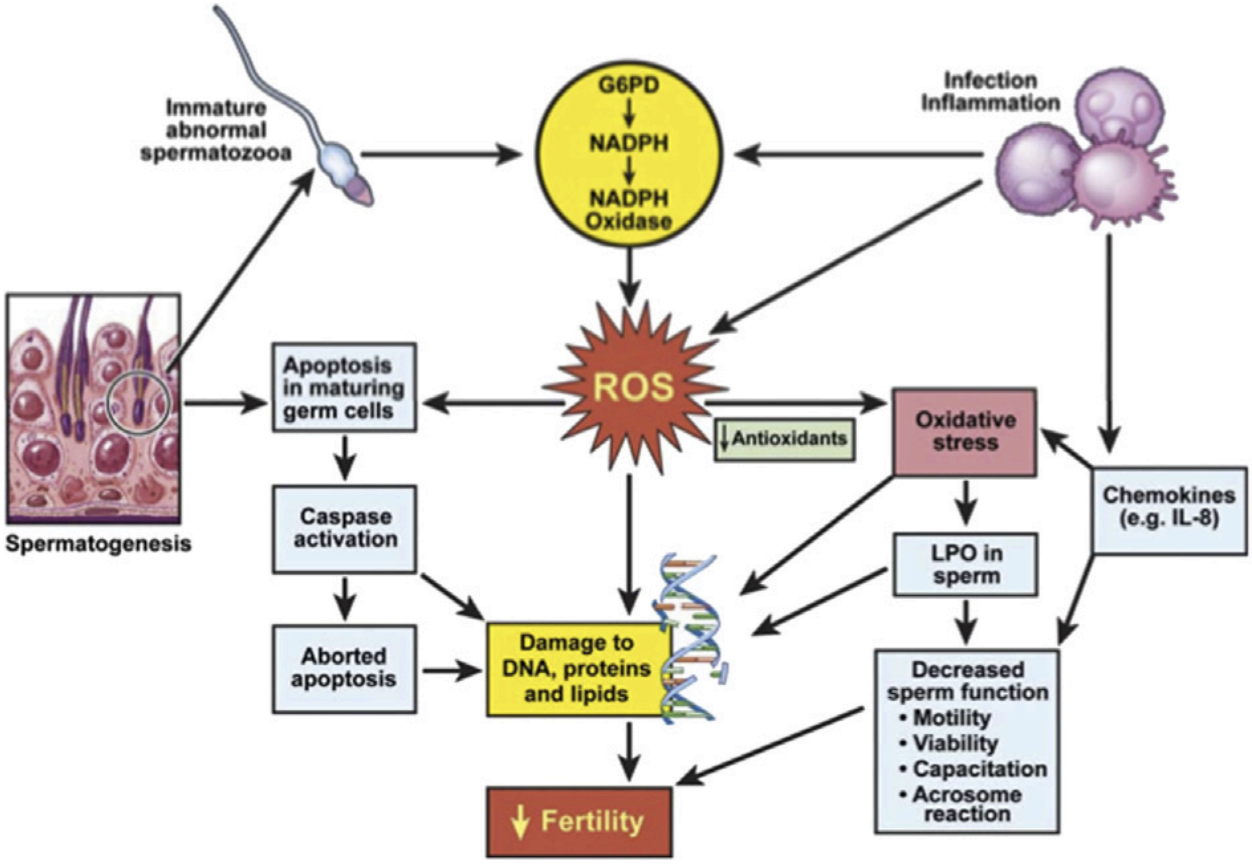
The prevalence of reactive oxygen species (ROS)-induced damage to sperm was reported to be in the range of 30–80% in infertile men (1, 2). ROS are chemically unstable and include reactive oxygen ion, peroxide, and free radical compounds (3). ROS are physiological byproducts of cell metabolism under aerobic conditions, in which 1–5% of the oxygen leaks out and is released as highly reactive free radicals (4). The primary form of ROS is the superoxide anion radical ($\cdot O_2^-$), and, this anion can be converted to other ROS such as hydrogen peroxide (H_2O_2), peroxyl radical ($ROO^{\cdot}$), and hydroxyl radical ($\cdot OH$) (1). ROS exhibit half-life times in the nanosecond range and they have detrimental effects on proteins and lipids, which result in protein modifications and lipid peroxidation. Moreover, ROS can cause nuclear DNA damage in sperm (5).

Although ROS compounds cause cellular damage at high concentrations, which can affect sperm function and result in infertility, they also have physiological functions in terms of regulating vascular tone and gene events within the testes. Furthermore, a controlled physiologic level of production by the sperm cell is required for sperm functional effects including hyperactivation, capacitation, and acrosome reaction (6).

A-2: Seminal free radicals and antioxidants

The main sources of free radicals or ROS within semen are leukocytes and immature sperm (Figure 1).

Figure 1. Mechanism of oxidative stress in semen



The mechanism of oxidative stress in human semen. From: Agarwal A, Sekhon LH. Oxidative stress and antioxidants for idiopathic oligoasthenoteratospermia: is it justified? Indian J Urol 2011; 27:74–85.

ROS production by leukocytes can be 1000 times higher than in sperm under normal conditions (7,8). However, the association between the seminal leukocyte number and ROS-induced infertility remains controversial (9). There is an inverse relationship between ROS production by sperm and their maturation state. During spermatogenesis, the sperm membrane is modified considerably, which results in loss of cytoplasm and produces a morphologically normal sperm. Any remaining cytoplasmic residue can result in abnormal sperm morphology, which leads to creation of ROS via NADPH oxidase (1,7, 10). Thus, teratozoospermic sperms are directly correlated with higher ROS production (11).

ROS are finely balanced by seminal plasma antioxidants that protect and support the sperm. The sperm are reliant on the surrounding seminal plasma for antioxidant support because it has been shown that the sperm themselves have low levels of antioxidants (12). Endogenous antioxidants can be categorized as enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX). SOD inactivates the superoxide anion radical ($\cdot O_2^-$) and catalase inactivates hydrogen peroxide (H_2O_2) by converting them into water and oxygen (2). GPX uses glutathione that scavenges free radicals. GPX activities against oxidative stress (OS) depend on the oxidized glutathione that is reduced by glutathione reductase (13). Non-enzymatic antioxidants include ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione, amino acids (taurine, hypotaurine), albumin, carnitine, carotenoids, flavenoids, urate, pyruvate, zinc, ubiquinol, and prostasomes (14). Ejaculated sperm are well protected from OS by seminal antioxidants. However, during spermatogenesis and epididymal storage, sperm depend on testicular and epidydimal antioxidants (1).

A-3: Causes oxidative stress

Idiopathic

Men with normozoospermia and idiopathic infertility have higher ROS levels in the semen and lower antioxidant capacity when compared to fertile men (15).

Iatrogenic

In vitro fertilization (IVF), intrauterine insemination (IUI), and cryopreservation have been linked with sperm OS (16,17). Moreover, chemotherapy and radiation therapy for cancer patients can induce OS. Heat shock proteins play a central role in accelerating OS response to chemotherapy. Radiation is a known risk factor for infertility through its effect on spermatogonia, while the Sertoli and Leydig cells are known to be resistant to radiation because of the high concentration of antioxidants in these cells (7, 18). Additionally, aspirin and paracetamol have the potential to cause sperm OS through P450 activity (19).

Lifestyle

Smoking has been found to increase ROS production, with a 48% increase in seminal plasma leukocytes accompanied by a decrease in seminal antioxidants (20). ROS have been associated with excessive alcohol consumption, excessive exercise, obesity (through proinflammatory cytokines originating from adipose tissue), psychological stress, and advancing age (through an age-related decline in steroidogenesis) (21-25).

Environmental

Several environmental toxins have been connected to an increase in ROS levels in the testicles. These include methoxyethanol (found in paints and brake fluid), phthalates (a plastic softener in food packaging), sulfur dioxide (a byproduct of petroleum and used as a preservative), pesticides, herbicides, and heavy metals (e.g. cadmium and lead) (1, 7, 26-30). Gennart et al. observed an increase in infertility and miscarriage in the partners of paint and welding factory workers (31).

Infection, inflammation, and autoimmune

Genitourinary infection and inflammation can increase OS by increasing the influx of leukocytes in the local environment. The inflammatory response leads to an increase testicular tissue proinflammatory cytokines, such as interleukin (IL)-1β, cyclooxygenase 2, interferons (IFN)-γ, and tumor necrosis alpha (TNF)-α, which eventually leads to an increase the leukocyte concentration and a decrease in antioxidant enzymes leading to increased OS within the testicle (32). For example, chronic prostatitis is associated with an increase in seminal OS through the inflammatory response cascade (33). Moreover, vasectomy leads to autoimmune-antibody response toward the sperm, which may result in infertility after vasectomy reversal through an increase in proinflammatory cytokines that mediate ROS damage and the OS state in the semen (1, 34, 35).

Testicular

Varicocele was found to be associated with OS and elevated ROS production in the semen. The mechanism of varicocele-induced OS is scrotal hyperthermia (36). Similarly, cryptorchidism leads to an elevation in testicular temperature with elevated sperm ROS production (37). Additionally, spermatic cord torsion can lead to OS through ischemia-reperfusion injury, which can affect the torted and the untorted testes (1, 38). Torsion with associated OS can result in germ cell necrosis and loss of seminiferous tubules (7, 38).

Chronic disease

OS has been linked to several chronic conditions including diabetes, chronic kidney disease, end-stage renal disease, renal transplant, beta-thalassemia, and homocysteinemia (39-42).

A-4: Oxidative stress and sperm dysfunction: impact on sperm, DNA integrity, and ART

ROS can originate from leukocytes (extrinsic ROS) or from immature abnormal sperm (intrinsic ROS). Extrinsic ROS affect the sperm plasma membrane, which is rich in polyunsaturated fatty acids, via a lipid peroxidation. Eventually, lipid peroxidation impairs sperm motility and sperm membrane fusion functions, such as sperm-oocyte fusion and acrosome reaction (6). However, intrinsic ROS attack sperm nuclear and mitochondrial DNA, which leads to poor fertilization ability, a decrease in pregnancy rates, and embryo growth impairment (43). Experiments have shown that hydrogen peroxide is the most powerful ROS that attacks sperm

motility via reduction of intracellular adenosine triphosphate (ATP) and disruption of the sperm axoneme (44).

The effect of OS on sperm-oocyte fusion has a two-phase response. Low amounts of ROS increase sperm-oocyte fusion, which is evident by enhancing tyrosine phosphorylation during capacitation and sterol oxidation, which is associated with an influx of calcium during the acrosome reaction (44, 45). Increased OS can impair sperm-oocyte fusion by increasing lipid peroxidation, which causes alterations in the sperm plasma membrane and modifications in the proteins responsible for the fusion function (46).

Higher levels of OS can increase nuclear and mitochondrial DNA damage. ROS disrupt sperm DNA by attacking purine and pyrimidine bases (47). Additionally, ROS can provoke and alter the apoptotic mechanism in sperm, leading to initiation of the apoptotic cascade by caspase enzymes and resulting in DNA damage. Abnormality in the apoptotic mechanism can result in either oligozoospermia or teratozoospermia if abnormal sperm accumulate (48). Moreover, induction of sperm DNA strand breaks can decrease the fertilization potential, increase aneuploidy rates, increased frequency of genetic abnormalities, and increase the chance of passing defective paternal DNA to the offspring.

The association between ROS and the effect on assisted reproductive technology (ART) has been examined. The *in vitro* environment renders the embryo vulnerable to ROS effects without the presence of antioxidants that are normally found *in vivo* during fertilization (49) (Figure 2).

Figure 2. Effect of ROS on ART

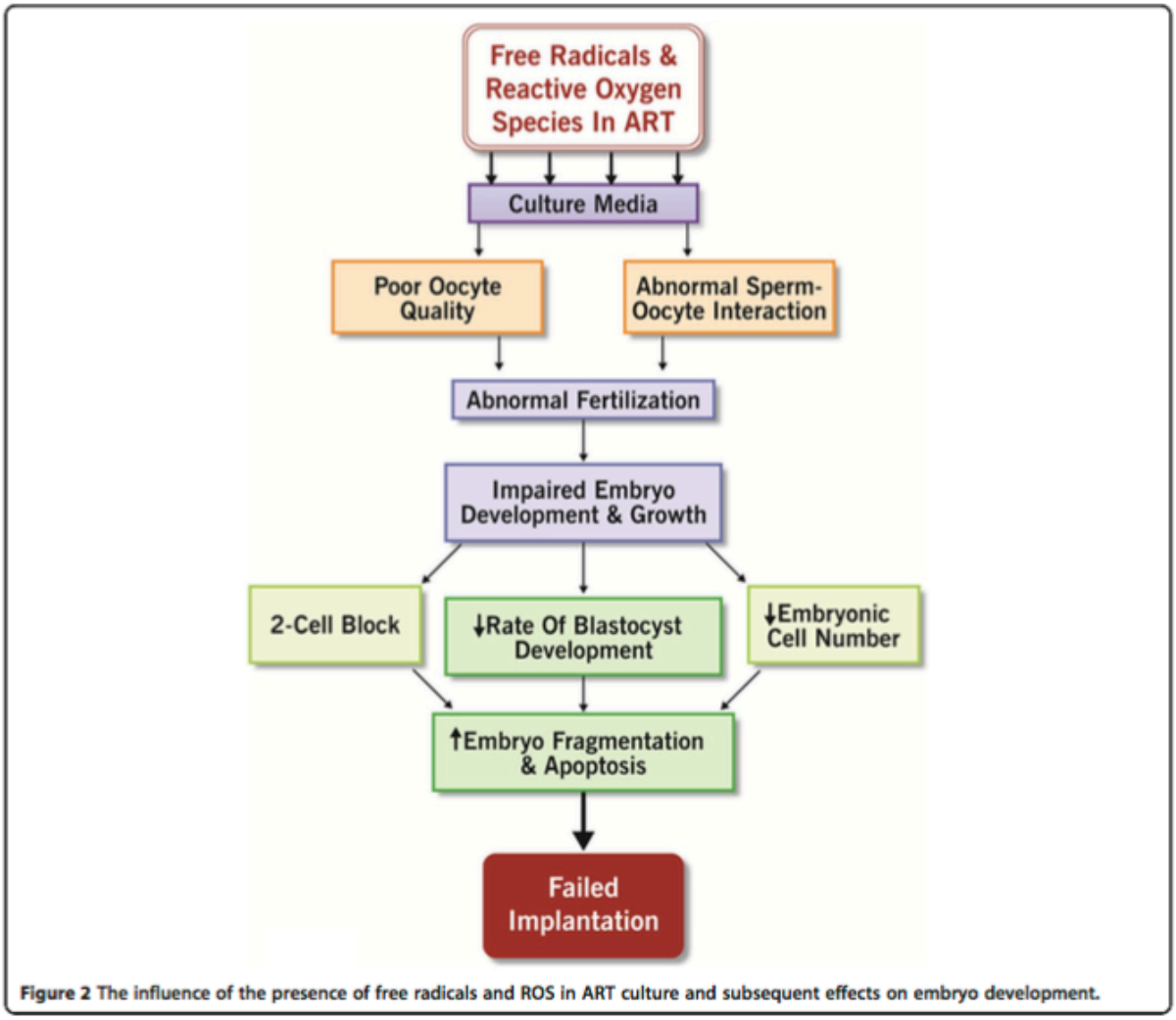


Figure 2 The influence of the presence of free radicals and ROS in ART culture and subsequent effects on embryo development.

From: Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, Gupta S. The effects of oxidative stress on female reproduction: a review. Reprod Biol Endocrinol 2012; 10:49.

In IVF, DNA-damaged sperm will be blocked from fertilization. Conversely, during intracytoplasmic sperm injection (ICSI), the sperm injected bypass this natural selection for fertilization, and this allows fertilization of damaged-DNA sperm, which can have serious consequences such as mesenchymal cancer in offspring and genetic defects. Commonly, these embryos with defective DNA will not make it past the blastocyst or early fetal phase (1, 7, 50).

There is little clear evidence regarding the detrimental effect of ROS on ART, which remains controversial. According to Yeung et al., there was no association between ROS and the IVF success rate (51). The same group showed that ROS may have a beneficial effect on IVF. However, other investigators have shown that ROS can lead to IVF failure (50, 52).

Agarwal et al. conducted a meta-analysis in 2005 to assess the correlation between ROS and IVF success (53). They evaluated nine studies and included only three studies for the final analysis, with a total of 122 study participants. They included studies that correlated N-formylmethionyl-leucyl-phenylalanine (FMLP)-stimulated ROS levels with the fertilization rate. They concluded that ROS has a statistically significant negative consequence on the fertilization rate following IVF. They also added some limitations in their meta-analysis, which include missing statistical data in some of the studies, heterogeneity in the inclusion criteria among the studies, and absence of cutoff values of physiologic versus pathologic ROS (7, 53).

A-5: Management of oxidative stress

A-5.1: Lifestyle

Lifestyle modification strategies are the first step in patients with OS. It is important to counsel the patient about eating a healthy diet, avoiding obesity, quitting smoking, and decreasing alcohol use. These measures would have a positive effect on the fertility potential (1,6).

A-5.2: Environmental

There are many environmental factors (heat, heavy metals, herbicides, pesticides, toxins) that have been linked to oxidative DNA damage. Physicians should counsel patients who work in factories with exposure to toxins, such as paint and welding factories, to follow the control measures to meet exposure limits. Moreover, heat exposure should be avoided by, for example, avoiding working near the oven in restaurants, prolonged hot baths, and saunas (1, 26-31).

A-5.3: Treatment of infection/inflammation

A variety of infectious organisms can initiate OS including: Streptococci, coagulase-negative Staphylococci, *Escherichia coli*, Herpes simplex virus, cytomegalovirus, and Epstein-Barr virus (54). The strongest association between infection and OS was found in cases of chlamydia and ureaplasma infections (54, 55). Multiple studies have shown the beneficial effects of antibiotics in these infections in terms of reducing ROS production and OS (1, 56, 57).

A-5.4: Treatment of oxidative pathology (varicocele)

Varicocelectomy was suggested to be valuable for reducing ROS and OS-induced DNA damage (58). Several investigators have shown a decrease in ROS levels and an improvement in seminal antioxidant capacity after varicocele repair. However, well-designed randomized controlled trials are needed to confirm these findings (59).

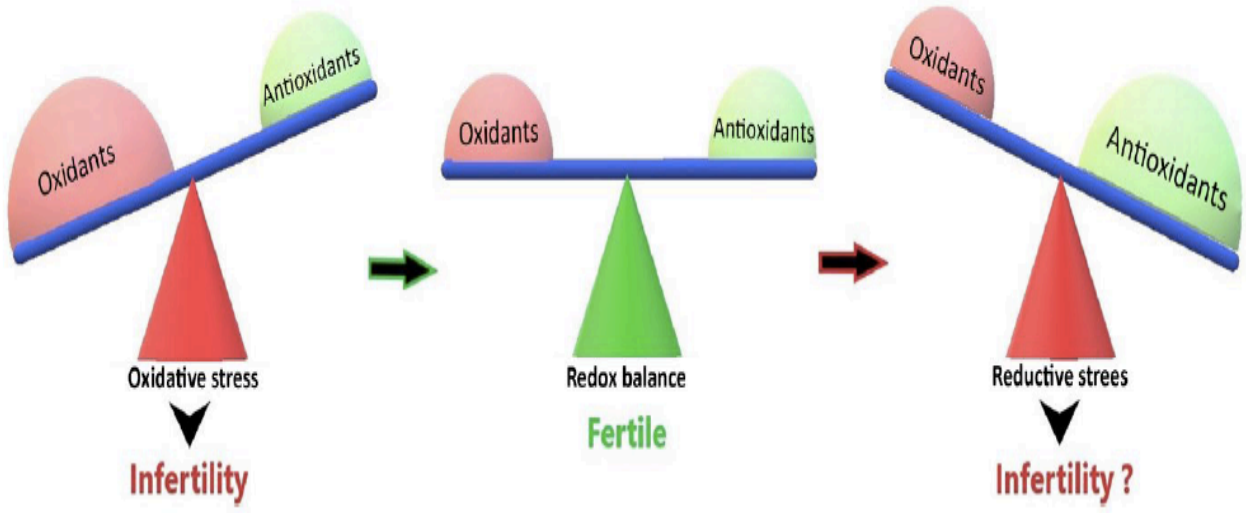
A-6: Overview of antioxidants

Antioxidants include diverse endogenous and exogenous compounds that can neutralize free radical activity and protect sperm from OS. Endogenous (physiologic) antioxidants can be categorized as enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include SOD, catalase, and (GPX). The non-enzymatic antioxidants include ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione, amino acids (taurine, hypotaurine), albumin, carnitine, carotenoids, flavenoids, urate, pyruvate, zinc, ubiquinol, and prostasomes, and lycopene. Exogenous antioxidants have two forms: dietary and oral supplements (natural extracts, herbs, and synthetic). The most frequently used compounds include vitamin E, vitamin C, carnitine, N-acetylcysteine (NAC), folic acid, lycopene, coenzyme Q10 (CoQ10), selenium, and zinc (60).

There are risks and benefits to using these supplements. Antioxidants have been shown to decrease ROS, OS, and protect against DNA damage. However, a controlled balance between oxidation and reduction is of paramount importance for cellular function (5). Overexposure to antioxidants may result in a “reductive stress” where a shift occurs from the redox status balance into a more reduced status, which has a detrimental effect that is similar to effects observed with OS. Reductive stress can result in cancers, cardiomyopathy, Alzheimer disease, and male

infertility. However, excessive antioxidants can result in an “antioxidant paradox,” where a shift occurs from the redox balance to a more oxidized status, which leads to OS (Figure 3) (5).

Figure 3. Redox balance: effects of oxidants and antioxidants



From: Henkel R, Sandhu IS, Agarwal A. The excessive use of antioxidant therapy: A possible cause of male infertility? Andrologia. 2019;51(1):e13162.

Other risks associated with antioxidant overconsumption include increasing all-cause mortality with vitamin E overuse (61); high L-carnitine levels are associated with atherosclerosis (62); vitamin A has been shown to increase mortality from cardiovascular disease and lung cancer (63); and selenium + high dose vitamin E has been found to increase the risk of high grade prostate cancer (7, 64).

A-7: Mechanism of action

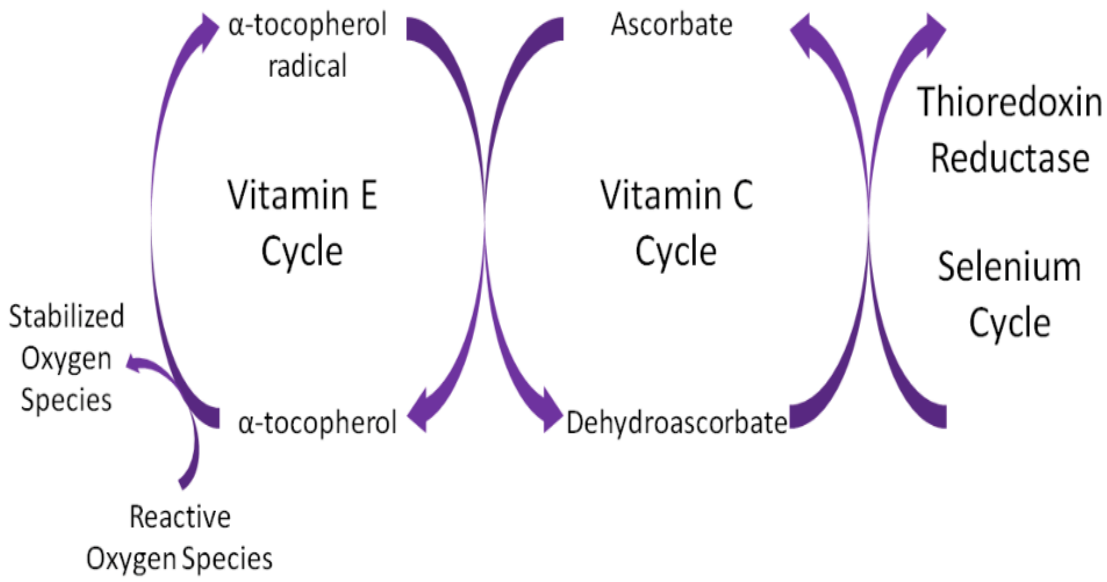
Vitamin C (ascorbic acid)

Vitamin C is a water-soluble vitamin with diverse biological functions. It is found in high concentrations in the seminal plasma. It acts as neutralizer for hydroxyl radicals, assists in regenerating vitamin E, and acts as a cofactor in amidation reactions (Figure 4) (65).

Vitamin E (α-tocopherol)

Vitamin E is a fat-soluble vitamin with strong activity in lipid environment. It reduces lipid peroxidation, protects cellular membrane from free radical damage, and fosters the effect of other antioxidants (Figure 4) (66).

Figure 4. Regeneration of α-tocopherol from the tocopheroxyl radical by ascorbate



α-Tocopherol (the main form of vitamin E in the body) is oxidized, forming an α-tocopherol radical. This donation of an electron stabilizes ROS. Ascorbate (vitamin C) is then oxidized, forming dehydroascorbate to regenerate (reduce) α-tocopherol. Ascorbate is then regenerated by the selenoenzyme thioredoxin reductase. (From: Packer L, Weber SU, Rimbach G. (2001) Molecular aspects of alpha-tocotrienol antioxidant action and cell signalling. J Nutr 131(2): 369S-373S).

Carnitines

L-carnitine (LC) or 3-aminobutyric acid is a water-soluble compound that is required for sperm metabolism and bioenergetic reactions. It exists at a concentration 2000 times higher in epididymis than in the whole blood (67). LC influences the initial and subsequent maintenance of sperm motility. It also neutralises free radicals and prevents ROS production (5).

Coenzyme Q10

This vitamin-like compound is also known as ubiquinone. It is found in high concentrations in sperm mitochondria and it participates in electron transport and energy production. It is also a free radical scavenger (68).

N-acetylcysteine

NAC functions as a precursor of glutathione that neutralises free radicals and maintains sperm stability and motility (69).

Selenium

Selenium is a component and a cofactor of glutathione peroxidase and thioredoxin reductase antioxidant enzymes. It is mainly involved in reducing OS and protecting sperm DNA. Moreover, it is essential for maintaining the structural integrity of sperm (68, 70).

Zinc

Zinc is an essential mineral and it is considered to be the second most abundant trace metal in humans. Zinc is crucial for activating signalling pathways, DNA and RNA metabolism,

protein synthesis, and apoptosis regulation. It also plays a fundamental role in maintaining sperm integrity, sperm maturation, sperm quality and motility through the ATP system, and phospholipids (71).

Folic acid

Folic acid is required for vital steps during spermatogenesis and for proper sperm function such as DNA methylation, purine and pyrimidine synthesis, and methionine production (72).

Lycopene

Lycopene is a lipophilic carotenoid that protects lipids against peroxidation, regulates gene expression, and acts as a free radical scavenger (73).

A-8: Effect of *in vitro* antioxidants (exogenous ROS, endogenous ROS, semen processing, cryopreservation and thawing)

Effect of in vitro antioxidants in shielding spermatozoa from exogenous ROS

Exogenous ROS can originate from leukocytes and affect the sperm plasma membrane via a lipid peroxidation mechanism. Lipid peroxidation then impairs sperm motility and induces sperm DNA damage. Hydrogen peroxide was shown to be the most powerful ROS affecting sperm motility (44). Catalase, vitamin E, and glutathione preserve motility and protect the sperm against lipid peroxidation through scavenging free radicals. Superoxide dismutase was found to be either less effective or ineffective in protecting sperm motility from ROS (6, 74, 75). *In vitro*

use of glutathione, hypotaurine, catalase, and NAC protect the sperm against DNA damage (76). Moreover, the *in vitro* combination of vitamins C and E significantly reduces the effect of hydrogen peroxide and protects the sperm DNA from its effect (77).

Effect of in vitro antioxidants in shielding spermatozoa from endogenous ROS

Antioxidants were found to be less effective in protecting spermatozoa functions and DNA integrity induced by endogenous ROS compared to their beneficial effect in the exogenous ROS setting (6). However, albumin is reported to demonstrate an ability to protect sperm motility in the presence of endogenous ROS (78). Investigators have shown that antioxidants may provide protection from endogenous ROS only if the sperm is abnormal (poor chromatin compaction) (79-81).

Effect of in vitro antioxidants in shielding spermatozoa from semen processing

Semen samples from infertile men are more vulnerable to OS compared with samples from fertile men during *in vitro* semen processing because of the ability of abnormal sperm to generate large amount of ROS compared with the normal sperm (82). Gentle semen processing is important because it reduces sperm DNA damage (78). The studies that addressed the effect of *in vitro* antioxidants on sperm motility and DNA integrity had inconsistent results. Some investigators have shown a beneficial effect of antioxidants (dithiotreitol, NAC, vitamin E, folic acid, glutathione, catalase) on protecting sperm motility and DNA integrity during semen processing (75, 83-87). However, others found no valuable effect of antioxidants (catalase, glutathione, hypotaurine) in preserving motility and DNA integrity during semen processing (87-89).

Effect of in vitro antioxidants in shielding spermatozoa from cryopreservation and thawing

Cryopreservation and thawing can induce lipid peroxidation of the sperm membrane with associated DNA damage and motility impairment. Adding antioxidants could neutralize free radicals resulting from these processes. Several antioxidants have been investigated to examine their role in protecting sperm DNA and improving post-thaw motility, which include vitamin C, vitamin E, tempol, quercetin, catalase, resveratrol, genistein, and selenium. Most studies have demonstrated that supplementation of *in vitro* antioxidants during cryopreservation have a favorable effect on DNA integrity and post-thaw motility (90-94).

A-9: Effects of antioxidants on semen parameters

Several published studies have assessed the impact of various antioxidant treatments on semen parameters. The main limitations of the available evidence include small sample sizes, short follow up, different antioxidant regimens with variable doses, and lack of a proper control in the baseline demographic data. A recent Cochrane Collaboration that included 48 randomized controlled trials assessing the effect of antioxidants in subfertile men revealed a significant variability in the effect of antioxidants on sperm parameters (95). Further studies are needed to delineate the real role of antioxidants in infertile male.

Vitamin C

In 1992, Dawson et al. included 75 male smokers and observed that vitamin C supplementation with a daily dose of 200 mg or 1000 mg resulted in an improvement in sperm quality compared to the placebo group (96). Another study explored the effect of vitamin C (500 mg daily) in 115 infertile men for 3 months following varicocelectomy and they showed an improvement in sperm motility and morphology, but there was no improvement in sperm count (97). Similarly, Gual-Frau et al. included 20 infertile patients with asthenoteratozoospermia and clinical grade 1 varicocele to examine the effect of multivitamin supplementation containing vitamin C (60 mg) for 3 months and observed a statistically significant improvement in the total sperm number, but other sperm parameters were unchanged (98). Moreover, the effect of a vitamin C-containing regimen (vitamin C 80 mg, vitamin E 40 mg, CoQ10 120 mg) was examined in 169 patients with oligoasthenozoospermia after 3 and 6 months of supplementation (99). The authors reported an improvement in sperm concentration and motility. Omu et al. investigated the effect of administering a combination of zinc (200 mg), vitamin E (10 mg), and vitamin C (10 mg) in 45 asthenozoospermic men, and a statistically significant increase in sperm motility was noted (100). Furthermore, Akmal et al. conducted a prospective study on infertile patients with oligozoospermia and reported an improvement in sperm motility, sperm count, and sperm morphology after 2 months of vitamin C supplementation (2000 mg daily) (101).

Many investigators have found that vitamin C supplementation improved semen parameters. However, other studies failed to show such findings. Rolf et al. conducted a prospective randomized placebo-controlled double-blind trial of 31 infertile men with asthenozoospermia (102). Patients were randomized to receive either 1000 mg vitamin C and 800 mg vitamin E or placebo pills for 56 days. The trial showed no improvement in semen

parameters after supplementation compared to placebo. Similarly, Greco et al. evaluated the effect of 2 months of treatment with 1 g vitamin C and 1 g vitamin E daily in men with idiopathic infertility and found that antioxidants had no effect on sperm parameters compared to the placebo group (103).

Vitamin E

A study by Suleiman et al. (1996) showed that 87 infertile patients with asthenozoospermia were included and randomized to vitamin E (300 mg daily) or placebo for 6 months (104). The authors reported an improvement in sperm motility. Subsequent studies examined the combination of vitamin E with other minerals and vitamins. ElSheik et al. performed a prospective randomized trial enrolling 90 infertile men and reported that the combination of vitamin E (400 mg daily) and clomiphene citrate (25 mg daily) for 6 months resulted in an improvement is sperm motility and concentration (105). In another prospective study, the combination of vitamin E (400 units) and selenium (200 μg) for 100 days led to 52.6% improvement in sperm motility and morphology (106).

However, two other randomized double-blind placebo-controlled studies showed no improvement in semen parameters after vitamin E ingestion. Moilanen et al. reported that treatment with vitamin E (300 mg daily) for 3 months did not reveal any significant improvement in sperm concentration, motility, and morphology (107). Additionally, Kessopoulou et al. conducted a study that enrolled 30 infertile men with high ROS and showed that there were no improvement in the semen parameters after vitamin E (600 mg daily) supplementation for 3 months (108).

Carnitines

One of the earliest reports was published by Lenzi et al. when they performed a double-blind cross-over trial and they included 100 infertile men to assess the effect of LC (2 g daily) and they showed an improvement in sperm concentration and motility (109). The improvement in motility was more pronounced in men with a lower baseline sperm motility. One year later, the same group published their experience using LC (2 g daily) combined with L-acetyl-carnitine (LAC) (1 g daily), an improvement in all sperm parameters was noted with a similar trend between motility and low initial values (110). Similarly, Balercia et al. examined the effect of an LC and LAC combination and found a significant increase in sperm motility and total oxygen radical scavenging capacity (111). Moreover, a link was found between the physiologic function of mitochondria, which is reflected by phospholipid hydroperoxide glutathione peroxidase (PHGPx) and carnitine supplementation (112). Garolla et al. observed that carnitine treatment improved sperm motility in infertile men with a normal PHGPx level (112). Additionally, a combination of LC (1 g), LAC (500 mg), fumarate (725 mg), fructose (1 g), CoQ10 (20 mg), vitamin C (90 mg), zinc (10 mg), folic acid (200 μg), and vitamin B12 (1.5 μg) was examined in patients with oligoasthenoteratozoospermia (OAT) with and without varicocele (113). The authors found a significant increase in sperm concentration and motility compared to placebo and the effect was more apparent in the varicocele group (113).

However, a prospective randomized double-blind placebo-controlled trial by Sigman et al. failed to show any significant effect on sperm motility after LC and LAC supplementation in men with idiopathic asthenozoospermia (114).

Coenzyme Q10

CoQ10 was assessed in a randomized double-blind placebo-controlled trial where 60 men with idiopathic infertility were randomized to receive CoQ10 (200 mg daily) or placebo for 6 months (115). The result revealed a statistically significant increase in motility especially in patients with a lower initial motility value (115). Another study examined the effect of CoQ10 (300 mg daily) in 212 infertile men compared to placebo and they showed an improvement in sperm concentration and motility after 26 weeks of supplementation (116). Nadjarzadeh et al. investigated the effect of CoQ10 on OS and antioxidant enzymes in the semen and they observed an improvement in catalase and superoxide dismutase activities that were associated with an increase in sperm morphology (117). Similarly, Thakur et al. showed that daily supplementation of 150 mg CoQ10 significantly improved all sperm parameters in infertile patients (118). However, a double-blind placebo-controlled randomized trial was performed and included 47 men with idiopathic OAT. The patients were randomized to receive CoQ10 (200 mg daily) or placebo for 12 weeks, and they found an increase in total antioxidant capacity with a decrease in lipid peroxidation but with no effect on semen parameters (119).

N-acetylcysteine

NAC (600 mg daily) was shown to improve semen volume, motility, and viscosity in 120 men with idiopathic infertility compared to placebo (120). Moreover, Safarinejad et al. explored the effect of (600 mg NAC + 200 μg selenium) on 468 patients with idiopathic OAT for 26 weeks (121). The authors found an improvement in all sperm parameters and the combination had an additive effect (121).

Selenium

Selenium has been examined in combination with either vitamin E or NAC. The vitamin E (400 mg) and selenium (225 μg) combination for 3 months produced an improvement in sperm motility and a decrease in malondialdehyde (MDA), which is a marker for lipid peroxidation (122). Another clinical trial was performed by Moslemi et al. where the combination of vitamin E (400 units) and selenium (200 μg) for 100 days resulted in a 52.6% improvement in sperm motility and morphology (106). As mentioned in the NAC section, combination of 600 mg NAC + 200 μg selenium for 26 weeks improved all semen parameters. However, Hawkes et al. showed no improvement in semen parameters after 48 weeks of selenium supplementation (300 μg daily) in healthy normozoospermic men (123).

Zinc

A study by Omu et al. (1998) examined the effect of zinc (500 mg daily) for 3 months in 100 patients with astenozoospermia and randomized them to receive zinc or no treatment (124). The study showed an improvement in sperm concentration and motility with a decline in antisperm antibodies (124). Hadwan et al. published two studies in 2014 and 2015, which included infertile men with asthenozoopsermia and compared them to a fertile group. They were given zinc (440 mg daily) for 3 months and the results showed an improvement in sperm concentration and motility. They also observed that catalase-like activity, peroxynitrite levels, nitric oxide synthase activity, and arginase activity were restored to normal ranges in infertile men (125, 126). Moreover, zinc has been examined in combination with either vitamin E and vitamin C or folic acid. In 2008, Omu et al. assessed the effect of administering a combination of

zinc (200 mg), vitamin E (10 mg), and vitamin C (10 mg) in 45 asthenozoospermic men, and they reported a statistically significant increase in sperm motility (100). Additionally, the combination of zinc (66 mg) and folic acid (5 mg) for 26 weeks in fertile and infertile men resulted in a 74% increase in total sperm count in the infertile group (128). A similar regimen was used in another study with a similar duration and it showed an improvement in sperm concentration only, with no effect on other semen parameters (129).

Folic acid

As previously shown in the zinc section, two studies by Wong et al. and Ebisch et al. have revealed the beneficial effect of combining zinc and folic acid on total sperm count and concentration (128, 129). However, Raigani et al. did not show any improvement in sperm concentration following zinc (220 mg daily) and folic acid (5 mg daily) treatment for 16 weeks in 83 men with OAT (127).

Lycopene

Lycopene was shown to significantly improve sperm concentration and motility in two reports that included patients with idiopathic OAT (130, 131).

A-10: Combination of oral antioxidants

The theory behind the current practice of using combination antioxidants is to obtain a synergetic effect in the treatment of male infertility. One of the earliest studies was performed by Comhaire et al. (2000) and they found that the combination of NAC or vitamins A and E and essential fatty acids resulted in a decrease in ROS activity with an increase in sperm

concentration, but no improvement in sperm motility or morphology (132). Similarly, another study included men with persistent oligozoospermia 6 months after varicocele embolization. The patients were given NAC, vitamin C, vitamin E, vitamin A, thiamine, riboflavin, biotin, B12, zinc, and other minerals. Results showed an improvement in sperm count with no effect on sperm motility and morphology (133). Piomboni et al. conducted a study on 36 infertile men with asthenoteratozoospermia and leukocytospermia and noticed a significant improvement in motility and morphology with a decrease in leukocytospermia after 90 days of antioxidant supplementation (beta-glucan, papaya, lactoferrin, 60 mg vitamin C, and 10 mg vitamin E) (134). Similarly, Wirleitner et al. observed an improvement in all semen parameters after an antioxidant combination (200 mg vitamin C, 200 mg vitamin E, 1 mg folic acid, 50 mg zinc, 200 μg selenium, 100 mg NAC, and 600 mg LC) for at least 2 months (135). Similar results were found with using the same regimen but at a lower dose in other studies (136, 137).

However, other studies did not show an improvement in semen parameters after combination antioxidant supplementation (102, 103, 127). The Cochrane review that included 48 randomized controlled trials examining the effect of antioxidants in subfertile men showed significant variability in the effect of antioxidants on sperm parameters (95). More recently, Stenqvist et al. conducted a double-blind placebo controlled randomized trial that included 77 infertile men with a high DNA fragmentation index (DFI) > 25% (138). Subjects were randomized to antioxidant treatment twice per day (30 mg vitamin C, 5 mg vitamin E, 100 μg folic acid, 5 mg zinc, 25 μg selenium, 0.5 μg vitamin B12, 750 mg LC, and 10 mg CoQ10) or placebo for 6 months. The authors showed no statistically significant improvement in any of the semen parameters after 6 months (138).

A-11: Effects of antioxidants on advanced sperm function tests: SDF, HDS, and OS

The fertilization process and subsequent embryo development depends partially on the inherent integrity of the sperm DNA. There may be a threshold of sperm DNA damage (i.e. sperm DNA fragmentation (SDF), abnormal chromatin packaging, and protamine deficiency) beyond which fertilization and embryo development are impaired (139). Several studies have investigated the effect of antioxidants on sperm DNA integrity.

One of the first reports that examined the association between antioxidants and sperm DNA was published by Fraga et al. (1991) when they assessed the effect of vitamin C repletion in 10 subjects who were maintained on a controlled intake of vitamin C for 15 weeks (140). The study showed a significant decrease by 36% in 8-hydroxy-2′-deoxyguanosine (8-OHdG), which is a measure of oxidative DNA damage (140). Similarly, Kodama et al. found a significant reduction in 8-OHdG in 14 infertile men after 2 months of antioxidant supplementation (200 mg vitamin C, 200 mg vitamin E, and 400 mg glutathione) (141). In another study by Greco et al., the combination of 1 g vitamin C and vitamin E daily for 2 months in men with idiopathic infertility and high SDF resulted in a significant decrease in SDF compared to placebo group (103). They used terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) to assess SDF. Moreover, a prospective study showed a decrease in SDF (measured by sperm chromatin structure assay-SCSA) but there was an unexpected increase in %high DNA stainability (%HDS) after 3 months of vitamin C and E (400 mg), zinc (500 μmol), selenium (1 μmol), and β-carotene supplementation (142). Tunc et al. examined the effect of Menevit (100 mg vitamin C, 400 IU vitamin E, 25 mg zinc, 26 μg selenium, 0.5 mg folic acid, lycopene, and garlic oil) in 45 infertile men. The authors revealed a significant decrease in SDF (measured

using TUNEL), an increase sperm protamination, and a decrease in ROS production (143). Omu et al. also assessed the effect of administering the combination of zinc (200 mg), vitamin E (10 mg), and vitamin C (10 mg) in 45 asthenozoospermic men, and they found a statistically significant decrease in SDF (measured using SCSA) (100). Two other prospective studies that used the sperm chromatin dispersion test (SCD) reported a significant decrease in SDF and an improvement in DNA degraded sperm (DDS) after antioxidant supplementation (98, 137).

Conversely, other studies failed to show an effect of high SDF by antioxidants. Silver et al. (2005) included 87 healthy men to study the effect of antioxidant intake (vitamin C, vitamin, E, and β-carotene) on sperm chromatin integrity and they observed no association exist between antioxidants and SDF (measured using SCSA) and HDS. Only subjects with moderate β-carotene intake had high SDF and HDS as a side effect (144). Similar effect on HDS has been shown by Arabi et al. after high-dose folic acid (72). Piomboni et al. also performed a study on 36 infertile men with asthenoteratozoospermia and leukocytospermia and showed no significant decrease in SDF (measured with SCSA) after 90 days of antioxidants supplementation (beta-glucan, papaya, lactoferrin, 60 mg vitamin C, and 10 mg vitamin E) (134). Furthermore, Raigani et al. conducted a double-blind randomized trial in 83 men with OAT who received zinc (220 mg daily) and folic acid (5 mg daily) supplementation for 16 weeks (127). The study showed a trend towards decrease in SDF (measured using SCSA), but a statistically significant increase in aniline blue staining (AB). Recently, Stenqvist et al. conducted a double-blind placebo-controlled randomized trial that included 77 infertile men with a high DFI > 25% (138). Participants were randomized to receive antioxidant treatment twice per day (30 mg vitamin C, 5 mg vitamin E, 100 μg folic acid, 5 mg zinc, 25 μg selenium, 0.5 μg vitamin B12, 750 mg LC,

and 10 mg CoQ10) or placebo for 6 months. The authors showed no statistically significant change in SDF (measured using SCSA) after 6 months (138).

Several investigators have assessed the effect of antioxidants on seminal OS activity. Various antioxidants led to a reduction in MDA, which is a marker for lipid peroxidation (100, 104, 120, 122). Additionally, antioxidants have been found to decrease seminal ROS levels in many reports (132, 143, 145).

A-12: Effects of antioxidants on spontaneous clinical pregnancy, live birth rate (LBR) and ART outcomes

The beneficial effects of antioxidants on semen parameters and sperm quality that have been established in many studies, but their effect in pregnancy is not well-defined (1). Kobori et al. reported a pregnancy rate of 28% in 169 patients with oligoasthenozoospermia after 3 and 6 months of supplementation (vitamin C 80 mg, vitamin E 40 mg, and CoQ10 120 mg) (99). Similarly, vitamin E supplementation resulted in a pregnancy rate of 11% and 21% in two studies (104,106). Moreover, individual supplementation of zinc and carnitine was found to improve the pregnancy rate by over 13% (110, 124). Furthermore, a combination of LC (1 g), LAC (500 mg), fumarate (725 mg), fructose (1 g), CoQ10 (20 mg), vitamin C (90 mg), zinc (10 mg), folic acid (200 μg), and vitamin B12 (1.5 μg) was assessed in patients with OAT with and without varicocele (113). Although the pregnancy rate was not an endpoint in the study, the authors found ten pregnancies in the treatment group compared to two pregnancies in the placebo group (113). However, Rolf et al. reported that vitamin C and E supplementation did not result in pregnancy in a randomized placebo-controlled study, as follows: 0% (0/15) vs. 0% (0/16) (102). Although antioxidant supplementation resulted in an improved pregnancy rate in two other

studies, the difference between treatment and untreated/placebo groups was not statistically significant (133, 146). The Cochrane collaboration that included 48 randomized controlled trials that assessed the effect of antioxidants in subfertile men, which showed a statistically significant increase in the live birth rate and clinical pregnancy rate (95).

The effect of antioxidants on ART was examined in a few studies. Vitamin E was found to improve IVF fertilization rates and sperm zona pellucida binding (104, 108, 147). Additionally, Greco et al. evaluated the effect of 2 months of treatment with 1 g vitamin C and vitamin E daily on the second ICSI attempt in men with high SDF and who had a failed first ICSI attempt (148). The study found no difference in cleavage and fertilization rates, but a significant increase in the clinical pregnancy rate (6.9% to 48.2%) and the implantation rate (2.2 to 19.6%) was noted (148). Tremellen et al. conducted a randomized placebo-controlled double-blind trial comparing the Menevit formulation with placebo in infertile men before their partner's ICSI cycle (149). They reported a significant improvement in the clinical pregnancy rate compared to the placebo group but with no improvement in the fertilization rate or embryo quality between the two groups (149). Finally, CoQ10 resulted in an increase in the fertilization rate in ICSI in subjects with a low fertilization rate in a previous ICSI cycle (10.3% vs. 26.3%, $P < 0.05$) (150).

A-13: Adverse effects of antioxidants: "Antioxidant paradox"

Excessive use of antioxidants should be avoided because it can result in an oxidative state called the "antioxidant paradox", where a shift happens from the redox balance to a more oxidized status, leading to OS (5) (Figure 3). The "Antioxidant paradox" has a detrimental effect at the cellular level, which is similar to the effect seen with OS. Adverse effects that can result

from this condition include infertility, aging, neurodegenerative disease, chromosomal abnormalities, and teratogenicity (5).

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B. Aim of thesis

1) To evaluate the effect of *in vitro* OS on sperm parameters (progressive motility and viability), sperm DNA fragmentation, and chromatin integrity in fertile donors.

2) To evaluate the impact of *in vivo* antioxidant treatment on sperm parameters (progressive motility and concentration), sperm DNA fragmentation, and chromatin integrity in men with idiopathic infertility.

C. MANUSCRIPT 1: EFFECT OF IN VITRO OXIDATIVE STRESS ON SPERM PARAMETERS, SPERM DNA, AND CHROMATIN INTEGRITY: A PROSPECTIVE STUDY

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C-1: ABSTRACT

Background: Reactive oxygen species (ROS) can induce sperm DNA damage, impair sperm motility, and influence assisted reproductive technology (ART) outcomes.

Objectives: To evaluate the effect of in vitro oxidative stress (OS) on sperm parameters (progressive motility and viability), sperm DNA fragmentation (SDF), and chromatin integrity in fertile donors.

Materials and methods: We conducted a prospective study of 10 fertile donors at McGill University Health Center in Montreal. Semen samples were collected and Sperm parameters (progressive motility and viability), DNA fragmentation index (DFI), high DNA stainability (HDS), and iodoacetamide fluorescein (IAF) were measured at baseline and at increasing doses of hydrogen peroxide (H_2O_2): 100, 250, and 500 μM H_2O_2.

Results: The mean viability, mean % progressive motility, and mean % DFI showed a statistically significant differences among the groups ($P < 0.05$). H_2O_2 induced a statistically significant increase in %DFI as the dose of H_2O_2 increased ($P < 0.05$). % positive IAF increased with increasing the dose of H_2O_2 but without statistical significance ($P > 0.05$). In term of the effect of H_2O_2 on % HDS, there was an improvement with increasing H_2O_2 concentrations, but it was not of statistical significance ($P > 0.05$).

Conclusion: In vitro oxidative stress (OS) resulted in a significant increase in DFI with impaired motility at higher concentration of H_2O_2 with no impact on chromatin compaction.

C-2: Introduction

There is a lack of full knowledge pertaining to the nature, extent, and etiology of sperm DNA damage in humans. Sperm chromatin is very tightly compacted by links between the DNA, the nuclear matrix and sperm nuclear proteins (protamines). During spermiogenesis, most of the histones are replaced by transition nuclear proteins (TP-1, TP-2) and then by protamines (1, 2). About 15% of histones remain in sperm chromatin (3). The DNA strands are tightly wrapped around the protamine molecules to form toroidal structures and these protamines are cross-linked by inter- and intra-molecular disulfide bonds which results in compaction of sperm DNA, a step that is important to protect the paternal genome from oxidative stress (OS) (4). Oxidation of sulfhydryl groups associated with protamines is important for the formation of disulfide bonds (5). Any imbalance in the histone to protamine ratio or in oxidation of sulfhydryl groups can result in poor sperm DNA compaction which in turn increases the vulnerability to DNA damage.

Reactive oxygen species (ROS) are unstable compounds derived from oxygen ions, peroxides, and free radicals (6). ROS include superoxide anion radical ($\cdot O_2^-$), which can be converted to other ROS such as hydrogen peroxide (H_2O_2), peroxyl radical ($ROO^\cdot$), and hydroxyl radical ($\cdot OH$) (7). ROS have a very short half-life and they can initiate oxidative attack on lipids present in plasma membrane which results in a process known as "lipid peroxidation". Also, ROS can induce sperm apoptosis which activates caspase enzymes leading to nuclear and mitochondrial DNA damage (8). It has been shown that hydrogen peroxide is one of the most powerful ROS, as it can impair sperm motility via depletion of intracellular adenosine

triphosphate (ATP) and disruption of sperm axoneme (26). Furthermore, abnormally formed sperm can generate more ROS through NADPH oxidase. Thus, there is a correlation between teratozoospermic sperm and higher ROS generation (9).

Assessing OS in vitro has relevant clinical implications, particularly, during assisted reproductive technology (ART) procedures as there is sperm manipulation in vitro that can induce oxidative damage to the sperm DNA. ROS-induced sperm dysfunction can result from serial centrifugation from excess generation of ROS by spermatozoa and semen leukocytes. On the other hand, gentler sperm separation permits good sperm recovery with minimal sperm dysfunction (10).

The purpose of this study was to prospectively evaluate the effect of in vitro OS on sperm parameters, sperm DNA fragmentation (SDF), and chromatin integrity in fertile donors.

C-3: Materials and methods

Materials:

We purchased Acridine orange (AO) from PolySciences (Warrington, PA, USA). We purchased IAF (5-iodoacetamide-fluorescein) from Invitrogen (Burlington, ON, Canada). Unless otherwise stated, all other chemicals were acquired from Sigma Chemical Co (St. Louis, MO, USA) and were at least of reagent grade. Also, we purchased Percoll from GE Healthcare (Baie d'Urfe´, Canada).

Population:

Semen samples were collected from 10 fertile donors. Sperm parameters (progressive motility and viability), DNA fragmentation index (DFI), high DNA stainability (HDS), and iodoacetamide fluorescein (IAF) were measured at baseline and at different hydrogen peroxide (H_2O_2) concentrations. All subjects were informed about the study and signed consent forms. The ethics board of the McGill University Health Centre approved this study.

Semen handling:

Healthy volunteers were chosen for their good semen quality; their semen samples were normal (11). Samples collection was done by masturbation after 3–5 days of sexual abstinence, and we allowed the samples to liquefy for 30 minutes at 37^0 C. Then, spermatozoa were purified by Percoll density gradient centrifugation (95%–65%–40%–20% layers, made isotonic with HEPES-balanced saline) (12, 13). This is followed by centrifugation for 25 min at 2300 xg. Spermatozoa in the 95% Percoll layer and those that in the 65%–95% interface were pooled and diluted to 200 x 10^6 cells/mL with 95% Percoll. These preparations have a progressive sperm motility of >70% and are largely devoid of immature or abnormal cells and of white blood cells (14). Then stocks of 100x H_2O_2: 250 µM and 500 µM were prepared and mixed with sperm in "Biggers, Whitten, and Whittingham" (BWW) medium and incubation with increasing doses of H_2O_2 (at final concentrations of 100, 250 and 500 µM H_2O_2 at 37 °C for 2 hours). After the incubation period was over, bovine serum albumin (BSA) was added (4 mg/ml) and motility measurement done using a computer-assisted semen analyzer –CASA. Then a top up of the remaining tubes with HBS was done, with centrifugation at 1, 500 rpm for 5 min and removing supernatant. The pellet was dissolved in 95% ethanol (for cytochemical tests) and stored at -20 °C. For sperm chromatin structure assay (SCSA), the pellet was dissolved in TNE and stored at -

80 °C. Vitality Assessment was done using (Eosin-Nigrosin (E-N) test). Stain was prepared by dissolving 0.67g Eosin Y in 100 ml distilled water and heated gently. Then 10 g of Nigrosin was added and dissolved. The E-N solution was brought to a boil and the stain was allowed to cool before filtering and then stored in the fridge. After that, it was warmed up to RT before use. The test is done by mixing equal volumes of washed sperm and E-N stain and then smearing the samples on glass slides. After air-drying, we observed under high power magnification and counted at least 200 sperm and those that are white (unstained) were classified as live and those showing any degree of pink or red coloration were classified as dead.

Sperm DFI and HDS:

We assessed sperm DNA damage using the SCSA and we expressed the results as sperm percentage HDS (a measure of sperm chromatin compaction) and sperm percentage DFI (an index of DNA damage). Frozen semen samples were thawed on ice, TNE was used as a dilution buffer (0.1 M TRIS buffer, 0.15 M NaCl, 1 mMEDTA, pH 7.4) and then treatment with 0.40 ml of acid–detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% Triton X-100, pH 1.2) was done. The samples were stained after 30 seconds by adding 1.2 ml acridine orange (AO) stain solution containing 6 µg AO (chromatographically purified; cat. # 04539, Polysciences Inc., Warrington, PA, USA) per ml buffer (0.037 M citric acid, 0.126 M Na2HPO4, 0.0011 M EDTA (di-sodium), 0.15 M NaCl, pH 6.0). Fluorescence was measured after 3 minutes for a minimum of 5000 cells using a MACSQuant Analyzer (Miltenyi Biotech GmBH, Teterow, Germany) equipped with argon laser. Excitation arisen at 488 nm, green and red fluorescence signified double-stranded DNA and single-stranded DNA, respectively. WinList software (Verity Softwarehouse Inc., Topsham, ME, USA) was utilised to generate the cytogram (red vs. green fluorescence) and

histogram (total cells vs. DFI) plots, as well as, %HDS and %DFI readings. We reported the mean of the two sperm percentage DFI and percentage HDS values. The variability of the replicate SCSA measures (percentage DFI and percentage HDS) was <5%. Testing of the increasing dosage of H_2O_2 on sperm samples from a specific donor was always carried out on the same run. We have shown that comparable results (<5% variability) were obtained with SCSA whether tested on fresh or frozen-thawed samples and that repeat assessments of reference semen samples showed that the inter-assay variability of sperm percentage DFI is low (<5%) (41, 42). Over 300 aliquots of the same semen sample ('reference sample') have been stored at −80 °C for ongoing assessment of inter-assay variability. To validate our measures of DNA damage, we have previously observed a strong association (r = 0.71) between terminal nucleotidyl transferase dUTP nick end labeling – TUNEL assay in parallel with sperm percentage DFI (15).

Cytochemical test of sperm chromatin: iodoacetamide fluorescein

We fixed the semen samples with 95% ethanol and we kept them at −20 °C before further processing. Then, we prepared the smears from the fixed samples, left them to air-dry at 20°C for 30 min and immediately stained. Iodoacetamide-fluorescein (IAF) fluorescence (IAF, targets free sulfhydryl group), we incubated the smears with 0.1 M Tris (pH 6.8) for 5 min and then with 0.1 mM IAF for 15 min (16). A brief rinse of the IAF-stained smears with dH2O was done, then we washed with Tris and mounted with glycerol containing 1,4-diazabicyclo [2.2.2] octane (DABCO).

To overcome the subjective variation, we counted at least 200 sperm per slide. We followed the same grading systems adapted by de Lamirande et al. and divided the

counted sperm into three categories: pale (very pale fluorescence over the entire head), intermediate (bright fluorescence over post-acrosomal region) or intense (bright fluorescence over the entire head) (16). In our study, the results of the IAF positive fluorescence was reported as %IAF fluorescence (the % of cells with bright fluorescence of the whole head).

Data analysis:

Results are expressed as mean ± SD. Inter-group differences (BWW and different concentrations of H_2O_2) were assessed using one-way ANOVA. IBM Statistical Package for the Social Sciences (SPSS, version 20; SPSS Inc., IBM Corp., Armonk, NY, USA) was used to collect data and perform statistical analysis. A p value <0.05 was considered statistically significant.

C-4: Results

We enrolled 10 fertile donors. The sperm parameters (progressive motility and viability), DFI, and chromatin integrity measures at baseline and at different H_2O_2 concentrations are shown in Table 1. BWW served as a control medium (baseline) and H_2O_2 served as an in vitro agent to induce OS. The analysis for the DFI and chromatin integrity measures were done for all donors. The analysis for progressive motility and viability was done for 7 & 5 donors, respectively. The mean viability and mean % progressive motility showed a statistically significant differences among the groups ($P < 0.05$). There was an inverse relationship between higher concentrations of H_2O_2 and sperm parameters (progressive motility and viability). The effect of H_2O_2 on mean % positive IAF fluorescence and % HDS was not consistent. H_2O_2 induced a statistically significant increase in %DFI as the dose of H_2O_2 increased ($P < 0.05$).

Moreover, % positive IAF increased with increasing the dose of H_2O_2 but without statistical significance ($P > 0.05$). Interestingly, the effect of H_2O_2 on % HDS was unexpected as there was an improvement with increasing H_2O_2 concentrations, but it was not of statistical significance ($P > 0.05$).

C-5: Discussion

We have performed an in vitro study to assess the impact of OS on sperm parameters (progressive motility and viability), DFI, and chromatin integrity measures at baseline and at different H_2O_2 concentrations. In this study, we have found a great variability in the effect of in vitro OS. There was a statistically significant adverse effect on %DFI, progressive motility and viability. On the other hand, the effect on % positive IAF fluorescence and % HDS was not significant.

Assessment of sperm DNA and chromatin integrity is becoming more popular as it may help predict outcomes and dictate appropriate treatment. Sperm DFI (measured with SCSA) is a measure of DNA damage and susceptibility to denaturation. Moreover, sperm HDS and IAF are measures of sperm chromatin compaction. Specifically, HDS is a measure for sperm decondensation, with high percentages indicating easy accessibility of the stain to DNA and less chromatin compaction (17). IAF fluorescence is another assay for chromatin compaction that targets sulfhydryl group and histone content, with a high positive stain indicating less disulfide (S-S) bonds crosslinking including those of protamines (16). Although, the in vitro OS in our study affected the sperm parameters and sperm DNA, this effect was not reflected at the chromatin compaction level which indicates that the relationship between sperm DNA integrity, nuclear protein content, and sperm nuclear sulfhydryl group status is more complex than we

thought. Even the chromatin compaction measures did not act in the same manner in this experiment. A possible explanation for that is the difference in the mechanisms by which IAF and HDS assess chromatin integrity. Also, it is possible that higher level of OS are needed to see a change in chromatin compaction, especially in case of HDS assay.

In this study, an increase in DFI was associated with a decrease in progressive motility and viability after in vitro OS. Notably, at the highest dose of H_2O_2 (500 μM H_2O_2), there was a dramatic increase in %DFI with a massive decrease in motility, but with no significant effect on IAF and HDS. Several studies have shown an inverse relationship between DFI and sperm parameters with motility being the most affected parameter as OS induces lipid peroxidation and damage in mitochondrial membrane that impairs sperm motility (18-23). In term of the effect of H_2O_2 on DFI and sperm parameters, our findings are in keeping with the results from Aitken et al., Kemal Duru et al. showing a dose-dependent relationship between H_2O_2, DFI, and motility (24, 25).

In our study, we noticed an improvement in %HDS. Although, it was not statistically significant, it supports the observations of previous reports that low level of ROS have physiologic functions and may protect DNA and boost sperm functional activities like capacitation and acrosome reaction (24, 25).

There is paucity of studies that examined effect of in vitro OS on chromatin integrity measures (IAF and HDS). This study indicated that the effect of in vitro OS on sperm DNA and chromatin compaction is variable and more research is needed to understand the nature and the impact of OS on sperm integrity.

In conclusion, we have shown in this prospective study that in vitro OS resulted in a significant increase in DFI with impaired motility at higher concentration of H_2O_2 but with no impact on chromatin compaction.

Table 1 Sperm parameters (progressive motility and viability), DFI, and chromatin integrity measures at baseline (BWW- control medium) and at different H_2O_2 concentrations (in vitro OS)

Parameter	BWW	100 µM H_2O_2	250 µM H_2O_2	500 µM H_2O_2	P value[a]
% positive IAF	4.9±2.33	5.7±2.4	5.9±2.7	6.2±1.9	0.4
%DFI	8.3±4.1	11.7±5.1	16.2±6.5	21.2±7	<0.001
%HDS	2.3±1.5	4.1±4.6	3.8±4.3	2.2±1.7	0.53
Progressive motility (%)	76±11.7	60.6±7.2	53.8±5.1	1.7±2.3	0.0001
Viability (%)	94.4±1.8	89.6±1.8	85.6±3.4	77±3.5	<0.001

Values are mean±SD

BWW: Biggers, Whitten, and Whittingham medium, *H_2O_2: Hydrogen peroxide, OS: oxidative stress, IAF: positive iodoacetamide fluorescence (percentage of intense fluorescence), % DFI: DNA fragmentation index, % HDS: high DNA stainability*

[a] (Kruskal-Wallis one-way ANOVA on ranks)

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D. MANUSCRIPT 2: EFFECT OF ANTIOXIDANT TREATMENT ON SPERM PARAMETERS, SPERM DNA, AND CHROMATIN INTEGRITY: A PROSPECTIVE TRIAL

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D-1: ABSTRACT

Background: There is no clear conclusion that can be drawn from the studies that address the effect of antioxidants on sperm parameters and chromatin integrity. There is a need for larger well-designed clinical studies and for experimental studies.

Objectives: To evaluate the impact of antioxidant treatment on sperm parameters, sperm DNA fragmentation (SDF), and chromatin integrity in men with idiopathic infertility.

Materials and methods: We conducted a prospective study of 24 men presenting with idiopathic infertility at the OVO fertility clinic in Montreal between January 2016 and January 2018. We included 6 healthy fertile sperm donors as a control. All patients received 6 months of antioxidants treatment. We assessed sperm parameters, DNA fragmentation index (DFI), high DNA stainability (HDS), iodoacetamide fluorescein (IAF), and aniline blue (AB) staining before and 6 months after antioxidant treatment.

Results: The fertile donor group had a higher mean sperm concentration and mean % progressive motility compared to the antioxidant group ($P < 0.05$). The mean % DFI, % positive IAF fluorescence, % positive AB staining of antioxidant treatment group were significantly higher than that of the fertile control group ($P < 0.05$). Conversely, the mean % HDS in the antioxidant group was not significantly different than the control group. There was an improvement in sperm parameters after antioxidant treatment, but the difference was not statistically significant ($P > 0.05$). Unexpectedly, the chromatin integrity measures (% HDS, % positive IAF fluorescence, % positive AB) worsened after 6 months of antioxidant treatment, but the changes were not statistically significant ($P > 0.05$). There was a trend toward improvement in % DFI after antioxidants supplementation with borderline statistical significance (mean: 23.4 vs. 19.1; $P = 0.06$).

Conclusion: The study suggests that 6 months of antioxidant supplementation had no significant impact on sperm parameters, DFI, and chromatin integrity measures.

D-2: Introduction

Sperm DNA damage either in form of high SDF or abnormal chromatin packaging can affect fertilization and embryo development. There is now clinical evidence to show that human sperm DNA damage may negatively affect reproductive outcomes and that spermatozoa of infertile men retain more DNA damage than spermatozoa of fertile men (1, 2). Oxidative stress (OS) plays a central role in sperm DNA damage-induced male infertility (3). The prevalence of reactive oxygen species (ROS)-induced damage to sperm has been found to be between 30-80% in infertile men (4). Despite the harmful effect of ROS, a controlled physiologic balance of their production by the sperm cell is essential for some functional aspects including: acrosome reaction, hyperactivation and capacitation (5). High ROS levels and low antioxidant capacity has been reported in normozoospermic men with idiopathic infertility compared to fertile men (6). Moreover, ROS induces sperm DNA damage, sperm membrane fusion defects, and sperm motility impairment via multiple mechanisms (lipid peroxidation process, initiation of apoptosis, and alteration in DNA integrity) (7, 8). As such, ROS is counterbalanced by seminal plasma which is rich in enzymatic and non-enzymatic antioxidants that contribute to sperm protection and support (9).

Oral antioxidants may have a favorable effect in decreasing ROS and protecting the sperm against DNA damage. Nevertheless, a balance between oxidation and reduction is vital for sperm function. As such, excessive antioxidants can cause a shift from the redox status to either a

reduced status “reductive stress” or oxidized status “antioxidant paradox” (10). Antioxidants exert their beneficial effect through neutralizing ROS, preventing lipid peroxidation, maintaining sperm integrity, regulating gene expression, and preservation of sperm motility (11, 12, 13).

Studies have shown that several antioxidants had a beneficial impact on semen parameters, SDF, OS, and clinical pregnancy. However, other investigators did not find such association. Although, a Cochrane Collaboration that comprised 48 randomized controlled trials assessing the effect of antioxidant in subfertile men have shown a statistically significant increase in live birth rate and clinical pregnancy rate. It revealed a significant variability in the effect of antioxidants on sperm parameters (14). The available evidence contains several limitations including short follow up, small sample size, different regimens of antioxidants with variable doses and lack of a proper control of baseline demographic data (15).

The purpose of this study was to prospectively evaluate the effect of antioxidant supplementation on sperm parameters, SDF, and chromatin integrity in men with idiopathic infertility.

D-3: Materials and methods

Materials:

We purchased Acridine orange (AO) from PolySciences (Warrington, PA, USA). Also, We purchased 5-iodoacetamide-fluorescein (IAF) from Invitrogen (Burlington, ON, Canada). Unless otherwise stated, all other chemicals were acquired from Sigma Chemical Co (St. Louis, MO, USA) and were at least of reagent grade.

Study Population:

We performed a prospective study of 24 men presenting with idiopathic infertility at the OVO fertility clinic in Montreal between January 2016 and January 2018. Patients with varicocele, abnormal hormonal levels and history of smoking were excluded. All 24 men submitted semen samples before and 6 months after antioxidant treatment. Antioxidant treatment was supplemented once daily and consisted of 5 mg folic acid, 500 mg vitamin C, 400 IU vitamin E, 100 μg selenium, and 50 mg zinc. Sperm parameters, DNA fragmentation index (DFI), high DNA stainability (HDS), iodoacetamide fluorescein (IAF), and aniline blue (AB) staining were measured before and 6 months after antioxidant supplementation. The controls were 6 healthy fertile sperm donors. All subjects were informed about the study and signed a consent form. The ethics board of the McGill University Health Centre approved this study.

Semen handling:

Semen samples were provided by all participants by masturbation after 3–5 days of sexual abstinence, and were allowed to liquefy for 30 minutes at $37\ ^{0}$C. Standard semen parameters (volume, concentration, motility) were determined using a computer-assisted semen analyzer – CASA (Sperm vision HR software v1.01, Penetrating Innovations, Ingersoll, ON, Canada) . Motile sperm was found in all semen samples, and none of the semen samples had significant numbers of leukocytospermia or round cells or as per WHO guidelines (<1 million round cells/mL). After liquefaction, two 25–100 μL aliquots of raw semen (containing

approximately 1-2 million spermatozoa) were collected from the original sample and frozen at −80 °C for later evaluation of sperm chromatin structure assay (SCSA) parameters (%DFI and %HDS) and cytochemical tests of chromatin integrity (%IAF fluorescence and %AB staining).

Sperm DFI and HDS:

Sperm DNA damage was assessed using the SCSA and we expressed the results as sperm percentage HDS (a measure of sperm chromatin compaction) and sperm percentage DFI (an index of DNA damage). Frozen semen samples were thawed on ice, TNE was used as a dilution buffer (0.1 M TRIS buffer, 0.15 M NaCl, 1 mM EDTA, pH 7.4) and then treatment with 0.40 ml of acid–detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% Triton X-100, pH 1.2) was done. The samples were stained after 30 seconds by adding 1.2 ml acridine orange (AO) stain solution containing 6 μg AO (chromatographically purified; cat. # 04539, Polysciences Inc., Warrington, PA, USA) per ml buffer (0.037 M citric acid, 0.126 M Na2HPO4, 0.0011 M EDTA (di-sodium), 0.15 M NaCl, pH 6.0) (42). Fluorescence was measured after 3 minutes for a minimum of 5000 cells using a MACSQuant Analyzer (Miltenyi Biotech GmBH, Teterow, Germany) equipped with argon laser. Excitation occurred at 488 nm, green and red fluorescence signified double-stranded DNA and single-stranded DNA, respectively. WinList software (Verity Softwarehouse Inc., Topsham, ME, USA) was used to generate the cytogram (red vs. green fluorescence) and histogram (total cells vs. DFI) plots, as well as, %HDS and %DFI readings. We reported the mean of the two sperm % DFI and % HDS values. The variability of the replicate SCSA measures (% DFI and % HDS) was <5%. Testing of paired samples (pre- and post-antioxidants) was always carried out on the same run. We have shown that comparable results (<5% variability) were obtained with SCSA whether tested on fresh or frozen-thawed samples and that

repeat assessments of reference semen samples showed that the inter-assay variability of sperm percentage DFI is low (<5%) (41, 42). Over 300 aliquots of the same semen sample ('reference sample') have been stored at −80 °C for ongoing assessment of inter-assay variability. To validate our measures of DNA damage, we have previously observed a strong association (r = 0.71) between terminal nucleotidyl transferase dUTP nick end labeling – TUNEL assay in parallel with sperm percentage DFI (43).

Cytochemical tests of sperm chromatin: aniline blue and iodoacetamide fluorescein

We fixed the thawed semen samples with 95% ethanol and we kept them at −20 °C before further processing. Then, we prepared the smears from the fixed samples, left them to air-dry at 20°C for 30 min and immediately stained. For evaluation of aniline blue (AB) staining (AB is specific to histone lysines), smears are incubated with the dye (5% AB in 4% acetic acid) for 5 minutes, then washed 3 times with dH2O and mounted with glycerol (39). For assessment of iodoacetamide-fluorescein (IAF) fluorescence (IAF, target sperm protamine sulfhydryl group), we incubated the smears with 0.1 M Tris (pH 6.8) for 5 min and then with 0.1 mM IAF for 15 min (39). A brief rinse of the IAF-stained smears with dH2O was done, then we washed with Tris and mounted with glycerol containing 1,4-diazabicyclo [2.2.2] octane (DABCO).

To overcome the subjective variation, we counted at least 200 sperm per slide. We used the same grading systems adapted by de Lamirande et al. and divided the counted sperm into three categories: Dark blue (dark blue over the whole head), pale (whole head pale staining), or intermediate (post acrosomal region intensely stained) for AB staining (39). For IAF, the fluorescence is graded as pale (very pale fluorescence over the entire head), intermediate (bright fluorescence over post-acrosomal region) or intense (bright

fluorescence over the entire head) (39). In this study, the results of the IAF positive fluorescence and AB positive staining were reported as %IAF fluorescence (the % of cells with bright fluorescence of the whole head) and %AB staining (the % of cells with dark blue stain over the whole head), respectively.

Data analysis:

Results were expressed as mean ± SD. Inter-group (fertile controls and antioxidant group) differences were assessed by Mann–Whitney rank sum test. Differences between before and 6 months after antioxidant treatment parameters were assessed by Wilcoxon signed-rank test. IBM Statistical Package for the Social Sciences (SPSS, version 20; SPSS Inc., IBM Corp., Armonk, NY, USA) was used to collect data and perform statistical analysis. A p value <0.05 was considered statistically significant.

D-4: Results

We enrolled 24 men with idiopathic infertility. The baseline sperm parameters and chromatin integrity measures are shown in Table 1. The mean sperm concentration and mean % progressive motility were significantly higher in the fertile donor group compared to the antioxidant group ($P < 0.05$). The mean % DFI, % positive IAF fluorescence and % positive AB staining of antioxidant group were significantly higher than that of the fertile control group ($P < 0.05$). However, the mean % HDS in the antioxidant group was not significantly different than the control group.

We detected no significant improvement in sperm concentration and chromatin integrity 6 months after antioxidant supplementation (see Table 2). Although, sperm parameters improved

after antioxidant treatment, the difference was not statistically significant ($P > 0.05$). Surprisingly, the chromatin integrity measures (% HDS, % positive IAF fluorescence, % positive AB) were indicative of poorer chromatin compaction after 6 months of antioxidant treatment, but the effect was not statistically significant ($P > 0.05$).

The only beneficial effect of antioxidant supplementation was noted on the % DFI. There was a near significant improvement in % DFI after antioxidants supplementation (mean: 23.4 vs. 19.1; $P = 0.06$).

D-5: Discussion

We have conducted a prospective study to determine the impact of antioxidants on sperm parameters and chromatin integrity measures in men with idiopathic infertility. We have observed that 6 months of antioxidant treatment did not result in a significant beneficial effect. Apart from %HDS, the control group had better conventional sperm parameters and better sperm chromatin integrity.

Several investigators have demonstrated that antioxidant supplementation can improve sperm parameters (16-22). Nevertheless, the Cochrane review along with other studies showed that antioxidants had variable effect on semen parameters (14, 23-25). The lack of significant improvement in sperm parameters after antioxidants in this study is in keeping with multiple reports that failed to show any association between antioxidant intake and effects on semen parameters (26-33). More recently, Stenqvist et al. conducted a randomized controlled trial that included 77 infertile men (34). They observed no statistically significant improvement in any of the semen parameters after 6 months of antioxidant supplementation.

We observed an improvement in %DFI after antioxidant treatment but the difference was not statistically significant. Antioxidants are required for maintenance of sperm DNA and chromatin integrity (5, 19). Nonetheless, combining several antioxidants with different mechanisms might preclude a possible beneficial effect of one or more antioxidants on DNA fragmentation due to a contrary effect (35). A study by Silver et al. that assessed the effect of antioxidants intake (vitamin C, vitamin, E, and β-carotene) on sperm chromatin integrity, found no association between SDF and antioxidant intake (36). Piomboni et al, similarly reported no significant decrease in SDF after 90 days of antioxidants supplementation (35). Raigani et al. performed a double-blind randomized trial in 83 infertile men and treated them with zinc and folic acid supplements for 16 weeks (33). They found a decrease in SDF, but it was not statistically significant. Furthermore, a randomized controlled trial that included men with high DFI > 25% failed to show any significant change in DFI after 6 months of antioxidants compared to placebo (34).

It has been reported that antioxidants may increase sperm DNA decondensation by opening protamine disulphide bonds and this phenomenon is more evident with vitamin C (37). The unwanted effect of antioxidants in attenuating chromatin compaction has been found by some investigators. Menezo et al. observed a decrease in SDF after 3 months of antioxidants treatment, but an unanticipated increase in %HDS was reported in that study (37). In our study, a nonsignificant increase in %HDS was noted and a similar trend was reported by Arabi et al after folic acid supplementation (38).

Aniline blue is an assay for sperm chromatin maturity and condensation that targets lysin-rich histones (33). It is rarely explored in antioxidant studies. The only study that used this assay to evaluate chromatin integrity men with antioxidants was done by Raigani et al. and it

revealed an increase in AB staining in some of the arms in that study but without a statistical significance (33). Moreover, IAF fluorescence is another assay for chromatin compaction that targets sulfhydryl group (39). To our knowledge, no published studies have used the IAF test to study sulfhydryl group status before and after antioxidant therapy. We have previously observed a correlation between % IAF fluorescence and % AB staining and have highlighted the importance of using these tests in assessing chromatin integrity (40).

The findings of this study suggest that the antioxidant effect on sperm DNA and chromatin integrity is not similar. The observed increase in sperm decondensation with less chromatin compaction support the hypothesis of interference of antioxidants with sperm protamine interchain disulphide bonds. In turn, high DNA decondensation can result in in vitro fertilization (IVF) failures. Moreover, antioxidants can induce a shift from the redox status to a "reductive stress" or "antioxidant paradox" which has a damaging effect similar to the one observed with oxidative stress.

The main challenge in assessing antioxidants effect is that there are no known cutoff values for normal redux balance. Thus, prescribing antioxidants should be based on individual characteristic of each infertile patient. Patients with certain risks such as smoking, obesity and high SDF may benefit more from antioxidants and have less risk of "antioxidant paradox" state. More studies are needed to define the target group that will benefit the most from antioxidants with fewer adverse effects.

Our study is one of the very few studies that incorporated sperm parameters with DFI and chromatin integrity tests in the setting of antioxidants and it is the first study that included IAF as a measure of effect of antioxidant on chromatin compaction. An important limitation of our study is the sample size. Clearly, there is a need for larger, randomized

controlled trials to assess the true effect of antioxidants on sperm parameters and chromatin integrity.

In conclusion, we have shown in this prospective study of men with idiopathic infertility that 6 months of antioxidant supplementation had no significant impact on sperm parameters, %DFI and chromatin integrity measures. Future studies are required to determine the best candidate for antioxidants, the ideal regimen, and the optimal duration. Finally, there is a need for development of new tests that evaluate oxidative stress levels, antioxidant capacity, and redox balance to provide a clear indication of when and how to use antioxidants.

Table 1 Sperm parameters and chromatin integrity measures in antioxidant group (before supplement) and fertile healthy donors (controls)

Parameter	Antioxidant	Controls	P value [a]
Concentration (million/ml)	118.5±127.7	256.1±119.7	0.01
Progressive motility (%)	36.1±26.2	72.8±13.9	0.001
%DFI	23.4±12.9	6.7±1.3	0.0003
%HDS	2.1±1.6	2.1±1.1	0.6
%Positive IAF	13±6.8	7±2.9	0.012
%Positive AB	25.7±12.8	9.3±1.7	0.0008

Values are mean±SD

AB: positive aniline blue staining (percentage of dark staining), IAF: positive iodoacetamide fluorescence (percentage of intense fluorescence), % DFI: DNA fragmentation index, % HDS: high DNA stainability

[a] Mann-Whitney rank sum test

Table 2 Sperm parameters and chromatin integrity measures before and 6 months after treatment with antioxidants

Parameter	Before treatment	After treatment	P value [a]
Concentration (million/ml)	118.5±127.7	133.4±164	0.9
Progressive motility (%)	36.1±26.2	37.3±22.4	0.7
%DFI	23.4±12.9	19.1±7.7	0.06
%HDS	2.1±1.6	2.3±1.7	0.12
%Positive IAF	13±6.8	15.7±8.2	0.2
%Positive AB	25.7±12.8	26.6±9.3	0.5

Values are mean±SD

AB: positive aniline blue staining (percentage of dark staining), IAF: positive iodoacetamide fluorescence (percentage of intense fluorescence), % DFI: DNA fragmentation index, % HDS: high DNA stainability

[a] Wilcoxon signed-rank test

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E. Conclusion and Summary

We performed two prospective studies examining the effect of *in vitro* OS and *in vivo* antioxidants on sperm parameters, sperm DNA, and chromatin integrity using various assays. Both studies showed variable effects on theses assays which indicate the complexity of the relation between sperm parameters, DNA integrity, and chromatin compaction. One of the limitations of our studies is the relatively small sample size. Future large randomised controlled trials are needed to determine the real impact of OS and antioxidants on DNA integrity and chromatin compaction.