IJAS Iranian Journal of Applied Animal Science

Impact of Reactive Oxygen Species on Spermatozoa: A Balancing Act between Beneficial and Detrimental Effects

Review Article

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Received on: 20 Jan 2013
Revised on: 5 Mar 2013
Accepted on: 16 Mar 2013
Online Published on: Jun 2014

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Online version is available on: www.ijas.ir

ABSTRACT

Reactive oxygen species (ROS) plays an important role in sperm motility. The physiological generation at low concentration induces beneficial effects on sperm functions and plays a significant role in sperm metabolism. Meanwhile, the excessive generation of reactive oxygen species can overwhelm protective mechanism and triggers changes in lipid and protein layers of sperm plasma membrane, which induces lipid damage, protein damage, DNA damage, motility impairment and alteration in capacitation and acrosome reaction. Reactive oxygen species (ROS) can be measured by variable biomarkers as melondialdehyde (MDA) concentration, individual free radicals, etc. The quantification of free radicals in livestock semen is also briefly reported.

KEY WORDS reactive oxygen species, semen.

INTRODUCTION

Mammalian sperm cells present highly specific lipidic composition, high content of polyunsaturated fatty acids, plasmalogenes and sphingomyelins. This unusual structure of sperm membrane is responsible for its flexibility and the functional ability of sperm cells. Therefore, sperm cells are highly susceptible to reactive oxygen species (ROS) attack. When manipulated *in vitro* during assisted reproductive techniques, these cells run the risk of generating and being exposed to increased levels of ROS. Studies have shown that ROS play a significant role in male infertility (Makker *et al.* 2009).

ROS, including hydrogen peroxide (H_2O_2), superoxide anion (O^{2-}) and hydroxyl radicals (OH^-) are formed as natural by products of the normal metabolism of aerobic organisms.

During metabolism, ROS are unstable and highly reactive, becoming stable by acquiring electrons from nucleic acids, lipids, proteins, carbohydrates or any nearby molecule causing a cascade of chain reactions resulting in cellular damage and disease (Agarwal *et al.* 2005). ROS are a double-edged sword. They are involved in diverse physiological functions of sperm including capacitation, acrosome reaction and binding to the zona pellucida at physiological concentrations (Bucak *et al.* 2007; Zhang *et al.* 2012). Under normal conditions, scavenging molecules known as antioxidants convert ROS to safe by-products to prevent damage caused by ROS. However, when the balance between ROS production and detoxification is disrupted, ROS accumulation elicits oxidative stress that can damage the sperm cell membrane (Ford, 2004), adversely affect DNA integrity (Baumber *et al.* 2003), block oxidative metabolism (Makker *et al.* 2009), reduce the ability for sperm oocyte

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fusion (Griveau and Le Lannou, 1997) and reduce sperm motility and viability (Bucak *et al*. 2007).

Free radicals

ROS are highly reactive oxidizing agents belonging to the class of free radicals. Free radicals can be defined as any atom or molecule (not necessarily derived from oxygen) with one or more unpaired electrons in its outer orbit, which is highly unstable. To become stable, it promptly reacts with other free radicals or non-radicals in its surroundings; this is represented by a superscript bold dot ($R^{\bullet}$). Free radicals can be formed either by processes of homolytic bond fission or by electron transfer reactions (Sikka *et al*. 1995).

Homolytic bond fission

$$A{:}\ B^{\bullet} \longrightarrow A^{\bullet} + B$$

Electron transfer reactions

$$A^{-}{:} + B \longrightarrow A^{\bullet} + B^{-\bullet}$$

The above reaction continues either through absorption of radiations, such as ionizing, UV. visible and thermal radiations, or by redox reactions as non enzymatic electron transfer reactions, metal catalyzed reactions or enzyme catalyzed processes.

Common ROS in reproductive biology

ROS represent a broad category of molecules that indicate the collection of radicals (hydroxyl ion, superoxide, nitric oxide, peroxyl, etc.) and non radicals (ozone, single oxygen, lipid peroxides, hydrogen peroxide) and oxygen derivatives.

The nitrogen derived on free radicals such as nitric oxide ($NO^{\bullet}$) and peroxinitrite anion ($ONOO^{-}$) play a significant role in reproduction and fertilization. In sperm pathology, importance has been given to only oxyradicals such as super oxide anion, hydroxyl radical and nitrous oxide. Sometimes hydrogen peroxide, singlet oxygen ($^1O_2$) and hydrochlorous acid (HOCl) are also included in the category of oxyradicals. But these cannot be considered as free radicals because of its possession of paired electrons in their orbit. Hence the term reactive oxygen species is used to cover all of these chemical species (listed below); (Table 1).

Singlet oxygen: ($^1O_2$).
Super oxide anion radical: ($O_2^{\bullet -}$).
Hydroxyl radical: (OH^{-}).
Perhydroxyl radical: ($HO_2^{\bullet -}$).
Hydrogen peroxide: (H_2O_2).
Peroxyl radical: (ROO^{-}).
Hydro peroxyl radical: (HOO^{-}).
Hypochlorous acid: (HOCl).

Common sources of ROS

The most common sources of ROS are mainly:
Sperm cells themselves (Immature/defective/damaged/dead sperms).
Leucocytes and other inflammatory cells.
Sperm preparation techniques and egg yolk in diluted semen.

Formation of reactive oxygen species

The generation of reactive oxygen species occurs at minute quantities physiologically or at excessive quantities pathologically due to ionizing and solar radiations, xenobiotics, presence of inflammatory cells other than polymorphonuclear cells, increased cellular metabolism and activation of oxidases as well as oxygenases (Babior *et al*. 1973). There is stronger evidence that animal sperm can originate ROS although these may be mainly due to mitochondrial origin (Kumar *et al*. 2002). Superoxide anion is generated continuously by several cellular processes; the most important sources of this are electron transport chain in mitochondria and the endoplasmic reticulum. For every four electrons fed into a cytochrome oxidase complex, a molecule of oxygen is normally reduced to two molecules of water. Some components of electron transport chain especially NaDH-coenzyme Q (CoQ) reductase complex and the reduced form of CoQ, leak few electron on producing univalent reduction of oxygen successively to give super oxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$) (Mazzilli *et al*. 1994). Therefore, the mitochondrial respiration is the main biological source of superoxide anion radicals under normal physiological conditions.

The rate of univalent pathway and the consequent formation of ROS is significantly increased by uncouplers of oxidative phosporylation or hyperbaric oxygen treatment during pathological conditions such as ischaemia, ageing and by alteration of mitochondrial lipids during deficiency of polyunsaturated fatty acids (PUFA) and lipid peroxidation processes. Super oxide anion radicals are also generated in microsomes and plasma membranes under normal conditions (Griveau *et al*. 1995). ROS is also produced through reduction of oxygen by plasma membrane redox system (PMRS) designed to transport reducing power across the cell plasma membrane besides NADPH oxidase activity.

In the microenvironment of cell membranes, hydroperoxyl radicals are formed from superoxide anion. Hydrogen peroxide is the most stable intermediate of successive oxygen reduction and it is generated by the same sources that produce superoxide anion since two molecules of superoxide anion dismute to hydrogen peroxide and oxygen. It can also be produced directly by different cells involved in the inflammatory processes, by a membrane NADH oxidase system and by xanthine / xanthine oxidase system.

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Table 1 Characteristics of reactive oxygen species (ROS)

Reactive oxygen species	Characteristics
($O2^{-}$)	Not crosses plasma membranes but it can diffuse considerable distance from its site of production Half life: 0.4-1000 µs Form both inside and outside the cell
(H_2O_2)	Fragmentation and cross-linking of soluble proteins Damage of membrane integral and peripheral proteins
(OH^{-})	Protein fragmentation Amino acid modification and cross linking (especially when O^2 is absent or limiting) Radicals generated by local amino acid oxidation and fragmentation of polypeptides Fenton's reaction with metal bound to proteins
(HOO^{-})	Oxidation of critical-SH group and intra and inter molecular cross-linking

A membrane bound enzyme aromatic amino acid oxidase (AAAO) oxidatively deaminates the aromatic amino acids (egg yolk is an excellent source) like L-phenylalanine, L-tyrosine, L-tryptophan etc., which is the main source of H_2O_2 in bovine and ram semen at ambient temperature (Tselkas *et al*. 2000). While, hydroxyl radicals are generated during the respiratory burst of neutrophils and macrophages and are cytotoxic products of the less toxic superoxide anion radicals and hydrogen peroxide. Lipid hydroperoxides degradation, super oxide anion radicals or hydrogen peroxide transition metals such as iron or copper yields hydroxyl radicals are responsible of the formation of ROS (Mazzilli *et al.* 1994), (Figure 1).

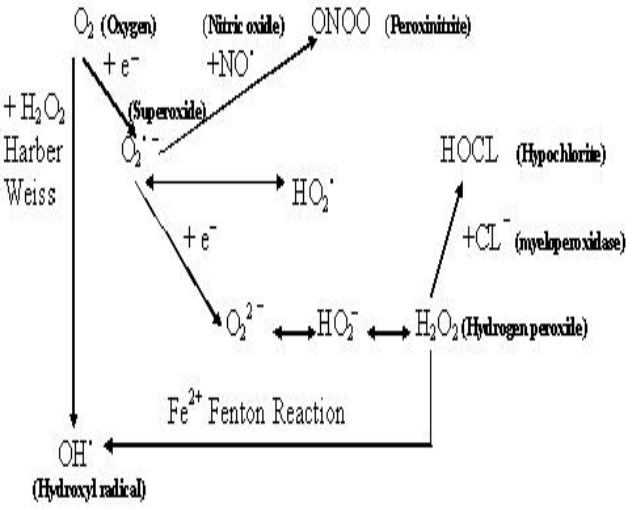

Figure 1 The successive reduction of oxygen to form reactive oxygen species

Physiology and mode of action

Mammalian sperm plasma membrane, rich in polyunsaturated fatty acids (PUFA) can be easily damaged by the reaction between ROS and the polyunsaturated fatty acids. This mechanism is widely known as the lipid peroxidation reaction (Agarwal *et al.* 2003). Lipid peroxidation of sperm membrane is considered to be the key mechanism of this ROS induced sperm damage leading to chromatin destabilization associated with marked alterations in the DNA-protein complex (Surai *et al*. 2001). It also affects the functions of various enzymes, including cytochrome oxidase, lactase dehydrogenase and glucose-phosphate dehydrogenase (Ferrandi *et al*. 1992). Furthermore, ROS distrupts mitochondria functions, inhibit the synthesis of DNA, RNA and proteins, increase DNA fragmentation, modify the cytoskeleton, affect the axoneme (De Lamirande and Gagnon, 1992), through the oxidative stress and the production of cytotoxic aldehydes (Aitken *et al*. 1993), composed of three major steps: initiation, propagation and termination (Nogushi and Niki, 1999). The initiation step is the process of producing lipid radicals by ROS. In the propagation step the lipid radicals from the initiation step attack other unsaturated fatty acid molecules on the cell membrane or steal electrons from oxygen (O_2) to become hydrogen peroxide (H_2O_2). Hydrogen peroxide (H_2O_2) can continue to attack other PUFA on the cell membrane. This step can continue so the lipid peroxidation reaction is also known as the chain reaction (Herrera and Barbas, 2001). In the termination step, which is the end stage of the lipid peroxidation reaction, there is a combination of free radicals to form paired stable electrons. This step can be stopped earlier by antioxidants that can trap free radicals (Silva, 2006; Saraswat *et al*. 2012a). The lipid peroxidation reaction results in changes on sperm membrane fluidity, loss of membrane integrity as well as irreversible loss of sperm motility (Storey, 1997). It has been demonstrated that free oxygen radicals such as superoxide radicals ($O_2^{\bullet -}$) are produced during the freezing-thawing process in human (Mazzilli *et al.* 1994), bovine (Chatterjee and Gagnon, 2001) and dog spermatozoa (Tselkas *et al.* 2000; Michael *et al.* 2007). In addition, the cycle of freezing and thawing has been reported to be responsible for a decrease in the level of antioxidants such as glutathione (GSH) or superoxide dimutase (SOD) in bovine (Bilodeau *et al.* 2000) and human (Alvarez and Storey, 1992) spermatozoa. Therefore, ROS cause sperm damage during the process of sperm freezing. Moreover, their role in spermatozoal function has been very difficult to perceive due to their dual roles i.e. beneficial as

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well as detrimental. In normal levels, ROS helps in many biochemical reactions but at higher levels, they exert detrimental effect on the cells. The higher levels of ROS in the cells may be either due to higher physiologically / pathologically production of ROS or inadequate antioxidant system of the cell to combat the ROS.

Detrimental effects

Lipid damage

ROS main target is sperm plasma membrane, which has double leaflets that are not simply a passive, bilayer, lipidic film in which receptors receive their molecular signals, but these receptors are specialized and its structure consists of a specific composition of phospholipids and a significant concentration of PUFA. Phospholipids are the most representative lipid fraction of sperm plasma membrane especially phosphatidylcholine (lecithin), phosphatidylethanolamine (cephalin) (Michael *et al*. 2007; Michael *et al*. 2009) and the plasmalogen, which is special kind of phospholipid exclusively found in spermatozoa and other cells with the capacity to react promptly to stimuli. In testicular spermatozoa, the prominent fatty acid within their membrane phosphide is palmitic acid (16 C saturated fatty acid) whereas in epididymal spermatozoa is docasahexanoic acid (22 C unsaturated fatty acid). Docasahexanoic acid is attacked by ROS (Kumaresan *et al*. 2009; Bucak *et al*. 2009) due to the presence of double bonds in it. ROS attacks mainly PUFA in sperm plasma membrane and it results in lipid peroxidation (LPO) that is the key mechanism of ROS induced sperm damage leading to infertility. The common types of LPO are no enzymatic lipid peroxidation and enzymatic lipid peroxidation, involves NADPH-cytochrome P-450 reductase and proceeds via ADP-Fe^3 + $O_2^{\cdot -}$ (perferryl complex). The end product of LPO is melondialdehyde (MDA) production induced by ion promoters and its formation can be measured by thiobarbituric acid (TBA) reaction, which is a simple and useful diagnostic tool for measurement of LPO for *in vitro* and *in vivo* systems. The most significant effect of LPO in all cells is the disturbed cellular and organelles membrane structure and its functions like transport process, maintenance of ion and metabolite gradients, receptor mediated signal transduction, etc.

Protein damage

Protein is the major component of seminal plasma and sperm cell organelles. The most of the enzymes responsible for various biochemical reactions of sperm are also proteins. Radicals like superoxide anion because sulphahydral oxidation along with other changes, perhydroxyl radical cause fragmentation and cross-linking of soluble proteins and also damage membrane integral and peripheral proteins. The hydroxyl radical causes protein fragmentation, amino acid modification and cross linking. The end products of lipid peroxidation cause oxidation of critical SH group and intra and inter molecular cross linking; (Table 2).

DNA damage

Lipid peroxides formed during lipid peroxidation and other ROS damage sperm DNA through oxidation of DNA bases though the sperm nuclear chromatin is highly condensed with the high resistance to denaturation. The lipid peroxyl or alkoxyl radicals primarily attack guanine (Upreti *et al*. 1994b) while hydroxyl radicals generate multiple products from all four bases, i.e. 5-hydroxy methyl uracil, 5-hydroxy adenine, thymidine glycol, etc (Griveau *et al*. 1997). The sperm cells before final stages of differentiation and maturation have the ability to repair damaged DNA by removing the damaged bases and nucleotides; however, the matured / ejaculated sperms lack the ability of repair damaged DNA. Damage to DNA can affect sperm functions like motility (Saraswat *et al*. 2012b) by declining the ATP required for the sperm movement. In the DNA repair mechanism, ATP levels of spermatozoa are significantly reduced due to the activation of poly (ADP-ribose) polymerase to repair damaged DNA. The ATP synthesis in the spermatozoa is also compromised greatly due to the depletion of NAD that is utilized as substrate by repair enzyme complex (Upreti *et al*. 1994a). In excessive DNA damage, the process of germ cell apoptosis is accelerated leading to the oligospermia (Babior *et al*. 1973); (Table 3).

Motility impairement

Apart from the reduction of sperm motility (Saraswat *et al*. 2012c) due to low ATP levels caused by DNA repair mechanisms, ROS cause alteration in glyceraldehyde 6-phosphate dehydrogenase (G-6-PDH; highly vulnerable to oxidative stress), responsible for production of ATP by synthesis and oxidation of NADPH in the sperm cell metabolism, thereby reducing the sperm motility due to low level of ATP (Slater, 1984). The end products of lipid peroxidation like MDA inhibit large number of cellular enzymes such as ATPase that may also affect sperm movement. Consequently, the ROS impair sperm movement is mainly produced by inhibition / alteration of one or more enzymes involved in sperm cell metabolism.

Carbohydrate damage

Carbohydrates seem to be relatively less damaged by ROS (Lenzi *et al*. 1996) vis-a-vis lipids and proteins. They react with carbohydrate polymers thus induces fragmentation. Moreover, they affect membrane glycoprotein which can change the surface receptors thus hindering in sperm zona binding at the time of fertilization.

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Table 2 Different radicals responsible for protein damage in semen

Free Radicals	Protein damage
($O2^{\bullet -}$)	Sulphydryl oxidation along with other changes
($HO_2^{\bullet -}$)	Fragmentation and cross-linking of soluble proteins, damage of membrane integral and peripheral proteins
(OH^-) and other higher oxidation states of transition metals	Protein fragmentation, amino acid modification and cross-linking (especially when O^2 is absent or limiting). Radicals generated by local amino acid oxidation and fragmentation of polypeptides. Fenton's reaction with metal bound to proteins
End products of lipid peroxidation like MDA	Oxidation of critical-SH group and intra and inter molecular cross-linking

MDA: melondialdehyde.

Table 3 Methodology used for determination of reactive oxygen species in livestock semen

Species	Type of semen portion / seminal parameter	Methodology used	Free radical produced	Values	References
Goat	Frozen thawed semen	TBA reaction	-	0.6±0.2; 14.2±0.8 (nmol/L)	Bucak *et al*. (2009); Priyadharshni *et al*. (2012)
Ram	Frozen thawed semen	TBA reaction	-	9.2±0.5 (nmol/L)	Bucak *et al*. (2007)
Boar	Liquid semen	Determining MDA production using TBA	-	99.83±2.69 (n*M*\10^9 sperm)	Kumaresan *et al.* (2009)
	seminal plasma			191.98±11.58 (n*M*\10^9 sperm)	
Canine	Liquid semen	By spectrophotometric method based on the dismutase inhibitiable reduction of cytochrome C	Superoxide ($O_2^{\bullet -}$)	0.27±0.07 (nmol/mL min)	Michael *et al*. (2007); Michael *et al*. (2009)
	Frozen semen			0.066±0.16 (nmol/mL min)	
	Liquid semen	Determination of formaldehyde produced by the oxidation of DMSO	Hydroxyl radical (OH^-)	14.2±1.9 (%)	
	Frozen semen			9.23±1.04 (%)	
	Liquid semen	Spectrophotometrically with the use of luminal	tROS	51.6±5.6 ε	
	Frozen semen			136.40±14.41 ε	
Equine	Equine spermatozoa separated from seminal plasma	LPO-586 kit	-	0.43±0.19 m	Baumber *et al*. (2000)

TBA: thiobarbituric acid; MDA: melondialdehyde and DMSO: dimethyl sulphoxide.

Alteration in capacitation and acrosome reaction

The level of ROS present in the cell and in the medium affects both capacitation and acrosome reaction processes. Excessive ROS can play a negative role either in the male genital tract itself by altering the substances present in the plasma that prevent sperm from undergoing capacitation and inducing premature capacitation. Therefore, sperms become much more permeable to calcium ion and thus undergo premature capacitation due to alteration in plasma membrane.

Beneficial effects

ROS interfere physiologically in the regulation of sperm function. Super oxide anion formation at physiological concentration is necessary for the events, like initiation of hyperactivation, capacitation and acrosome reaction of sperms preceding fertilization (Forman and Boveris, 1984). It has been observed that low amount of free radicals in human semen enhance spermatozoa ability to bind zona pellucida (Kumar and Das, 2005).

ROS apart of promoting the capacitation, play a significant role in the regulation of sperm maturation. Phospholipid hydroperoxide glutathione peroxidase (GPX4) can utilize thiol groups in nuclear proteins as an alternative to glutathione. Generation of lipid peroxides by ROS might provide a substrate for GPX4 to drive the oxidation of these proteins and to facilitate nuclear condensation (Aitken, 1995). ROS might also be involved in motility initiation by enhancing the cAMP synthesis and protein phosphorylation at ejaculation (Baumber *et al*. 2000). Antioxidant concentration and storage period had a significant effect on sperm motility, viability, acrosomal integrity and membrane integrity (Upreti *et al.* 1994b).

Assessment of oxidative stress

The measurement of the rate of ROS generation by luminol-induced chemiluminescence is the most common method for quantitating reactive oxygen species. The methods commonly used for measuring ROS can be categorized into: 1) reactions involving nitroblue tetrazolium or cyto-

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chrome c-Fe^{3+} complexes that measure ROS on the cell membrane surface; 2) reactions that measure ROS (generated inside or outside the cell) utilizing luminol-dependent chemiluminescence and 3) the electron spin resonance methods, which are more sensitive and can identify the type of ROS generated inside the cell, require skillful operation, accurate interpretations and expensive instrumentation.

Assessment of lipid peroxidation in spermatozoa

Lipid peroxidation (LPO) is the most extensively studied manifestation of oxygen activation in biology. The most common types of LPO are non-enzymatic membrane LPO and enzymatic (NADPH and adenosine diphosphate (ADP) dependent) LPO.

The enzymatic reaction involves NADPH-cytochrome P-450 reductase and proceeds via an ADP-Fe^3 + $O_2^{\cdot-}$ (perferryl) complex.

In spermatozoa, it is common the production of malondialdehyde (MDA), an end-product of LPO induced by ferrous ion promoters. Formation of MDA can be assayed by the thiobarbituric acid reaction (Shannon and Curson, 1972; Priyadharshni *et al*. 2012) which is a simple and useful diagnostic tool for the measurement of LPO for *in vitro* and *in vivo* systems.

Assessment of superoxide anion radical

Extracellular superoxide anion ($O_2^{\bullet-}$) in semen can be measured by a spectrophotometric method based on the dismutase-inhibitable reduction of cytochrome C (Nash, 1953; Blake *et al*. 1987).

1) Incubate aliquots of diluted semen (0.1 mL) in triplicate samples for 30 min at 37 ˚C with a solution containing the following: 0.36 mL modified Krebs-Ringer phosphate buffer (KRP, Merck, Germany) with 0.04 µmol cytochrome C (type VI, from horse heart, Sigma, Germany); 0.02 mL Tris buffer with or without 150U SOD (from bovine erythrocytes, Fluka, Germany); 0.02 mL Tris buffer with 50U catalase (from bovine liver, Sigma, Germany).

2) Terminate the incubation by the addition of 2 mL of ice-cold KRP containing 1m*M* *N*-ethylmaleimide.

3) Centrifuge the test tubes at 600 × *g* for 10 min.

4) Superoxide anion dependent cytochrome C reduction was calculated by subtracting the reduction occurring in the presence of SOD from that occurring in its absence.

For the calculation, the resulting absorption (obtained by subtracting sample absorption 550-468 with SOD from sample absorption 550-468 without SOD) was multiplied by 2.5 (dilution factor) and divided by 0.0242 (micromolar extinction coefficient of cytochrome C at 550-468), thus giving the nano moles of the $O2^-$ produced by 0.1 mL of semen. The results are expressed as nanomoles $O^{2\bullet-}$ / (mL×min).

Assessment of hydroxial radical

Hydroxyl radical ($OH^{\bullet}$) can be measured based on the determination of formaldehyde produced by the oxidation of dimethyl sulphoxide (DMSO) (Pontiki *et al*. 2006; Schraufstatter *et al*. 1986) modified in order to measure the existing $OH^{\bullet}$ in semen.

1) Prior to $OH^{\bullet}$ measurement, it was washed the sperm by mixing 500 µL of semen or extender in 1ml of Tris-fructose-citric acid buffer (TFC buffer; Tris 3.025 g, fructose 1.25 g, citric acid 1.7 g, distilled water 100 mL, pH 7.0) and then centrifuged at 700 × *g* for 8 min.

2) Discard 1 mL of the supernatant using vortex by adding 1 mL of TFC buffer and gentlymix to the resulting mixture.

3) Incubate the aliquots of washed semen and extender (0.1 mL) in triplicate samples for 30 min at 37 ˚C with a reaction mixture that contains the following: 500 µL phosphate buffer (50 m*M*, pH 7.4), 200 µL ethylene diaminetetraacetic acid (EDTA; 0.1 m*M* in phosphate buffer), 200 µL DMSO (33 m*M* in phosphate buffer).

4) Stop the reaction with 250 µL trichloroacetic acid (CCl_3COOH; 17.5% w/v) and measure the formaldehyde production spectrophotometrically at 412 nm after addition of 1 mL Nash solution, at 60 ˚C for 10 min. Nash solution contains 45 g ammonium acetate (CH_3COONH_4), 0.9 mL acetic acid (CH_3COOH) and 0.6 mL acetylacetone ($CH_3COCH_2COCH_3$) in 100 mL distilled water.

5) Calculate the $OH^{\bullet}$ content by subtracting the absorbance of the extender from the absorbance of the relevant semen sample. Transform the value taken in percentage. The difference in absorbance of mixtures incubated, which contained phosphate buffer, EDTA, $FeCl_3$ and ascorbic acid (10 m*M*), in the presence or absence of DMSO was defined as 100%.

Determination of total reactive oxygen species (tROS)

Total reactive oxygen species (tROS) can be measured with the use of luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione), which is an extremely sensitive chemiluminescent probe that reacts with a variety of ROS.

1) Dilute the aliquots (100 µL) of semen and its relevant extender in triplicate samples using 890µl distilled water.

2) Add 10 µL of luminol (1 m*M* in DMSO) to it.

3) Measure the absorbance at 380 nm.

4) Subtract the absorbance of the extender from the absorbance of the relevant semen sample and express the results as "absorbance coefficient price ε", which can be computed according to the spectro photometric equation of Lambert Beer's law. This relationship may be expressed as:

$A = \varepsilon dc$

Where:

A: absorbance.

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ε: molar extinction coefficient.
d: path length in cm.
c: molar concentration.

Strategies to reduce oxidative stress

Spermatozoa are protected by various antioxidants and antioxidant enzymes in the seminal plasma or in spermatozoa itself to prevent oxidative damage (Kim and Parthasarathy, 2008). An antioxidant that reduces oxidative stress and improves sperm motility could be useful in the management of male infertility (Bansal and Bilaspuri, 2008). Vitamin E (antioxidant) may directly quench the free radicals such as peroxyl and alkoxyl ($ROO^{\cdot}$) generated during ferrous ascorbate-induced LPO, thus it is suggested to act as the major chain breaking antioxidant (Bansal and Bilaspuri, 2009).

Thiol groups also play an important role in detoxification and antioxidation of ROS, besides maintaining the intracellular redox status. These groups serve as defense mechanisms of sperm cells to fight against oxidative stress (Bansal and Bilaspuri, 2008). A variety of biological and chemical antioxidants that attack reactive oxygen species (ROS) and lipid peroxidation (LPO) are presently under investigation (Sikka, 1996).

Recent studies demonstrate that supplementation of cryopreservation extenders with antioxidants has been shown to provide a cryoprotective effect on bull, ram, goat, boar, canine and human sperm quality, thus improving semen parameters, for example, sperm motility and membrane integrity after thawing (Bucak *et al*. 2010). Supplementation with these antioxidants prior to the cryopreservation process may be recommended to facilitate the enhancement of sperm cryopreservation technique for the goat breeding industry (Bucak *et al*. 2010).

Antioxidant defense mechanisms against reactive oxygen species

Antioxidant defense mechanism in semen includes three level of protection

Prevention

Prevention of ROS formation is the first line of defense against oxidative stress. An example is the binding of metal ions, iron and copper ions in particular, which prevents them from initiating a chain reaction.

Interception

To break chain reaction by formation of non-radical end products because "radical begets radical". For example, α-tocopherol (vitamin E), considered a chain breaking antioxidant produces tocopheryl radicals during its oxidation, which can then be reduced by ubiquinone or by ascorbic acid.

The oxidation of vitamin C gives rise to ascorbyl radicals which can be reduced by glutathione, producing thiyl radicals and oxidizing glutathione until this last step. This process can be reversed by glutathione reductase. The defense system of enzymatic antioxidants indicates that catalase (CAT) is a hydrogen peroxide (H_2O_2) detoxifier and also gluthathione peroxidase (GPx) decomposes hydrogen peroxide (H_2O_2) to water and glutathione disulphide which is an important cellular antioxidant (Nogushi and Niki, 1999; Silva, 2006). The defense system of non- enzymatic antioxidants indicates that α-tocopherol is the most important lipid soluble antioxidant and that it protects cell membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Herrera and Barbas, 2001; Traber and Atkinson, 2007). This would remove the free radical intermediates and prevent the oxidation reaction from continuing. The oxidised α-tocopheroxyl radicals produced may be recycled back to the active reduced form through reduction by other antioxidants, such as ascorbate, retinol or ubiquinol (Wang Quinn, 1999). While ascorbic acid on oxidation decompose to form DHAA, ascorbyl radical and H_2O_2. DHAA and ascorbyl radicals on reduction and in presence of glutathione reductase form thiyl radicals and hydrogen peroxide form oxidized glutathione (Nogushi and Niki, 1999; Silva, 2006).

Repair

Damage caused by oxidants may be repaired by fortification of extender with antioxidant. From the aforesaid study it is clear that antioxidants are beneficial in protecting damaging effects of ROS on sperm movement and against oxidative damage caused due to cryopreservation.

CONCLUSION

The mechanism should be standardized and widely be used to maintain a perfect equilibrium between reactive oxygen species production and its detoxification, which is essential for maintaining the normal physiological phenomenon like survival and *in vivo* functions of spermatozoa. This balance will also be necessary during different stages of semen handling and freezing to cause excessive production of reactive oxygen species along with reducing the total antioxidant concentration due to dilution, for harvesting optimum post thaw recovery of fertile sperms for higher conception rate.

ACKNOWLEDGEMENT

We thank the Director, Head of Division (PRSM), of Central Institute for Research on Goats, Makhdoom Farah, Mathura 281122 and India for providing necessary help in the laboratory.

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