

Review

Reactive oxygen species and sperm cells

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Abstract

There is a dynamic interplay between pro- and anti-oxidant substances in human ejaculate. Excessive reactive oxygen species (ROS) generation can overwhelm protective mechanism and initiate changes in lipid and/or protein layers of sperm plasma membranes. Additionally, changes in DNA can be induced. The essential steps of lipid peroxidation have been listed as well as antioxidant substances of semen. A variety of detection techniques of lipid peroxidation have been summarized together with the lipid components of sperm membranes that can be subjected to stress. It is unsolved, a threshold for ROS levels that may induce functional sperm ability or may lead to male infertility.

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. However, spermatozoa's lipids are the main substrates for peroxidation, what may provoke severe functional disorder of sperm. On the other hand, low (physiological) levels of lipid peroxidation reflect the influence of reactive oxygen species (ROS) on sperm metabolism enhancing the ability of human spermatozoa to interact with zona pellucida [1]. A reason for higher, pathological lipid peroxidation of sperm membranes can be unbalanced oxidative stress. In this review we will discuss the influence of reactive oxygen species on sperm function mainly in aspect of lipid peroxidation.

Free radicals

Free radicals are short-lived reactive chemical intermediates, which contain one or more electrons with unpaired spin (Table 1). They are highly reactive and oxidize lipids,

amino acids and carbohydrates as well as causing DNA mutations. Reactive oxygen species therefore may have been implicated as an etiological factor of a very wide range of diseases [2-15]. Enhanced, pathological ROS generation in living organisms may be caused by several mechanisms like: ionizing radiation [16,17], bioactivation of xenobiotics [18], inflammatory cells [19], increased cellular metabolism [20], decompartmentalisation of transition metal ions [21], activation of oxidases and oxygenases [22] and loss of antioxidant capacity [23,24].

Membrane lipids

A characteristic feature of most, if not all, biological membranes is an asymmetrical arrangement of lipids within the bilayer. The lipid composition of plasma membrane of mammalian spermatozoa is markedly different from those of mammalian somatic cells. They have very high levels of phospholipids, sterols, saturated and polyunsaturated fatty acids therefore sperm cells are particularly susceptible to the damage induced by excessive ROS release (Table 2) [25-30].

Table 1: Examples of free radicals

Free Radical	Structure
Nitric oxide	NO•
Nitric dioxide	NO ₂ •
Hypochlorous acid	ClOH
Hypobromous acid	BrOH
Hypoiodous acid	IOH
Alcoyl radical	
	$\begin{array}{c} \text{C} \\ \\ \text{R}-\text{C}-\text{O}\bullet \end{array}$
Peroxy radical	ROO•
Peroxide	ROOH
Superoxide anion	O ₂ •-
Hydrogen peroxide	H ₂ O ₂
Hydroxyl radical	OH•

Table 2: Lipid components in human sperm cells.

Component	nmol/10 ⁸ cells
Phospholipid	
Choline diacylglycerophospholipid	37.0
Ethanolamine diacylglycerophospholipid	31.5
Choline plasmalogen	12.5
Ethanolamine plasmalogen	20.0
Phosphatidilserine	8.5
Phosphatidylinositol	6.1
Phosphatidyloglycerol	0.6
Sphingomyelin	20.0
Cardiolipin	2.1
Total phospholipids	138.3
Fatty acids	
<u>Saturated fatty acids</u>	
Hexadecanoic (palmitic)	105.5
Octadecanoic (stearic)	35.9
<u>Unsaturated fatty acids</u>	
Octadenoic (oleic)	32.6
Octadecadienoic (linoleic)	23.2
Icosatrienoic	14.9
Icosatetraenoic (arachidonic)	20.1
Docosahexaenoic	108.0
Sterols	
Cholesterol	133.0
Desmosterol	78.5
Total sterols	211.5
Glycolipids	6.4

Adapted from Alvarez and Storey [25] and Mack et al. [26]

Lipids are the major substances responsible for the fluidity of membrane lipid bilayers, and changes in composition of the plasma membranes of sperm cells from their epididymal maturation to their capacitation in the female reproductive tract. They are also involved as intermediates in the cell fusion [31-38].

Sperm cells undergo changes in lipid content during their passage through the epididymis. As a consequence of these changes the plasmalogenes (ether-linked lipids) become a major phospholipid component in the cauda epididymis and 2-fold increase of cholesterol/phospholipid molar ratio is observed during sperm migration from the seminiferous tubules [35].

Very high amounts of polyunsaturated fatty acids- especially docosahexaenoic (DHA) are found in the plasma membrane of human sperm (Table 2). DHA is thought to play a major role in regulating membrane fluidity in sperm and in the regulation of spermatogenesis [39-41]. DHA content is significantly higher in immature germ cells and immature spermatozoa as compared to mature sperm and indicates that there is a net decrease in DHA content during the process of sperm maturation [41].

Another sperm lipid, phosphatidylserine, is known to translocate during capacitation. Before capacitation this phospholipid was found mainly in the midpiece but after capacitation, the localisation of phosphatidylserine was changed and it was identified also in the acrosomal region but never in the equatorial area [42].

Human sperm also contains desmosterol, which is lost during capacitation [43]. Other phospholipid, sphingomyelin in the sperm influences the rate of capacitation by slowing down the loss of sterols, and the exogenous sphingomyelinase accelerates capacitation promoting the loss of sterols and generating ceramide [44].

Cholesterol is known to regulate the fluidity and the permeability of cell membrane. Cholesterol efflux during capacitation enables the massive influx of extracellular Ca^{2+} . Increased intracellular Ca^{2+} concentration plays an important role in the acrosome reaction [45-47]. The acrosome reaction is a crucial step during gamete interaction in all species, including man. It allows spermatozoa to penetrate the zona pellucida and fuse with the oocyte membrane. Spermatozoa unable to undergo the acrosome reaction will not fertilize intact oocytes. Zona pellucida binds to at least two different receptors in the plasma membrane. One is a G_i -coupled receptor that activates phospholipase C beta 1. The other one is a tyrosine kinase receptor coupled to phospholipase C gamma. Binding to

the receptor would regulate adenylyl cyclase leading to elevation of cAMP and protein kinase activation. The protein kinase activates a voltage-dependent Ca^{2+} channel in the outer acrosomal membrane which releases Ca^{2+} from the interior of the acrosome to the cytosol. This is the first, relatively small rise in Ca^{2+} , which leads to activation of the phospholipase C gamma. The products of phosphatidyl-inositol bisphosphate hydrolysis by phospholipase C diacylglycerol and inositol-trisphosphate will lead to protein kinase translocation to the plasma membrane and its activation. Protein kinase opens a voltage-dependent Ca^{2+} channel in the plasma membrane, leading to the second increase in Ca^{2+} . The G_i or tyrosine kinase can also activate an Na^+/H^+ exchanger leading to alkalization of the cytosol. Protein kinase also activates phospholipase A2 to generate arachidonic acid from membrane phospholipids.

Arachidonic acid will be converted to prostaglandins and leukotriens by the enzymes, cyclooxygenase and lipoxygenase, respectively. The increase in Ca^{2+} and pH leads to membrane fusion and acrosomal exocytosis [48]. Acrosome reaction takes place in the anterior region of the sperm head. Acrosomal plasma membrane is probably prevented from fusion with the acrosomal outer membrane by its high concentration of anti-fusogenic sterols.

Sperm membrane lipids play also an important role regulating the polarized migration of sperm surface antigens during developmental processes such as maturation and capacitation. Moreover, lipid regionalization may also lead to protein regionalization by virtue of the preferential solubility of the proteins at different sites [49-51].

Furthermore, sperm membrane lipids are involved in gamete interactions. One of them is sulfogalactosylglycerolipid [52] and another one lysophosphatidylcholine [53], which stimulate the fertilizing ability of spermatozoa and induce the changes in composition of zona pellucida, and in oolemma promoting sperm-egg fusion.

In summary, the all-lipid components located in the sperm membranes are involved in regulation of sperm maturation, spermatogenesis, capacitation, acrosome reaction and eventually in membrane fusion. Obviously, peroxidation of sperm lipids may also disturb all the mentioned sperm functions, and in extreme cases even completely inhibit spermatogenesis.

Antioxidants

Every human ejaculate is contaminated with potential secretors of ROS, such as activated leukocytes, precursor germ cells or morphologically abnormal sperm cells. On the other hand, every human ejaculate has intra- and extracellular antioxidants of enzymatic and non-enzymatic

matic systems. Enzymic and low-molecular weight antioxidants exist in human semen to scavenge free radicals as self-protection mechanisms [29,54-56].

The most important antioxidants in human semen

Enzymic antioxidants:

Superoxide dismutase

Catalase

Glutathione peroxidase

Low molecular weight antioxidants:

α -tocopherol

β -carotene

ascorbate

urate

Transition-metal chelators

transferrin

lactoferrin

caeruloplasmin

In some pathological conditions (e.g., genital tract inflammation), the excessive ROS generation leads to seminal oxidative stress which may exhaust antioxidant activity. The final effect of peroxidation is observed among seminal lipids also by their elevated levels [57-59].

Peroxidation of membrane lipids

Peroxidation of polyunsaturated fatty acids (PUFAs) in sperm cell membranes is an autocatalytic, self-propagating reaction, which can give a rise to cell dysfunction associated with loss of membrane function and integrity. The first step in the peroxidation process, called – initiation – is the abstraction of a hydrogen atom from an unsaturated fatty acid. The second step – propagation – is the formation of a lipid alkyl radical followed then by its rapid reaction with oxygen to form a lipid peroxy radical. The peroxy radical is capable of abstracting a hydrogen atom from an unsaturated fatty acid with the concomitant formation of a lipid radical and lipid hydroperoxide. Since the peroxy and alkyl radicals are regenerated, the cycle of propagation could continue indefinitely or until one of the substrates is consumed or terminated in the radical-radical reaction (Figure. 1).

Lipid peroxidation products

Peroxidation of polyunsaturated fatty acids has been implicated in a wide variety of pathological conditions including infertility, cardiac and cerebral ischaemic-reperfusion injury, and inflammatory joint diseases amongst others. The most popular (but not the most important) product of lipid peroxidation is malondialdehyde (MDA). There are a lot of other products of lipid peroxidation such as: conjugated dienes, and secondary peroxidation products, which include saturated and unsaturated aldehydes, ketones, oxo- and hydroxy acids, and saturated and unsaturated hydrocarbons (e.g. ethane, pentane).

Lipid peroxidation in biological membranes causes impairment of membrane functioning, decreased fluidity, inactivation of membrane-bound receptors and enzymes, and increased non-specific permeability to ions. Moreover, lipid hydroperoxides decompose upon exposure to copper while iron chelates the other factors including metals as haem, haemoglobin or myoglobin. Cytotoxic aldehydes are formed as a consequence of lipid hydroperoxide degradation. Malondialdehyde (MDA) and 4-hydroxynonenal are hydrophilic, and are released from low density lipoproteins (LDL) into aqueous surroundings. Hydroxynonenal is biologically active and can cause severe cell dysfunction both on protein and DNA levels. Hydroxynonenal is chemotactic for polymorphonuclear leukocytes (PMNs) at picomolar concentrations, inhibits cell proliferation and is mutagenic [60-63].

In contrast to MDA and hydroxynonenal, the other aldehyde products of lipid peroxidation are hydrophobic and remain closely associated with LDL accumulating to millimolar concentrations. Aldehydes at these elevated levels react with the protein portion of the LDL molecule, called apolipoprotein B (apoB). Consequently, the protein takes a negative charge and its complete structural rearrangement results in the formation of ox-LDL. Ox-LDL is no longer recognized by the LDL receptor, and has several proinflammatory properties.

Methods used to detect and to measure lipid peroxidation [64]

TBA test

The spectrophotometric thiobarbituric acid (TBA) test has been frequently used for many years as an indicator of the peroxidation of polyunsaturated fatty acids. This test involves the reaction of aldehydes with TBA at 100°C under acidic conditions to produce a pink-colored chromogen, which strongly absorbs light at a wavelength of 532 nm.

Fatty acids analysis by GLC or high-performance liquid chromatography (HPLC)

The analysis of fatty acids by GLC or HPLC is very useful for assessment of lipid peroxidation stimulated by different metal complexes, that give different distribution. In this test the loss of unsaturated fatty acids is measured.

Oxygen electrode

Concentration of dissolved oxygen is defined by oxygen electrode when measuring uptake of oxygen by carbon-centered radicals and during peroxide decomposition reactions.

Glutathione peroxidase test

Glutathione peroxidase reacts with hydrogen peroxide oxidizing GSH to GSSG. Addition of glutathione reductase and NADPH to reduce GSSG to GSH results in consumption of NADPH which can be related to the peroxide content.

Cyclooxygenase activity test

Stimulation of cyclooxygenase activity can be used to measure trace amounts of peroxide in biological fluids.

GLC/mass spectrometry assay

Lipid peroxides are measured by extraction, reduction to alcohols, separation by GLC and identification by mass spectrometry.

Hydrocarbon gases assay

This assay can be used as a non-invasive *in vivo* detection of peroxidation. Pentane and ethane are formed during lipid peroxide decomposition and can be measured using GC method.

Light emission

Peroxyl radicals can be produced as a self-reaction of excited carbonyls and singlet oxygen. Singlet oxygen and excited carbonyls emit light as they decay to the resting state. Heam moiety of proteins can decompose lipid peroxides with concomitant formation of reactive intermediates which can react with producing luminol light. The light emission can be measured in chemiluminescent assays.

Fluorescence

Aldehydes such as malondialdehyde (MDA) can react at acidic pH with amino groups to form Schiff bases. At neutral pH, fluorescent dihydropyridines may be formed. Aldehydes can also polymerize to produce fluorescent products in the absence of amino groups.

HPLC/antibody techniques

Cytotoxic aldehydes (e.g. 4-hydroxynonenal) can be measured by HPLC. Moreover, several techniques have

been developed using antibodies to detect proteins modified by lipid peroxidation products.

Lipid peroxidation – detrimental effects on sperm functions

- Excessive generation of ROS in semen, mainly by neutrophils but also by abnormal spermatozoa, could be a cause for infertility [64]. High concentrations of hydrogen peroxide induce lipid peroxidation and result in cell death.
- Increased ROS production by spermatozoa is associated with a decreased mitochondrial membrane potential (MMP). The patients with abnormal semen parameters had a significantly lower MMP [65].
- Infertile men have decreased sperm variables induced by higher ROS levels in semen. A positive relationship exists between increased sperm damage by ROS and higher levels of cytochrome c, and caspases 9 and 3, which indicate apoptosis in patients with 'male factor' of infertility [66].
- Excess of free radical generation frequently involves an error in spermiogenesis resulting in the release of spermatozoa from the germinal epithelium exhibiting abnormally high levels of cytoplasmic retention. Redundant cytoplasm contains enzymes that fuel further generation of ROS by the spermatozoa's plasma membrane redox systems. The consequences of such oxidative stress include a loss of motility and fertilizing potential, and the induction of DNA damage in the sperm nucleus. The loss of sperm function is due to the peroxidation of unsaturated fatty acids in the sperm plasma membrane, as a consequence of which the latter loses its fluidity and the cells lose their function [67].
- H₂O₂ directly affects sperm functions critical at fertilization process in a dose- and time-dependent fashion. Low concentrations maintain capacitation, whereas high concentrations have deleterious effects, as determined by the end points of the capacitation process. These effects are probably dependent on modifications of plasma membrane and intracellular homeostasis by the oxidative process [68].
- The sublethal effects of oxidative stress on motility parameters are significantly associated with membrane translocation of phosphatidylserine in sperm cells membrane [69].
- Oxidative stress induced by white blood cells has a damaging effect on the polyunsaturated fatty acids of sperm phospholipids which may result, among the other effects, in decreased membrane fluidity [70].

Biopositive effects of free radicals

The generation of ROS occurs physiologically during normal cell metabolism. Mitochondrial respiration is the main biological source of superoxide anion radicals under physiological conditions. During the tetravalent reduction of oxygen to water by the mitochondrial cytochrome c oxidase, these radicals can leak to the cell. At low concentrations reactive oxidants have a biopositive effect and act selectively [71]. They act on the metabolism of prostanooids, in gene regulation or in the regulation of cellular growth, intracellular signaling, and the other types of signal transduction. Moreover, oxygen free radicals play an important role in regulation of vasotonus and in antimicrobial defense. Limited amounts of ROS can also interfere physiologically in the regulation of sperm functions. It has been observed that low amounts of free radicals in human semen enhance spermatozoa ability to bind zona pellucida. In addition, the incubation of sperm cells with low concentrations of hydrogen peroxide was found to stimulate sperm capacitation, hyperactivation, acrosome reaction and oocyte fusion [72-75].

Future directions

Future research efforts should be directed towards understanding the role of particular components of sperm membrane and its transformations. Unfortunately, it is still not clear if sperm membrane peroxidated products are indispensable or detrimental in this process. If sperm membrane lipid peroxidation can be considered as useful, the inhibition of endogenous antioxidant activity would be advantageous in special, physiologic conditions. In such situation the administration of exogenous antioxidants would be inappropriate. On the other hand, if sperm lipid peroxidation inhibits or alters the physiological processes of, for instance, sperm maturation, the therapeutic intervention would be beneficial. Determining the structure and functional changes in sperm membrane lipids during the process of peroxidation may be useful in understanding the role of lipid metabolism in spermatozoa physiology and may help to develop novel therapeutic strategies for male infertility.

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