

REVIEW

Leukocytes and oxidative stress: dilemma for sperm function and male fertility

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Spermatozoa are constantly exposed to the interphase between oxidation through high amounts of reactive oxygen species (ROS) and leukocytes, and reduction by means of scavengers and antioxidants. Considering the very special functions as being the only cells with such high polarization and exerting their functions outside the body, even in a different individual, the female genital tract, the membranes of these cells are chemically composed of an extraordinary high amount of polyunsaturated fatty acids. This in turn, renders them very susceptible to oxidative stress, which is defined as an imbalance between oxidation and reduction towards the oxidative status. As a result, ROS deriving from both leukocytes and the male germ cells themselves cause a process called 'lipid peroxidation' and other damages to the sperm cell. On the other hand, a certain limited amount of ROS is essential in order to trigger vital physiological reactions in cells, including capacitation or the acrosome reaction in sperm. The treatment of patients with antioxidants to compensate the oxidative status caused by oxidative stress is highly debated as uncontrolled antioxidative treatment might derail the system towards the reduced status, which is also unphysiological and can even induce cancer. This paradox is called the 'antioxidant paradox'. Therefore, a proper andrological diagnostic work-up, including the evaluation of ROS levels and the antioxidant capacity of the semen, has to be carried out beforehand, aimed at keeping the fine balance between oxidation and scavenging of vital amounts of ROS. *Asian Journal of Andrology* (2011) 13, 43–52; doi:10.1038/aja.2010.76; published online 15 November 2010

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INTRODUCTION

In 1943, the Scottish scientist John MacLeod made the ground-breaking observation that human spermatozoa produce reactive oxygen species (ROS), i.e., highly reactive derivatives of oxygen such as hydrogen peroxide (H_2O_2), and that these substances have harmful effects on various sperm functions.¹ In more recent years, scientists revealed that ROS not only negatively impacted on the fertilization process, but also raised serious questions about the health and well-being of the progeny if the male germ cell was exposed to high concentrations of oxidants.^{2–4} This concept that oxidative stress causes damage to spermatozoa by impairing various sperm functions like motility, acrosome reaction or DNA integrity is now widely accepted.^{5–10}

On the other hand, a considerable amount of work substantiated that very low, controlled amounts of oxidants appeared to be essential for normal physiological functioning of the male germ cell, thus having vital positive effects for various sperm functions like motility, capacitation or the induction of acrosome reaction.^{11–15} Hence, there is reason to believe that ROS also play an important role in cell signaling and specific activation of different physiological processes which often utilize redox-regulated signal transduction pathways.^{16–18}

Because of these ambivalent functions of reactive oxygen species on sperm physiology as well as the very special nature of the male germ cell, a risk–benefit estimation for these potentially pernicious molecules has to be performed. Therefore, it is the aim of this report to

highlight this delicate balance between beneficial and detrimental effects of ROS and shed light on the nature and origin of ROS, sperm morphology, functions and physiology as well as on the various mechanisms available in the male and female organism to keep this balance at physiological levels. For the comprehension of sperm physiology as well as the fertilization process, one needs to realize that a physiological or pathological imbalance of this redox equilibrium for spermatozoa can either activate the male germ cells or eliminate them from the system by making them dysfunctional or eliminate them totally by the phagocytotic action of activated leukocytes, which are present in the genital tract of both male and female. Essentially, one has to understand that oxygen is actually a double-edged sword; on the one hand, oxygen is crucial for life on earth and derivatives are fundamental for the induction of signal transduction cascades in certain physiological processes, but on the other hand, the element and its derivatives are detrimental to cells, including spermatozoa. This is called the 'oxygen paradox'.^{19,20}

SPERM MORPHOLOGY AND MEMBRANE LIPID COMPOSITION

Sperm morphology

During spermiogenesis, i.e., the process of sperm elongation, the male germ cell undergoes dramatic morphological changes whereby the spermatozoon gains its specific shape. Together with these morphological changes, the nucleus condenses and the flagellum extends

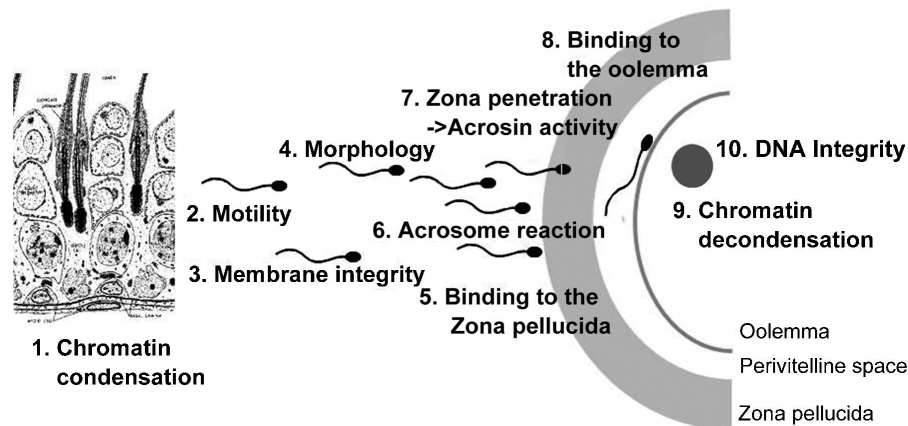


Figure 1 Schematic depiction of sperm functional parameters.

rendering the male germ cell not only the smallest cell in the human body, but also the most polarized cell (sperm head versus flagellum) with a diameter in the sperm head region of about 2.6–3.1 μm and an overall length of about 54.5–61.5 μm .^{21,22} Sperm elongation goes along with a dramatic loss of most of the cytoplasm, which in any cell contains intracellular scavengers to counteract oxidative stress, and is phagocytosed by Sertoli cells. Consequently, spermatozoa exhibit an inevitable lack of intrinsic antioxidative protection by ROS scavengers like catalase, glutathione peroxidase or superoxide dismutase as well as non-enzymatic molecules such as vitamins C or E or glutathione, which counteract the deleterious effects of oxidants in all aerobic cells.

Moreover, the male germ cell is the only cell that fulfills its functions extracorporally, even in a different individual, in the female genital tract whereby the male genetic information in the form of the genome is transported to the oocyte. For this, apart from covering a comparatively large distance of about 16–24 cm in the human from the vagina to the ampulla of the Fallopian tube, where fertilization takes place, the male germ cells have to interact with the oocyte and overcome various physiological barriers, namely, the cumulus oophorus, zona pellucida and oolemma. Thus, the sperm cells' obvious characteristics and functions (Figure 1) are their ability to move and to bind to and penetrate the oocyte vestments. These sperm functions are closely associated with the high compartmentalization of these cells (sperm head as 'container' for the genome and the acrosome as 'interacting device' with the oocyte; neck piece of the flagellum as 'connector'; mid-piece of the flagellum with mitochondria as 'power house' and the principal piece of the flagellum as main generator of sperm motility), which, compared with any other cell, comes along with a dramatic lack of cytoplasm as well as their specifically composed plasma membrane. Both these morphological and chemical characteristics are essential for normal sperm functions.

The extreme polarization of the male germ cell can only be properly maintained by a highly fluid plasma membrane.^{23,24} Since sperm functions mainly have to be regarded as membrane functions,^{25–27} membrane integrity is directly related to normal sperm functions.^{28–30} Consequently, normal sperm functions are, among others, determined by the composition of the plasma membrane lipids, which can be divided into phospholipids, neutral lipids and glycolipids.²⁴ The phospholipids can be subdivided into phosphoglycerolipids and sphingomyelin, of which the composition of phosphoglycerolipids varies considerably among different mammalian species and shows an extraordinary high content of polyunsaturated fatty acids (Table 1).³¹

Importance of sperm plasma membrane and lipids for sperm functions

Owing to its special functions, the plasma membrane of spermatozoa, in contrast to somatic cells, exhibits a very special composition of phospholipids, sterols and saturated as well as polyunsaturated fatty acids (PUFAs). Human sperm plasma membranes show a high content of unsaturated fatty acids, which amount to about 50% of the total content of fatty acids. Particularly high is the percentage of docosahexaenoic acid (DHA), a PUFA containing six double bonds per molecule,³² which makes up about 21% of the total fatty acid and 43% of the PUFA content of human spermatozoa (Table 1).³³

During recent years, the PUFA content in patients with oligozoospermia and/or asthenozoospermia was debated since different groups presented conflicting data with good arguments. On the one hand, Zalata *et al.*,²⁷ Lenzi *et al.*³⁴ and Tavilani *et al.*³⁵ reported that the PUFA levels in poorly motile sperm as well as patients' sperm were significantly lower, which would contribute to the poor motility in asthenozoospermic samples and even in patients with male genital tract infection. These data were corroborated by both the observations that

Table 1 Most important lipids present in the human sperm plasma membrane (according to Zalata *et al.*²⁷ and Sanocka and Kurpiz³³)

Component	nmol/10 ⁸ sperm	% of total fatty acids	%
Phospholipids			
Choline diacylglycerophospholipid	37.0	—	—
Ethanolamine diacylglycerophospholipid	31.5	—	—
Ethanolamine plasmalogen	20.0	—	—
Sphingomyelin	20.0	—	—
Choline plasmalogen	12.5	—	—
Fatty acid composition of phospholipids			
Saturated fatty acids (SFA)		49.95	of SFA
Palmitic acid (C16)	105.5	29.73	59.52
Stearic acid (C18)	35.9	11.35	22.72
Unsaturated fatty acids (UFA)		50.05	of UFA
Docosahexaenoic acid (C22:6; ω 3)	108.0	21.54	43.04
Oleic acid (C18:1; ω 9)	32.6	9.17	18.32
Linoleic acid (C18:2; ω 6)	23.2	3.91	7.81
Arachidonic acid (C20:4; ω 6)	20.1	2.39	4.77
Icosatrienoic acid (C20:3; ω 6)	14.9	2.71	5.41
Sterols (neutral lipids)			
Cholesterol	133.0	—	—
Desmosterol	78.5	—	—
Glycolipids	6.4	—	—

the mean melting point of fatty acids, an index of membrane fluidity, was significantly higher in these patients³² and saturated fatty acids predominate in the caput epididymis,³⁶ which, in turn, is in line with the fact that caput epididymal sperm are immotile.

In contrast, Ollero *et al.*³⁷ observed the highest levels of PUFAs, particularly DHA, in immature germ cells and spermatozoa. Similarly, Khosrowbeygi and Zarghami³⁸ revealed that pathological semen samples with poor motility have higher PUFA levels, especially DHA, than normozoospermic samples. These authors conclude that because spermatozoa from pathological samples with poor motility have such high contents of PUFAs, these spermatozoa are much more susceptible to oxidative damage by ROS. In a very recent report, Koppers *et al.*³⁹ confirmed these results and suggested that the reason for this high content of DHA in immature spermatozoa would lie in an aberrant transformation of defective germ cells during their morphogenesis leaving these cells with too high levels of PUFAs. Thus, an excess amount of unsaturated fatty acids appears to trigger mitochondrial ROS production and, therefore create oxidative stress for these spermatozoa, which results in functional damage. Koppers and co-workers³⁹ interpret this discrepancy with the different endpoints used in the different studies. While Zalata *et al.*²⁷ and Lenzi *et al.*³⁴ measured the free fatty acids as percentage of the content, Ollero and co-workers³⁷ determined the free fatty acid content per cell.

In light of the intrinsic lack of antioxidant protection combined with the extraordinary high lipid content of the plasma membrane, the male germ cell is extremely vulnerable to oxidative stress. Accordingly, these factors render these cells heavily dependent on antioxidative protection provided from the surrounding milieu, i.e., the epididymal fluid, seminal plasma as well as the fluids in the female genital tract. This is of particular importance since vagina and uterus are not immunologically privileged organs. Thus, specific mechanisms to protect the male germ cell and its functions must be provided by the male genital tract secretions in the seminal plasma as well as the female genital tract.

WHAT ARE RADICALS AND ROS?

From a chemical point of view, radicals are chemical intermediates that have one or more unpaired electrons. This chemical condition causes an electronically labile state and results in extreme reactivity of the respective molecules. Biologically important and relevant are free radical derivatives of oxygen (O_2) and nitrogen (N_2). These molecules are called ROS or reactive nitrogen species, of which only ROS are discussed in this report.

Molecular oxygen (O_2) itself has an unusual electronic structure; it is a paramagnetic diradical that has two unpaired electrons with parallel spins. Although oxygen is a diradical, its peculiar electronic structure makes molecular oxygen relatively non-reactive. Since a simultaneous introduction of two electrons into the molecule would contravene rules of quantum mechanics, additional electrons are introduced into the orbitals one by one resulting in the formation of reduced intermediates which are radicals, once this molecule reacts (Figure 2).

Examples of ROS relevant for spermatozoa are hydroxyl radicals ($\cdot OH$), superoxide anion ($\cdot O_2^-$) or hydrogen peroxide (H_2O_2). ROS have a high oxidative potential and therefore very short half-life times in the nanosecond (10^{-9} s) ($\cdot OH$; hydroxyl radicals) to millisecond range (10^{-3} s) ($\cdot O_2^-$; superoxide anion).⁴⁰ Therefore, these radicals practically react at the site of generation. On the contrary, some other oxygen intermediates like peroxy ($ROO\cdot$) and alkoxy ($RO\cdot$) radicals, which can be formed in plasma membranes in the process of lipid peroxidation (LPO, see below), show half-life times even in the second range ($RO\cdot$: 7 s) and are consequently comparatively stable. Thus,

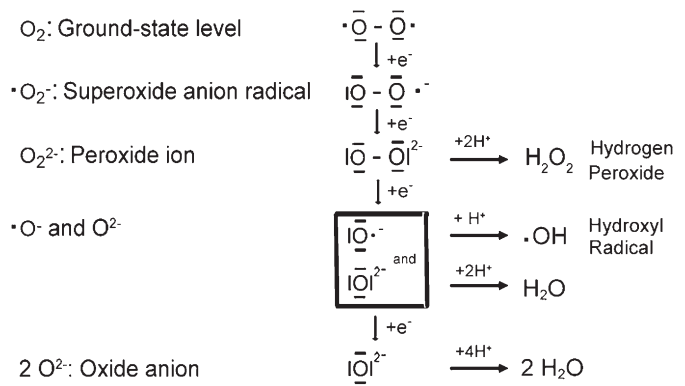


Figure 2 Oxidation forms of oxygen. If molecular oxygen, which is a diradical with two unpaired electrons, is reduced, it acquires four electrons and water (H_2O) is formed. The dashes around the oxygen (O) represent paired, the points represent unpaired electrons.

such molecules can travel some distances and exert their actions at other target sites.

In addition, another ROS, hydrogen peroxide (H_2O_2), which is chemically not a radical as it does not have unpaired electrons, but is also highly reactive, is persistent and can freely penetrate plasma membranes because it is electronically not charged. It is harmful to spermatozoa from different species and their functions at high concentrations.^{1,41–44} Hydrogen peroxide derives from spontaneous or enzymatic dismutation of superoxide. In contrast, other ROS like superoxide anion or the hydroxyl radical are non-membrane permeable.

ROS PRODUCTION IN HUMAN SPERMATOZOA

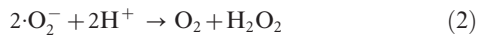
Like in any other living cell, in spermatozoa energy is largely aerobically produced by means of enzymatically controlled mitochondrial oxidative phosphorylation and oxidation of hydrogen in the form of nicotinamide adenine dinucleotide (NADH). Subsequently, the chemical energy is conserved as adenosine triphosphate. In the course of this stepwise process of the electron transfer chain, elementary oxygen (O_2) is taking up four electrons and is thus reduced to highly reactive free radicals as intermediate products. At the end of this process of oxygen reduction, water (H_2O) is formed (Figure 2). Yet, mammalian spermatozoa may also obtain the metabolic energy in form of adenosine triphosphate *via* the Embden–Meyerhof–Parnas pathway (glycolysis). However, this is species-specific and dependent on the demands set in the female genital tract.⁴⁵

In spermatozoa, a sperm-specific diaphorase (NADPH-dependent oxidoreductase)⁴⁶ is located in the sperm mid-piece of the flagellum.⁴⁷ Furthermore, mitochondria in somatic cells have been shown to possess at least nine sites capable of producing superoxide radicals (Equation (1)),⁴⁸ of which the Complex I (NADH dehydrogenase) and Complex III (coenzyme Q: cytochrome *c*—oxidoreductase) have been demonstrated in spermatozoa.^{5,49} Disruption and subsequent leakage of electrons from the mitochondrial electron transfer chain resulted in the generation of ROS from Complex I or III.^{49,50} Even in the course of normal physiological aerobic metabolism, about 1–5% of the consumed oxygen is converted into free radicals^{51,52} and ROS produced *via* this mechanism are normally regarded as cytotoxic byproducts that are involved in the etiology of disease and aging.⁵³



The superoxide anion, in turn, dismutates under the influence of a scavenging enzyme, superoxide dismutase, into hydrogen peroxide

(Equation (2)). Thus, spermatozoa are very professional and effective producers of ROS like superoxide and H_2O_2 .



Additionally, *in vivo* reactions of these ROS are coupled *via* the Fenton and Haber-Weiss reactions (Figure 3) in cycles where cofactors, particularly transition metal ions like $\text{Fe}^{2+}/\text{Fe}^{3+}$, accelerate the reaction in generating the most reactive hydroxyl radicals. Normally, the generation of these free radicals is regulated by scavengers like manganese superoxide dismutase, copper/zinc superoxide dismutase, glutathione peroxidase and catalase.

Considering that the spermatozoon's own ROS generation is fuelled by cytoplasmic glucose-6-phosphate dehydrogenase,⁵⁴ it is apparent that sperm exhibiting excess residual cytoplasm, which is one characteristic of poor sperm morphology due to immaturity and affects sperm fertilizing potential,⁵⁵ are deemed to generate excessive amounts of ROS themselves.^{54,56,57} There are also significant cell-to-cell differences in ROS production in subsets of spermatozoa at different maturational stages.⁵⁸ The clinical importance of the sperm's own ROS production was underlined by considerably stronger correlations between the percentage of ROS-producing spermatozoa and different parameters in the ejaculate including sperm motility.⁵⁹ This is particularly important, as excessively ROS-producing sperm seem to damage themselves, especially their DNA.

OTHER ORIGINS OF ROS AND OXIDATIVE STRESS

Leukocytes in the male genital tract

In the male genital tract and the ejaculate, ROS are not only deriving from the spermatozoa but can also be generated by leukocytes, which physiologically produce even up to 1000 times more ROS than spermatozoa.^{60,61} This high ROS production by leukocytes plays a major role in infections, inflammation and cellular defense mechanisms. Basically, the cellular mechanisms for the generation of ROS in leukocytes and spermatozoa are the same, yet in leukocytes it is a physiological necessity to release large amounts of superoxide into phagocytic vesicles during the killing action of pathogens.

Leukocytes are present in the genital tract of both male and female, even in healthy, fertile individuals not having an infection.⁶² In cases of male genital tract infections and inflammations, however, fertility including sperm functions^{63,64} is seriously affected as clinical findings show oligozoospermia, asthenozoospermia or even azoospermia.^{65,66} These changes can be triggered in various ways, namely, direct action of the pathogens on spermatozoa *via* agglutination⁶⁷ or indirectly by inducing inflammatory processes in the seminal tract by activating leukocytes.⁶⁸ In non-selected cases, the prevalence of male genital tract infection-related infertility varies between 10 and 20% and amounts to up to 35% in a large study comprising of more than 4000 patients consulting for infertility.⁶⁹ It also appears that bacterial infections have a more detrimental effect in fertility-compromised patients than in

fertile men,⁷⁰ indicating that the impact of such bacterial genital tract infections may have to be differentiated.

As a result of male genital tract infections/inflammations, activated leukocytes infiltrate the infected organs releasing high amounts of ROS,⁶⁰ which have been shown to be associated with infertility by induction and stimulation of membrane LPO through oxidative stress.^{9,25,71} Through this mechanism, infections/inflammations do not only damage sperm DNA and reduce sperm count and seminal volume, but also impair sperm functions like motility, acrosome reaction or acrosin activity.^{59,72–77}

Leukocytes in the female genital tract

Once they are ejaculated, spermatozoa are facing not only physical stress but also harsh environmental conditions in the female genital tract, which opens to the exterior and is therefore an entrance for pathogens to the female. Consequently, the vagina is well equipped with antimicrobial defense systems, which include a pH lower than 5 and immunological defense by leukocytes as spermatozoa are allo-genic to the female.⁷⁸ Although some antibacterial and antioxidative protection to spermatozoa is provided by the seminal plasma,^{79–82} and sperm initially outnumber the leukocytes present in vagina and cervix, they have to 'escape' the increasing number of leukocytes. Already 5–10 min after insemination spermatozoa have been recovered from the oviducts of inseminated women.⁸³ Hence, rapid sperm transport from the site of semen deposition to the Fallopian tube is aided by the female organism for the male germ cells to overcome the female immunological defense. In addition, once sperm move out of the protective seminal plasma, they are progressively loosing the protection in terms of scavengers for ROS like spermine^{79,80} or uric acid⁸⁴ which are abundantly provided in the seminal fluid. Spermatozoa therefore become increasingly susceptible to attack by leukocytes that eventually outnumber spermatozoa.⁸⁵ On the other hand, a recent report by Navarrete Gomez *et al.*⁸⁶ indicates that human tubular fluid appears to contain a protective compound against leukocyte-induced sperm DNA damage. Yet, the nature of this protective factor is still unknown.

Other sources of ROS

Besides ROS coming from leukocytic attacks to spermatozoa in the male and female genital tracts due to infection/inflammation and immunological stimulation, spermatozoa are facing ROS deriving from exogenous sources. These include environmental pollutants like heavy metals, pesticides or phthalate, a compound that is frequently found in plastic articles or cosmetics. Furthermore, smoking, alcohol, varicocele or spinal cord injuries contribute to such oxidative stress. For all these environmental and lifestyle factors, the impairment of fertility through ROS generation and subsequent oxidative stress has been reported.^{87–93}

OXIDATIVE STRESS

Considering that any living cell functions in a chemically rather reduced status, it is of utmost importance that the equilibrium between oxidants and antioxidants is finely balanced and kept in a steady state. This reduced status is maintained by various antioxidative systems, both enzymatic and non-enzymatic. While glutathione peroxidase and superoxide dismutase are most common among the enzymatic systems,⁹⁴ vitamins like vitamin A, C or E represent important non-enzymatic antioxidants.⁹⁵ Thus, cells are constantly facing the interface between the oxidative and reductive status. In cases where this steady state derails for whatever reason an imbalance in favor of

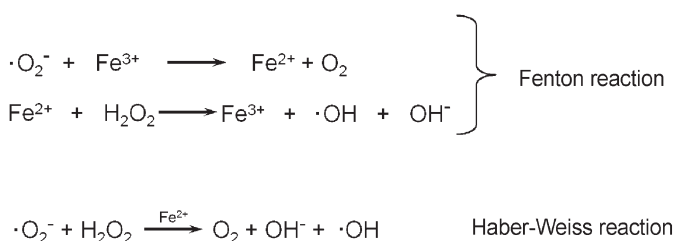


Figure 3 Fenton and Haber-Weiss reaction.

The propagation of LPO comes to an end when one radical reacts with another radical, thus producing a non-radical, stable product whereby the two free electrons from the two radicals form a covalent bond. This termination phase starts when a high concentration of lipid radicals is available so that the probability that two radicals can react is high. During LPO, lipid molecules are broken down and numerous stable carbonyl-containing by-products such as malondialdehyde and 4-hydroxy-2-alkenals such as 4-hydroxy-nonenal, resulting from $\omega 6$ fatty acids like docosahexaenoic acid, are formed. While MDA is highly mutagenic, 4-hydroxy-nonenal is most genotoxic.¹⁰¹ Consequently, the by-products of LPO pose another danger to spermatozoa, namely, cytotoxicity and DNA damage by forming DNA adducts.¹⁰² Thus, LPO is not only directly damaging membranes and therefore membrane functions, but also indirectly causing DNA damage (Figure 4b).

Recent studies clearly point out these relationships in different groups of patients. For instance, Khosrowbeygi and Zarghami³⁸ showed that sperm from asthenozoospermic, asthenoteratozoospermic and oligoasthenoteratozoospermic patients had significantly higher PUFA levels in their plasma membranes than normozoospermic men and were therefore much more susceptible for oxidative stress and LPO. Furthermore, abnormal semen samples showed significantly higher malondialdehyde levels compared to the controls and correlated negatively with the sperm count,¹⁰³ while in another study the sperm concentration of malondialdehyde (thiobarbituric acid reactive substance; TBARS concentration) was negatively correlated with fertilization rates in an *in vitro* fertilization program.¹⁰⁴

Under normal conditions living organisms have developed protective strategies and mechanisms to minimize or avoid this LPO-initiated damage by very quick termination of the radical chain reactions by scavenging the free radicals with antioxidants. The most important physiological scavengers are vitamins C and E as well as antioxidant enzymes including superoxide dismutase, catalase and glutathione peroxidase. However, due to the genotoxicity and mutagenicity of 2-alkenals and 4-hydroxy-2-alkenals and their ability to form DNA adducts, it is obvious that the assumption that the antioxidants completely scavenge and therefore balance the effects of ROS and LPO is no longer valid. Hence, the cellular defense system is not 100% efficient (reviewed in Ref. 105).

Other detrimental effects of ROS on sperm functions

Among other factors, the different physicochemical behavior of different ROS (H_2O_2 versus $OH\cdot$ or $O_2^{\cdot -}$) is the reason for a differentiated action of membrane permeable and non-membrane permeable ROS on different sperm functions like motility or DNA integrity. Also, the location of the production, extrinsic by leukocytes or intrinsic by the male germ cells themselves, appears to play a role as extrinsic ROS produced by leukocytes rather impairs sperm motility, while intrinsic ROS production seems to preferentially affect sperm DNA fragmentation.⁷⁵ On the other hand, a recent study (Mupfiga C, Henkel R, unpubl. data, 2010) revealed that the seminal leukocyte concentration not only correlated positively with ROS production in the ejaculate, but also with the sperm cell's own superoxide production ($r=0.336$; $P=0.0098$; $n=60$), spermatozoa activation of the effector caspases-3/-7 ($r=0.527$; $P<0.0001$; $n=58$) as well as the percentage of sperm with disrupted mitochondrial membrane potential ($r=0.465$; $P=0.043$; $n=20$). This gives evidence that a male genital tract infection/inflammation can trigger programmed cell death, apoptosis, by means of activating the caspase system.

In patients with chronic pelvic pain syndrome, apart from having impaired sperm motility or morphology,^{106–108} a significantly reduced

inducibility of the acrosome reaction has been shown in both categories, inflammatory (NIH IIIA) and non-inflammatory prostatitis.⁷⁶ As a possible cause for this acrosomal dysfunction, the detrimental influence of ROS and a reduced total antioxidative capacity in the semen of these patients has been suggested, while the mechanism of action would most probably function *via* the destabilization of the sperm plasma membrane by LPO.

BENEFICIAL EFFECTS OF ROS AND LEUKOCYTES

Although most reports published point out the detrimental effects of ROS, leukocytes and oxidative stress on sperm functions and thus on sperm fertilizing potential, pioneering work by the Gagnon and de Lamirande's group^{11,12} revealed that the superoxide anion in fact not only shows detrimental effects on sperm, but also plays an essential role in triggering cellular events like capacitation and hyperactivation, an essential requirement for sperm to penetrate the zona pellucida.¹⁰⁹ Later work by the same group^{15,43,110} as well as by others^{13,14,111} confirmed these proposed functions not only for superoxide but also for other ROS like nitric oxide or hydrogen peroxide. Moreover, it could be shown that besides capacitation and hyperactivation, acrosome reaction, sperm zona binding and oocyte fusion are also stimulated by various low levels of ROS even in other species.^{56,112–114} More in-depth investigations revealed sperm activation by modulation of protein phosphorylation, which is also a key element in triggering acrosome reaction.^{13,14,115,116} Thus, capacitating sperm physiologically produce their own controlled amounts of ROS that in turn regulate downstream events such as an increase in cAMP, and protein kinase A activation with subsequent phosphorylation of its substrates.¹¹⁷

Therefore, despite the fact that excessive seminal production of ROS, either by leukocytes or the male germ cells themselves, are negatively correlated with sperm motility and concentration,^{63,118} it makes sense that the concentration of ROS in the medium after sperm separation is positively correlated with fertilization *in vitro* ($r=0.148$; $P=0.0454$; $n=183$). In addition, a positive trend of the sperm ROS production after sperm separation was observed with the four-cell stage formation after *in vitro* fertilization ($r=0.135$; $P=0.0695$; $n=183$) (Henkel *et al.*, unpubl. data, 2005). In a subsequent ROC-curve analysis for fertilization, a cutoff value of 0.229×10^3 cpm/ 20×10^6 spermatozoa could be calculated.

If it is truly the case that even for fertilization *in vitro* a certain low amount of ROS is essential, one would have to be very careful in supplementing incubation media with antioxidants as it has been proposed recently.¹¹⁹ At least, the type of patient where such procedure would be beneficial would have to be selected very carefully beforehand because too high antioxidant concentrations in the insemination medium might then result in ROS concentrations that are too low for fertilization.

In contrast to the general view that leukocytes have a detrimental impact on sperm,^{74,120,121} even at concentrations as low as 2×10^4 ml⁻¹ after Percoll separation,¹²² and the sentiment that the current cutoff value for leukocytospermia of 1×10^6 ml⁻¹ given by the World Health Organization¹²³ is too high,^{75,124–126} a few reports indicate no detrimental effect of leukocytes.^{122,127} Some recent reports even attribute significant positive effects to seminal leukocytes.^{128–130} Despite uncertainty regarding the levels of leukocyte contamination that are critical, the argument is that a certain elevated number of leukocytes is beneficial to sperm concentration, motility and acrosome reaction because leukocytes might eliminate defective spermatozoa by phagocytosis and may even stimulate sperm functions through the release of ROS. This idea would be supported by data published by

Chakroun-Feki *et al.*¹³¹ who found that: (i) the presence of leukocytes in cervical mucus samples did not affect sperm motility; and (ii) the proportion of acrosome-reacted spermatozoa after sperm penetration of cervical mucus containing leukocytes was less pronounced, thus suggesting that low numbers of leukocytes in the cervical mucus could possibly influence or assist triggering capacitation.

Yet, high levels of leukocytes, particularly activated leukocytes, are still harmful to sperm functions. Consequently, one would have to see the presence of leukocytes in the ejaculate like ROS as a double-edged sword. This even more so as the negative impact of seminal ROS produced by leukocytes is depending on the total antioxidant capacity (TAC) of the ejaculate.^{132,133} In the light of individual variations in this parameter, one has to take the seminal redox status into account and future fertility analysis will have to take cognizance of this.

PROTECTION OF SPERMATOZOA AGAINST OXIDATIVE STRESS

Considering the vulnerability of spermatozoa to oxidative stress, the male germ cells have to be protected by relevant scavengers against these oxidants right from the site of sperm production, the testes, as developing spermatozoa during spermiogenesis have very limited ability to repair DNA and to replenish and regenerate glutathione.¹³⁴ Here, the protection is given by the Sertoli cell, which provides a high level of the glutathione-dependent defense.¹³⁵ Subsequently, in the epididymis, various members of the glutathione peroxidase family as well as catalase, indoleamine dioxygenase and superoxide dismutase have been identified as potential scavengers of ROS (reviewed in Ref. 136).

As mentioned before, seminal plasma contains large amounts of antioxidants to provide protection against oxidative stress in sperm cells. In fact, seminal fluid is the biological fluid that contains more antioxidant substances than any other physiological fluid does. The most important natural antioxidants in seminal plasma seem to be vitamins C and E,^{137,138} superoxide dismutase,¹³⁹ uric acid,¹⁴⁰ glutathione,¹⁴¹ glutathione peroxidase¹⁴² and the polyamine spermine (on average about 0.6 mg ml⁻¹) that acts directly as a free radical scavenger and inhibits LPO.^{79,80}

Apparently, the female genital tract is also providing antioxidant protection to spermatozoa against ROS and thereby sustaining normal sperm function, which is indeed a key element of the female reproductive system. Such a scavenger system has been shown in the Fallopian tube where epithelial cells protected sperm from ROS-induced damage.¹⁴³ Recently, Navarrete Gomez and co-workers⁸⁶ revealed that tubular fluid contains soluble, mainly non-enzymatic factors that inactivate leukocytic ROS.

ANTIOXIDANT SUPPLEMENTATION AND 'ANTIOXIDANT PARADOX'

Considering that modern lifestyle is characterized by a relatively low intake of natural antioxidants like vegetables or fruit, and a relatively high exposure to exogenous sources of ROS such as alcohol and cigarette consumption or environmental pollutants, many people are taking high dosages of antioxidants. This behavior is supported by advertising campaigns of companies and even doctors who prescribe antioxidants. Of course, this is done for a good reason to compensate the effects of lifestyle-derived ROS by artificial sources of antioxidants like vitamin tablets or other supplements. Yet, these antioxidative therapies to reduce oxidative stress caused by poor lifestyle, male genital tract infections/inflammations and defective spermatozoa are currently highly debated.^{144,145} While several studies using various

antioxidants alone or in combination have shown a significant reduction in seminal ROS levels^{146–148} and improvement in sperm count and motility,^{149–151} other studies found the opposite.^{152,153}

These apparently inconclusive results could be due to three reasons: Firstly, it is not only the administration of singular antioxidative substances that cause the beneficial effects, but the combination of different antioxidants at very specific concentrations. Secondly, normally no specific examinations of the redox status of such patients are carried out and therefore the redox status in these patients is unknown. Thirdly, due to the lack of information about the redox status, uncontrolled antioxidative treatment could then even be detrimental to the patient. The latter is attributable to the paradoxical effect of antioxidants, called the 'antioxidant paradox'.¹⁵⁴ This principle means that on the one hand, a certain amount of antioxidants is essential for normal cell function because cells generally function in a reduced state. On the other hand, however, a certain limited and localized level of ROS is also essential for cell function, for example, for normal function of several gene transcription factors¹⁵⁵ or the induction of sperm capacitation, hyperactivity and acrosome reaction.¹⁵⁶ In addition, apoptosis is accompanied by a shift towards a more oxidized status of the cell since caspases, as cysteine proteases, are sensitive to the redox status of the cell. Thus, changes to the intracellular redox status can either trigger or inhibit apoptosis.¹⁵⁷ Accordingly, the uncontrolled treatment with antioxidants can worsen conditions and even be a cause of cancer¹⁵⁴ or in the case of male infertility the reason for failed fertilization.

CONCLUSIONS: BALANCE BETWEEN OXIDANTS AND ANTIOXIDANTS

Considering the apparent possibility of dual, opposing functions of both leukocytes and ROS on sperm functions and therefore on male fertility, we have to accept the concept that ROS not only have detrimental effects on sperm functions, but also beneficial effects in terms of triggering essential cellular functions, probably in both spermatozoa and oocytes. For spermatozoa, this positive effect of ROS has been shown convincingly. Hence, a balance between ROS generation and scavenging, i.e., antioxidative capacity, is of critical importance for the physiology and functioning as well as the pathophysiology of these cells.^{43,156,158} While the evidence for the impact of ROS is clear at the moment, it is still under debate whether or not leukocytes might have direct beneficial effects on human sperm functions.

This has serious consequences for andrological diagnostics as well as for the treatment of male infertility. With regard to the andrological diagnostics, it is certainly not sufficient just to rely on the so-called classical sperm parameters, sperm concentration, motility and normal sperm morphology. In addition to some sperm function tests, the determination of the seminal redox status appears to be the way forward. This would then have to include the determination of ROS levels as well as the antioxidant capacity present in the ejaculate.^{132,133} This concept also explains the inconsistency reported in the literature about the impact and importance of ROS as well as that of leukocytes. It is therefore not sufficient just to measure only one parameter, the ROS levels or the seminal TAC, because both parameters may vary between different patients. For example, a patient might have high numbers of leukocytes present in the ejaculate, but if this patients also shows high levels of TAC, his fertility might not be compromised. On the other hand, a patient might have low numbers of activated seminal leukocytes, but very low TAC levels which do not scavenge ROS production sufficiently. As a result, this patient might be infertile. Thus, for spermatozoa this system is like a 'tightrope walk'; they will not have

functional competence if the system of seminal oxidants and anti-oxidants as a whole deviates to either side; it has to be finely balanced in order to function properly.

With regard to clinical treatment of patients with antioxidant, this concept requires clinicians to carefully choose patients that can benefit from antioxidative treatment. It is not enough just to treat the patient with antioxidants, but oxidative status and TAC must be evaluated first. The same care should be taken if sperm separation medium or insemination medium is supplemented with antioxidants. In instances where such determinations are not being done first, patients will not necessarily benefit from such treatment. In contrast, it can even cause harm.

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