Physiology of peroxidation processes in mammalian sperm

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“If a man will begin with certainties, he shall end in doubts; but if he will be content to begin with doubts, he shall end in certainties” (Bacon, *The advance of learning*)

“Many of life’s failures are people who did not realize how close they were to success when they gave up.” (T. Edison)

“Somewhere, something incredible is waiting to happen.” (C. Sagan)

“Science is organized knowledge. Wisdom is organized life.”(I. Kant)

“Imperfection is where a person has room for growth.” (author unkown)

A vida é um caminho
Com uma estrela a guiar
Andamos devagarinho
Na esperança de a encontrar. (P. Silva, 1998)

To God and N. Sra. de Fátima

To all alchemists in my live

To Johan

To my dear parents, António and Carlota

Contents

Preface

Molecular oxidation affects cell function and can lead to cell degeneration or cell death. Free radicals are a major factor in inducing this oxidation and they can attack and inactivate or alter the biological activity of molecules such as lipids and proteins that are essential for cell function. If the detrimental effects of oxidation are to be avoided, it is essential to understand the *modus operandi* of the various free radicals involved in these processes on cellular homeostasis and how this leads to pathology. Free radicals can be divided in two main groups: the Reactive Oxygen Species (ROS) and the Reactive Nitrogen Species (RNS).
Free radical biology has implications for many aspects of modern life ranging from the food industry, where preventing oxidation is crucial to food conservation, to areas of medical sciences such as neurology, cardiology and, more recently, reproductive medicine. It is becoming increasingly clear that molecular oxidation often plays a role in infertility, particularly when reproductive tissues or gametes are stored or manipulated in vitro.
This thesis focuses on the role of oxidation in male fertility, by studying the physiology of peroxidation in mammalian sperm, with special emphasis on bovine sperm.

In this thesis, the biological mechanisms that lead to the formation of ROS/RNS are reviewed together with the effects of oxidation introduced by reproductive techniques currently applied in the laboratory or in the field (Chapter 1). A normal sperm cell can withstand minor levels of peroxidation without appreciable loss of function. In fact, some molecular substrates must be oxidised in order for a sperm cell to acquire fertilizing ability and some of the free radicals that are formed act as signalling molecules essential to capacitation, the acrosome reaction and penetration of the oolema. Nevertheless, the extent of peroxidation is increased when sperm cells are submitted to cryopreservation and this excessive peroxidation of lipids, DNA and proteins is almost certainly detrimental to fertility.
In Chapters 2 and 3, new methods are developed for detecting and localizing (lipid) peroxidation in viable sperm cells subjected to various treatments e.g. following freeze/thawing.
Cholesterol is the most abundant molecule in the sperm plasma membrane and plays an important role in maintaining lipid bilayer stability. In Chapter 4, we investigated whether cholesterol is a target for oxidative stress, and how oxidised cholesterol is metabolised within the sperm cell. In addition, we investigated how different degrees of oxidative stress affect the sperm membrane and the integrity of

the cytosol, mitochondria and DNA and finally what influence these changes have on the fertilizing ability and subsequent embryo formation and quality (Chapter 5). Although sperm may have an intrinsic capacity for withstanding or tolerating certain levels of oxidative stress it is also possible that the oocyte plays an important role in repairing oxidative damage in the sperm components introduced during fertilization. Finally, the results of these studies and their possible implications for future research are summarized in Chapter 6. In total, we propose that these results may be useful in human reproductive medicine where sperm of subfertile men might be used in IVF programmes with improved success following the addition of specific antioxidant cocktails to prevent oxidation and thereby improve fertilization rates, embryo harvest, embryo quality and ultimately the likelihood of a successful pregnancy.

CHAPTER 1

Oxidative stress and fertility - aspects of molecular oxidation and their effects on the mammalian reproductive output

P.F.N. Silva, J.F. Brouwers, B.M. Gadella

In preparation

Introduction

The adverse effects of reactive oxygen species (ROS) in mammalian reproduction have been studied for over three decades and its relationship with human infertility has been established in literature. Possibly the additional processing steps involved with assisted reproductive techniques (ART), such as artificial insemination (AI), may introduce extra oxidative stress. Such techniques are used to overcome human infertility and may provide a way to optimize animal reproduction efficiency but additional oxidative stress may limit the success. Although, the knowledge in this area has evolved immensely in recent years, a rising concern about the detrimental effects that can occur during physiological processes and storage procedures still perseveres.

Oxidative processes related to spermatozoa are particularly of interest since ROS production during in vitro capacitation and during in vitro sperm storage may also aid the sperm to acquire full fertility competence. Both positive and negative effects of ROS are implied to correlate with the capacity of sperm to migrate through the oviduct, to meet the oocyte, to fertilize the oocyte and under considerable debate. The integrity of the DNA from the sperm cell and the oocyte at the moment of fertilization should be good enough not to compromise the normal development of the conceptus. Therefore, it is important during in vitro storage of germ cells and embryos to keep them at low metabolic rate to ensure minimal generation and activity of ROS.

The purpose of this review is to bring an updated understanding of ROS production and ROS induced molecular alterations in sperm cells, oocytes and embryos and its consequence on sperm functionality, oocyte and embryo viabilities and relationships with reproductive physiology.

Table 1 – Reactive oxygen species most commonly produced ($t_{½}$ denotes radical half-life)

ROS	Reaction
Superoxide radical ($O_2^{-\bullet}$) $t_{½}$= *1 x 10^{-6} s*	$O_2 + 1e^- \rightarrow O_2^{-\bullet}$, **NADPH oxidase** $NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^{-\bullet}$ Superoxide radical is the first intermediate in the reduction of molecular oxygen to water by the respiratory chain. It is membrane impermeable and relatively inert. During phagocytosis $O_2^{-\bullet}$ is generated through a NADPH oxidase system.
Hydroperoxyl radical ($HO_2^{\bullet}$)	$O_2 + H_2O \rightarrow HO_2^{\bullet} + OH^-$ Hydroperoxyl radical is the protonated form of $O_2^{-\bullet}$, being an intermediate radical form between $O_2^{-\bullet}$ and hydrogen peroxide (H_2O_2). In aqueous media the acid-base equilibrium is shifted from $O_2^{-\bullet}$ to its protonated form (pKa=4.8)[1].
Hydroxyl radical ($OH^{\bullet}$) $t_{½}$= *1 x 10^{-9} s*	**Fenton reaction:** $H_2O_2 + 1e^- (Fe^{2+}/Cu^+) \rightarrow Fe^{3+}/Cu^{2+} + OH^{\bullet} + OH^-$ **Haber-Weiss:** $O_2^{-\bullet} + H_2O_2 \rightarrow O_2 + H_2O + OH^{\bullet}$ Powerful oxidant, responsible for initiation of lipid peroxidation, where an atom of hydrogen is removed or added to a carbon atom adjacent to a double (unsaturated) bond[2] (see figure 1) and related to detrimental events such as lipid and protein oxidation[3,4].
Sulphur radical ($RS^{\bullet}$)	$RSH + >C^{\bullet} \rightarrow >CH + RS^{\bullet}$ $RSH + OH^{\bullet} \rightarrow RS^{\bullet} + H_2O$ $RSH + ROO^{\bullet} \rightarrow RS^{\bullet} + ROOH$ $RSH + Fe^{3+} \rightarrow RS^{\bullet} + Fe^{2+} + H^+$ or $RSH + Cu^{2+} \rightarrow RS^{\bullet} + Cu^+ + H^+$ Thiols are regarded as antioxidant agents (e.g.: reduced glutathione-GSH), by protecting protein –SH groups against oxidation. However, thiols can also react with a carbon centered radical ($C^{\bullet}$), $OH^{\bullet}$, $O_2^{-\bullet}$, alkoxyl ($RO^{\bullet}$) and peroxyl ($ROO^{\bullet}$) radicals, as well as, with transition metals Fe^{3+} and Cu^{2+}[5,6,7]. This will lead to the loss of functional proteins[1].
Singlet oxygen ($^1O_2$) $t_{½}$= *1 x 10^{-6} s*	In solution singlet oxygen atoms containing high-energy electrons become deactivated after the transfer of the electron's excitation energy to low energy electron on its substrate[8]. The singlet electron state on oxygen molecules are formed by light when a photon with a given energy hits one electron in one of the oxygen atoms. Common sensitizers such as acridine orange, methylene blue, rose Bengal and toluidine blue are examples of color substrates that react with singlet oxygen. Singlet oxygen may play a role in the regulation of cellular function, such as mitochondrial permeability transition, depending where its production in this organelle occurs[9].
Alkoxyl ($RO^{\bullet}$) and Peroxyl ($ROO^{\bullet}$) $t_{½}$= *1 x 10^{-6} s* and $t_{½}$= *1 x 10^{-2}* s, respectively	Oxygen-centered radicals are formed during breakdown of organic substrates such as lipids and proteins (see section 1.3).

Table 2 – Reactive nitrogen species most commonly produced

Nitric Oxide ($NO^{\bullet}$)	**NOS** **L-arginine + O_2 + 2 NADPH** → **Citrulline + $NO^{\bullet}$ +2 $NADP^+$ + H_2O** This reaction is catalyzed by nitric oxide synthase (NOS), producing $NO^{\bullet}$ and citrulline. NOS activity is Ca^{2+} dependent[10], involved in several signaling cascades pathways[11,12,13]
Nitrogen Dioxide ($NO_2^{\bullet}$)	**$NO^{\bullet} + O_2 \rightarrow NO_2^{\bullet}$** **$2\ NO_2^{\bullet} + H_2O \rightarrow HNO_3 + HNO_2$ (nitric and nitrous acid production)** **$RH + NO_2^{\bullet} \rightarrow R^{\bullet} + HNO_2$ (lipid peroxidation initiation)** This is a common air pollutant which is highly reactive and involved in respiratory problems (mainly lung damage). $NO_2^{\bullet}$ dissolves in water and then forms nitrous acid (HNO_2), which is potentially toxic as it leads to mutations. It may become carcinogenic when it reacts with secondary and tertiary amines. $NO_2^{\bullet}$ has also been shown to be a lipid peroxidation initiator by abstracting hydrogen atoms from polyunsaturated fatty acids (PUFAs)[14]

I. Reactive Oxygen Species and Defense Systems

ROS are instable compounds with a short half-life time and when present intracellularly can adversely affect certain cellular processes. ROS are products of aerobic metabolism and form part of the respiratory oxidation at the inner mitochondrial membrane. During this process protons may leak which will be used to form ROS (tables 1-3). Substrates for ROS production are present in every subcellular structure, from the plasma membrane proteins and lipids towards nuclear DNA. Cells have mechanisms to combat partially or totally this ROS production through antioxidant mechanisms, enzymatic or vitamin complexes in order to prevent excessive peroxidation of substrates (see fig. 1).

I.1 ROS/RNS production mechanisms

The following three tables summarize the reactions involved in production of ROS (Table 1), RNS (Table 2) and non-radical reactive species (Table 3).

Table 3 – Non-radical reactive species most commonly produced

Hydrogen peroxide (H_2O_2)	**SOD** **$2\ O_2^{-\bullet} + 2\ H^+ \rightarrow H_2O_2 + O_2$** This reaction is catalyzed by superoxide dismutase (SOD). H_2O_2 is an uncharged membrane permeable molecule that can diffuse over mitochondrial and other membranes. Spontaneous formation of H_2O_2 occurs by the reduction of hydroperoxyl radicals. **$HO_2^\bullet + e^- + H^+ \rightarrow H_2O_2$** **$H_2O_2$** is a major secretory product of phagocytes[15,16].
Hypochlorous acid (HOCl)	**MPO** **$H_2O_2 + Cl^- \rightarrow HOCl + OH^-$ (hypochlorous acid formation)** **$HOCl + O_2^{\bullet-} \rightarrow O_2 + Cl^- + OH^\bullet$ or $HOCl + Fe^{2+} \rightarrow Fe^{3+} + Cl^- + OH^\bullet$ (hydroxyl formation)** **$HOCl \leftrightarrows H^+ + OCl^-$ (hypochlorite formation)** **$HOCl + H^+ + Cl^- \leftrightarrows Cl_2 + H_2O$ (chlorine gas formation)** **R-NH2 + HOCl→ RNHCl + H_2O (taurine chloramines)** Activated neutrophils generate hypochlorous acid, in response to infectious agents[17]. This reaction is catalyzed by a "non-specific" peroxidase-myeloperoxidase (MPO), which is activated through a NADPH-complex. The generated $O_2^{-\bullet}$ consequently reacts with hypochlorous acid to form the more reactive hydroxyl radicals. The enzyme uses chloride (Cl^-) as co-substrate producing hypochlorous acid (HOCl) and 50% of this acid can be ionized (pKa=7.5) at pH=7.4[18]. Hypochlorous acid is highly toxic with detrimental effects for neighboring cells[19]. Hypochlorous acid is able to degrade biomolecules either directly or by decomposition into chlorine. The primary substrates are thiols and methionine-moieties. Protein and lipid chlorination reactions, protein carbonyl formation, aggregation and fragmentation are also products of this short-lived radical[20].
Peroxynitrite ($ONOO^-$) $t_{½}$= *3-5 x 10^{-3} s*	$NO^\bullet$ can interact with $O_2^{-\bullet}$ generating peroxynitrite ($ONOO^-$) in a iron dependent way: **$O_2^{-\bullet} + NO^\bullet \rightarrow ONOO^-$** **$ONOO^- + H^+ \rightarrow$ ONOOH**, this last reaction can result in the formation of nitrites (NO_2) and nitrates (NO_3)[21]. Nitric oxide related nitration reactions often result in altered protein structures and compromizes the functioning of modified proteins[22].
Ozone (O_3)	**$Cl^\bullet + O_3 \rightarrow O_2 + ClO$** Ozone is toxic because it reacts with aerosols at the stratosphere and troposphere with formation of chlorine radicals[23]. Reactivity of chlorine radicals is dealt with in this table under the hypochlorous acid section. Photon mediated dissociation of chlorofluorocarbon gases (CF_2Cl_2) may lead to the indicated reaction with ozone.

I.2 Antioxidant consortium

In general, antioxidant defense can be through enzymatic and non-enzymatic systems. Enzymes such as superoxide dismutase and catalase react with radicals $O_2^{-\bullet}$, H_2O_2, respectively. Glutathione peroxidases, especially phospholipid glutathione peroxidase, scavenge alkyl ($R^\bullet$), alkoxyl ($RO^\bullet$) and peroxyl ($ROO^\bullet$) radicals that may be formed from oxidized membrane components. Inhibition of oxidation pathways can equally be achieved via molecules such as vitamin C, vitamin E, glutathione (GSH) and co-enzyme Q (fig. 1).
This antioxidant consortium is a network of different elements that do work in a cooperative manner, very efficient in removing (most) radicals and preventing most somatic cells from massive oxidative damage. In table 4 the major enzymes involved in antioxidant defense are summarized.

I.2.1 Enzymatic antioxidant defense:

Table 4- Enzymatic defense:

Enzyme	Reaction
Superoxide dismutase (SOD)	**SOD** **$2\ O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$** Superoxide dismutase can have 3 isoforms: cytosolic copper/zinc superoxide dismutase (Cu/ZnSOD), intra-mitochondrial manganese superoxide dismutase (MnSOD)[24,25] and extracellular Cu/Zn SOD[26,27,28]. Cytosolic SOD constitutes the major proportion of total SOD activity and probably is responsible for removing $O_2^{\bullet-}$ radicals from the cytosol[1]. Mn-SOD is responsible for removing $O_2^{\bullet-}$ generated by oxidative metabolism in the mitochondria.
Catalase (CAT)	**CAT** **$2\ H_2O_2 \rightarrow 2H_2O + O_2$** Catalase is a hydrogen peroxide detoxifier. It removes this highly membrane permeable molecule from the media and inhibits NADPH oxidase and thus inhibits superoxide production[29].
Glutathione peroxidase family (GPx), including the monomeric phospholipid glutathione peroxidase (PHGPx)[30]	**GPx** **$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$** (selenium dependent) Removal of lipid hydroperoxides: **PHGPx** **$2\ GSH + R(OOH)COOH \rightarrow GSSG + R(OH)COOH + H_2O$**
Protein damage repair: **GSH reductase (thioredoxin)** **Peptide methionine sulphoxide reductase**	GSH reductase is involved in regenerating the intracellular thiol levels by reducing GSSG to GSH[31]. Methionine sulphoxide reductase is present in E. coli, protozoan, yeasts, higher plants and mammalian tissues (e.g.: methionine sulphoxide), its reducing power is at the expense between reduced thioredoxin and NADPH[32].
Indolamine dioxygenase (IDO) **Xanthine/xanthine oxidase**	Will be referred in the text but not described in detail in this table (see section II.2 for IDO and section capacitation and acrososme reaction for xanthine/xanthine-oxidase system)

I.2.2 Non-enzymatic antioxidant defense:

Members of non-enzymatic antioxidants are vitamin C and GSH (in the cytosol), vitamin E and co-enzyme Q (in membranes), and taurine, hypotaurine and pyruvate as extracellular and intracellular components. Vitamins A, C and E are well known for their role in preventing DNA and lipid peroxidation. In HIV infected patients vitamins A, C and E decreased the levels of modified DNA bases and malondialdehyde (MDA) formation[33]. Vitamin E and co-enzyme Q (also called ubiquinone) have lipophilic properties and can trap lipid-derived radicals in the membrane[34]. Co-enzyme Q is exclusively localized in the inner mitochondrial membrane. Both co-enzyme Q and vitamin C are capable to regenerate oxidized vitamin E[35], and can neutralize H_2O_2 that is produced and located in a hydrophilic environment (cytosol or extracellular fluids) (see fig. 1). Table 5 summarizes the major non-enzymatic antioxidant defense systems.

Table 5- Non-enzymatic defense:

Vitamin A (retinol)	Some carotenoids (e.g.: β-carotene) have been shown to function as antioxidants by protecting cells from lipid peroxidation inhibiting development of chronic diseases[36].
Vitamin E (alpha-tocopherol)	This molecule acts as a chain breaking antioxidant and is capable to neutralize peroxyl and other lipid derived radicals very efficiently. Normally these radicals are involved in propagation reactions[37,38,39]. Therefore, vitamin E is one of the most potent antioxidants that terminates lipid peroxidation propagations.
Vitamin C (ascorbic acid)	Vitamin C is a hydrophilic molecule and acts as an active antioxidant against lipid peroxidation by reacting with $O_2^{-\bullet}$, $OH^\bullet$ and lipid hydroperoxides. Vitamin C acts synergetically with vitamin E by reducing the α-tocopheryl radical (α-Toc$^\bullet$) to tocopherol[40].
Chelating proteins: Lactoferrin, transferrin, ferritin, ceruloplasm and (hypo)taurine	These metal binding and transporting proteins transfer metal ions (such as Fe^{3+} and Cu^{2+}, important in redox reactions involved in generation of ROS) from body fluids into[1]. Lactoferrin and transferrin are found in many secretory fluids such as seminal, oviductal fluids, saliva, milk, bile and nasal secretions[8]. Ferritin is responsible for the iron transference whereas ceruloplasm accounts for the copper binding[1]. Both hypotaurine and taurine are precusor and end-product, respectively of the metabolism of sulphur-containing aminoacids and are present intracellularly acting as radicals scavengers[18,41,42,43]
Uric acid, pyruvate, citrate	Will be referred in the text but not described in detail in this table (see section II.3)

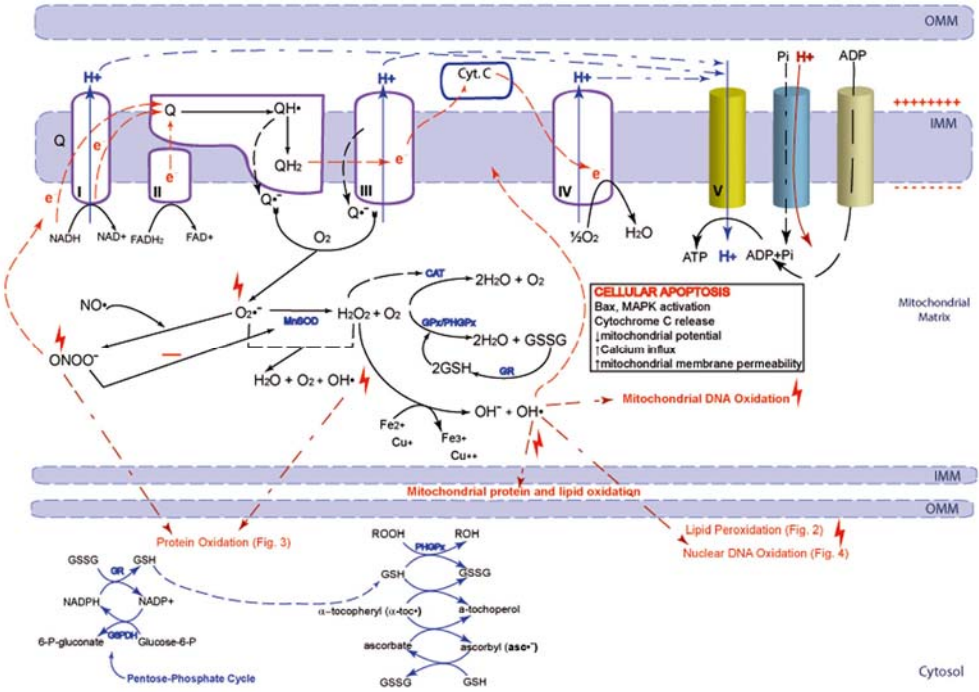

Figure 1- Generation of ROS and RNS within the cell. Quantitatively most important is the mitochondrial production of $O_2^{-\bullet}$ and H_2O_2 [72].
The cycles between fully oxidised- quinone Q first to the one-electron reduction product- semiquinone radical ($QH^{\bullet}$) and finally to the fully reduced- ubiquinol (QH_2) make it possible generate $O_2^{-\bullet}$ through a slip of the electron transport chain when electrons pass directely to oxygen instead of processing further towards the next electron carrier (complex III). This is the breaking point where oxidative radicals are generated. The close proximity to iron and copper redox pools in mitochondrial membranes drives the formation of $OH^{\bullet}$. Formation of this radical leads to lipid oxidation by reacting with cholesterol or with the unsaturated fatty acyl chains of phospholipids (see figure 2), with proteins (see figure 3) or with DNA causing base mutations (see figure 4). Peroxynitrite ($ONOO^-$) is a reaction product of $NO^{\bullet}$ and $O_2^{-\bullet}$ and can also react with protein and exerts a negative feedback on MnSOD. Peroxynitrite can directly attack functional centra of the electron transport chain (complexes I, II, III, IV). The immediate substrates for ROS/RNS are found within the mitochondria (lipids, proteins, mtDNA) but above threshold formation of radicals impair mitochondrial functioning. In a second phase a burst of radicals into the cytosol will occur. The phenomena are summarized in the text box cellular apoptosis. The major substrates for radical attack are lipids and proteins, and nuclear DNA.The radicals diffusing through the cytosol may trigger apoptotic pathways resulting in an increase of cytosolic calcium, a loss of mitochondrial potential and opening of outer mitochondrial membrane pores that potentiate the release of cytochrome c. The flow of electrons and protons across the inner mitochondrial membranes is considerd to secure a balanced mitochondrial potential, redox status and ATP production in the cell. The antioxidant enzymes MnSOD, CAT, GPx/PHGPx, GR, as well as, some non-enzymatic antioxidants (GSH, α-tocopherol, ascorbate) maintain the cellular redox homeostasis. One example is that after lipid peroxidation the generated vitamin E radical (α-tocopheryl) becomes regenerated by vitamin C (ascorbate) in synergy of the cycle with GSH/GSSG that occurs in the cytosol.
(I: Complex I- NADH-ubiquinone oxidoreductase; II: Complex II- succinate dehydrogenase; III: Complex III-ubiquinone:Cytochrome C oxidoreductase; IV: Complex IV-Cytochrome C oxidase; V- Complex V-ATP synthases).

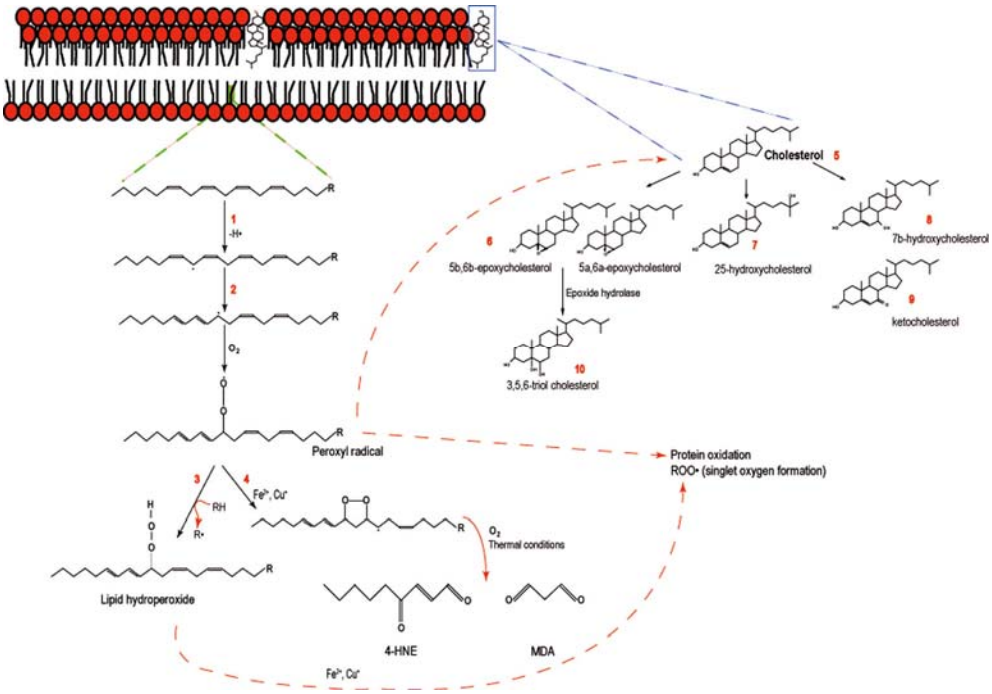

Figure 2- Lipid peroxidation pathways of cholesterol and of polyunsaturated fatty acid (PUFA) that are esterified to phospholipids.

1- Initiation: LPO starts with the extraction of one hydrogen from a methylene (-CH_2-) group (in bis-allylic groups, as indicated) **2- Molecular rearrangement:** The carbon radical is stabilized by the formation of conjugated diene bonds. Carbon radicals most likely react with O_2 to form a peroxyl radical ($ROO^{\bullet}$). **3- Propagation:** abstraction of hydrogen by a peroxyl radical of another methylene group from an adjacent fatty acid. **4-** $ROO^{\bullet}$ radicals can attack a double bond from the same chain generating cyclic peroxide radicals. **Termination:** If no scavenger system is present and metal ions (Fe^{2+}, Cu^{+}) are available, decomposition in alcohols, ketones, aldehydes and ethers is facilitated [97,1]. The end products of this propagation phase are the final products 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA) [177]. Peroxyl radicals can initiate a chain reaction (propagation) peroxidizing adjacent lipids, such as, cholesterol and produce with similar reactions cholesterol oxidation products (COPs- see structures **6 to 10**). $RO^{\bullet}$ and $ROO^{\bullet}$ radicals can extract a hydrogen atom from cholesterol structure **(5)** forming a cholesteryl radical. Under aerobic conditions the peroxyl radical can directly form ketocholesterol **(9)** or react with an adjacent PUFA from a phospholipid rendering cholesterol to its hydroperoxide structure. The latter can react with transition metal ions generating an alkoxyl radical structure, as well as, extract once more one hydrogen from adjacent PUFA´s originating 25 and 7β-hydroxycholesterol **(7 and 8)**. Similar reactions occur to form the epoxide forms **(6)** which can be metabolized by epoxide hydrolase to triol, one of the most toxic COPs [50,49,326].

I.3 Cellular Substrates of ROS

I.3.1 Lipids

As an example of lipid peroxidation pathway, figure 2 shows oxidation of a PUFA commonly found at the sn-2 position of the phospholipid glycerol backbone. In this figure the oxidation of cholesterol was also included and the predominant oxysterols formed are indicated[44]. Lipid peroxidation is characterized by a two-step process, initiation and propagation (fig. 2). Yu[1] proposed a third stage on lipid peroxidation designated termination where the last radical formed does not originate any further radicals. The two stable and detectable end products of lipid peroxidation (LPO) are malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). MDA, is very soluble in aqueous phases[45] and can further react with other substrates such as DNA (mutagenic agent) and proteins. Both MDA and 4-HNE can modify other molecules such as proteins or DNA and thus causing dysfunctioning of those substrates[46]. The molecule 4-HNE can be toxic by altering the functions of thiol-containing enzymes, or by affecting the levels of GSH[47]. Its stability and amphipathic properties allows 4-HNE to pass through several sub cellular compartments which enables it to interact with different cellular proteins, impairing their function[47]. Polyunsaturated fatty acids are the precursors for these toxic end products and especially sperm contains high levels of 22:5; 22:6 acyl chains[48]. In addition free sterols can be peroxidized by radicals or by auto oxidation[49]. Cholesterol oxidation is believed to be detrimental for myocytes[50], red blood cells[51], and leukocytes[52] on atherosclerosis, Alzheimer disease, immunosuppression and cancer[53,54].

I.3.2 Protein Peroxidation

Amino acids are arranged into a linear polypeptide chain in proteins. Individual peptides have different susceptibilities to radical mediated oxidation[55,56]. The oxidation of tyrosine is depicted in fig. 3. Recently, it was shown that proteins may be the initial targets of radicals rather than lipids or DNA. It remains unclear whether protein peroxides play a role on the latter processes or that the two are independent processes[56]

Hydroxyl reactive species have the ability to covalently modify proteins by cross-linkages causing protein aggregation. Subsequently, the modified proteins become sensitized for proteolysis either by the proteasome (ubiquitinylated proteins) or in the lysosome (other peptide modifications)[1]. If a transport protein or a channel protein is attacked by a radical its compromising functioning may alter the transmembrane gradient of ions and consequently impair cell function[57].

Oxidized tyrosine residues induce cross linking between proteins, changes in protein conformation and result in the loss of the physiological function of

determined effector proteins[58]. An example of this is the cross linking of two tyrosine residues which results in the formation of bityrosine. Bityrosine formation has been shown to inhibit tyrosine kinase mediated signal transduction by lowering tyrosine phosphorylation[8]. Oxidation of side chains of lysine, proline, arginine and threonine residues can directly form carbonyl groups on proteins[59]. Indirectly protein carbonyl groups can originate from reactive lipid peroxides that initiate protein oxidation by addition reactions of MDA and 4-HNE (Michael addition). MDA and 4HNE are end products of LPO[59,60] and the protein carbonyl group formation induced may indirectly affect enzymatic activity and consequent metabolic pathways[61]. The cell may switch from proteolysis to apoptosis above a certain threshold of accumulation in protein carbonyl groups. 4-HNE is believed to act as a signaling molecule to initiate or inhibit a determined pathway[46,60]. Detoxification of 4-HNE occurs via enzymatic activity of glutathione S-transferases (GSTs). This enzyme catalyzes the conjugation of GSH to 4-HNE via the Michael addition to C-3 carbon and thus quenches 4-HNE toxicity[47].

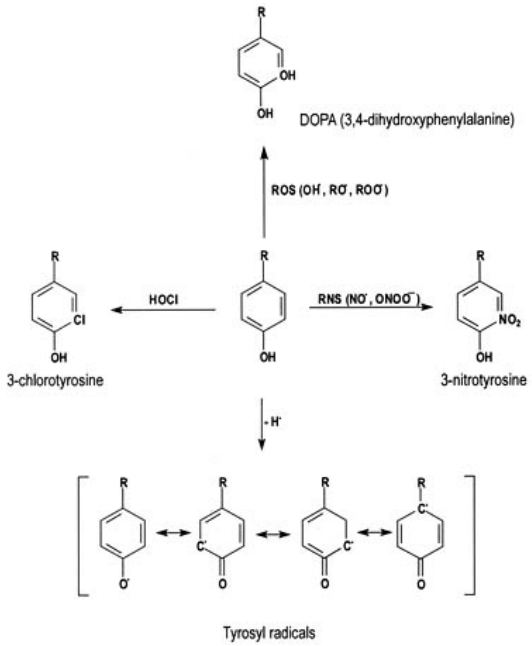

Figure 3- Oxidation products of tyrosine. This is the most common amino acid substrate for radical attack and its oxidation products are most routinely assessed. Peroxynitrite ($ONOO^-$) and $NO^{\bullet}$ [59] can modify tyrosine into 3-nitrotyrosine while hypochlorite radicals will produce 3-chlorotyrosine. Hydroxyl radicals will hydroxylate tyrosine to 3,4-dihydroxyphenylalanine (DOPA). Transition metals such as iron, copper or selenium are co-factors of several antioxidant enzymes (SOD, catalase, GPx, respectively) but also mediate of protein peroxidation [327], generating tyrosyl radicals through the Fenton, Haber-Weiss chemistry [328,62,329].

I.3.3 Carbohydrates

Sugar moieties (carbohydrates) attached either to proteins or to lipids are also substrates for peroxidation[1]. Transition metal ions can trigger auto-oxidation of carbohydrates and generate carbonyl radicals and H_2O_2[62]. Advanced glycation end products (AGEs) are formed by ROS during redox recycling of 3,4-dihydroxyphenylalanine (DOPA) or metal-catalyzed decomposition of amino acid hydroperoxides[63]. Thus protein oxidation and carbohydrate oxidation are intricated phenomena.

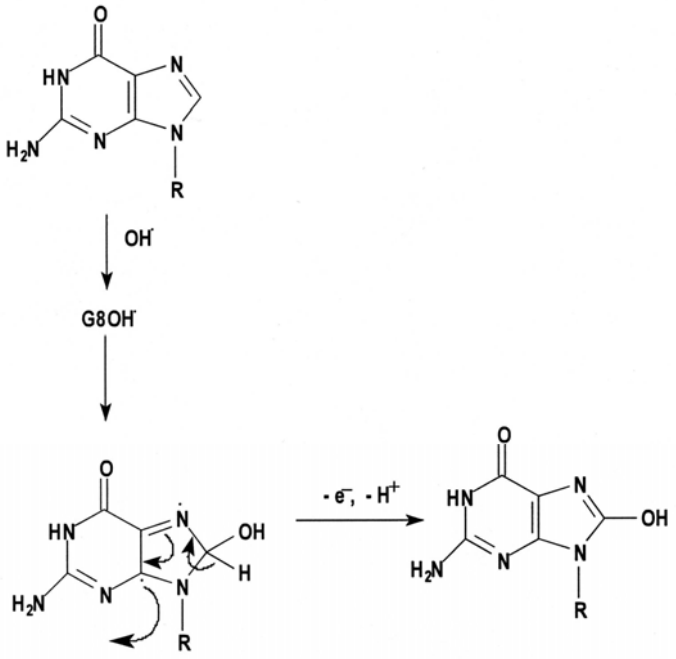

Figure 4- One of the possible pathways leading to oxidation of DNA base guanine and its molecular rearrangement to its common oxidation product 8-hydroxyguanine (8-OH-G).
Reaction of $OH^{\bullet}$ with carbon 8 of guanine in DNA conducts to formation of 8-hydroxyguanine radical which by loosing an electron forms 8-hydroxyguanine. There are possibility of reactions of $OH^{\bullet}$ with other guanine carbon positions (adapted from [330]).

I.3.4 DNA Oxidation

Reactive species such as $OH^{\bullet}$ can cause severe DNA damage with irreversible modification of DNA bases[58] which can conduct to mutations due to oxidation of deoxyribose moieties culminating in base release or DNA strand breaks[64] (see fig. 4). The end products of DNA damage 8-Oxoguanine (8-oxoG), 8-

hydroxydeoxyguanosine (8-OHdG) and thymine glycol (TG) can be easily detected in urine[65]. DNA oxidation may result in cross linkages between DNA and proteins (e.g.: thymine linking to tyrosine). These cross-links may interfere with chromatin unfolding, DNA repair, replication and transcription. The cross-links are formed in presence of Fe^{2+}, suggesting involvement of $OH^{\bullet}$ in this process (Fenton reaction)[66,67]. In line with this is the relation between the amount of 8-OHdG the concentrations of ferritin (an iron binding protein) in the blood[67]. DNA oxidation may lead to single or double strand breaks that may directly inactivate several genes[68], or lead to protein inactivation[69], or to apoptosis[70].

I.3.4.a Mitochondrial DNA (mtDNA)

Mitochondrial DNA is not coated with histone proteins, and typically has a permutation rates that are 10-100 times higher than DNA in the nucleus (which is folded twice over a necleosomal histone complex)[8]. The core proteins of mitochondrial complex I (NADH/ubiquinone oxireductase), III (ubiquinone/cytochrome c oxidoreductase) and IV (cytochrome oxidase) are encoded by mitochondrial DNA. These complexes are situated at the inner mitochondrial membrane and form the basic modules of the electron-transport chain. Complex III is believed to be the major source of ROS[71]. Note that mitochondria are the major site of superoxide formation and the most likely one. This may explain why the mitochondrial DNA is a target for free radical damage[1,72]. The mitochondria contain some antioxidants that protect substrates from radical destruction in this organelle. Examples are GSH, NADH and thioredoxin and enzymes like Mn-SOD, CAT, GR and GPx[69,73,71,74]. Hydrogen peroxide can be generated via dismutation of $O_2^{\bullet -}$ via the Mn-SOD but, paradoxically, components of the electron-transport chain like cytochrome c can convert H_2O_2 into $OH^{\bullet}$ (Fenton reaction). This short-lived highly reactive radical restricts its damage to a small radius of diffusion[75] but enough to trigger damage on macromolecules such as DNA.

I.3.5 Apoptosis

Apoptosis, also designated as programmed cell death, is a physiologically normal occurring event during (embryo) development, tissue homeostasis and during differentiation processes[76,77]. Initiation of apoptosis can result from extrinsic factors that bind to of cell-surface death receptors that switch on the caspase cascade. It can also result from factors such as perturbation/permeabilization of the outer mitochondrial membrane by chemical or physical agents [71,77]. The latter is often manifest in mammalian cells that are manipulated in vitro[78]. The cellular failure in ATP consumption, inadequate provision of ADP at the adenine nucleotide transporter with discharge of mitochondrial membrane potential and pH gradient,

are phenomena related to the mitochondrial permeability transition (MPT). MPT results in the release of cytochrome c from mitochondria into the cytosol and thus to cellular apoptosis. The release of cytochrome c (complex IV) from the mitochondria to the cytosol is a calcium-dependent response induced by oxidative stress. This leads to the formation of the apoptosome that induces nuclear apoptosis with participation of molecules such as ATP, pro-caspase 9 and at a later stage caspase 3[79]. Lipid peroxidation through the cardiolipin hydroperoxide cascade can stimulate dissociation of cytochrome c from the mitochondrial inner membrane[80] and contributes to the opening of mitochondrial permeability transition (MPT) pores. This leads to a collapse in proton and electron transport of complexes I and IV[81]. Another pro-apoptotic factor that leaks from the mitochondria is the apoptotic inducing factor (AIF) a 57 kDa flavoprotein homologous to oxidoreductases which is linked to apoptotic specific DNA degradation and condensation[78,79]. At the plasma membrane level exposure of PS and PE to the outer membrane leaflet[82,83] and production of ceramide through SM hydrolysis (via sphingomyelinase) are phenomena associated with cell apoptosis[84].

I.4 Methodologies Used To Detect and Measure Oxidation

I.4.1 ROS

Detection of ROS can be assessed indirectly by the use of fluorescence/chemiluminescence assays. Luminol is a probe that undergoes one electron oxidation before it becomes sensitized to the presence of ROS. Mostly horseradish peroxidase (HRP) is used for this electron donation. The production H_2O_2 can easily be monitored for instance after cell stimulation with phorbol 12-myristate, 13-acetate (PMA) (a protein kinase C activator)[85]. Another probe lucigenin is sensitive to superoxide ($O_2^{-\bullet}$) which is produced by NAD(P)H stimuli[86]. Superoxide can also be formed by the xanthine + xanthine-oxidase system which generates $O_2^{-\bullet}$ and can be used to study the effects on cellular molecular substrates[87]. These assays can be used to detect the efficiencies of antioxidants to inhibit hydrogen peroxides or superoxide formation.

A different type of detection of ROS production requires expensive equipment for assessing electron paramagnetic resonance properties of molecules in a given sample (EPR). The presence of unpaired electron(s) in a given radical species is detected with a spin trap compound. The spin trap is by definition a chemical substance that, by reacting covalently with an unstable radical, forms a stable and measurable free radical[88]. Thus, the radical species is “trapped” in a “long-lived” form which can be observed by EPR at room temperature[88]. This technique has already been used in human and livestock sperm[89,90].

I.4.2 Lipid peroxidation

Lipid peroxidation is routinely assessed with the thiobarbituric acid (TBA) reactive substances assay (TBARS). This assay is based on the reaction between malondialdehyde (MDA), an end-product of lipid peroxidation, and TBA molecules in a 1:2 ratio. The product is colored pink and can be measured in a photospectrometer using 532-535 nm wavelength[91]. This assay is most commonly used to quantify lipid peroxidation has been used by many investigators[92,93,94,95,96]. Despite its simplicity, the TBARS assay has several disadvantages. Firstly, several other aldehydes can react with TBA and the reaction products also colour pink[97] thus interfering with the spectrophotometer assay. Secondly, peroxidation of some sugars (e.g.: sucrose and 2-deoxyribose) can also lead to MDA production[98]. Both phenomena may lead to an overestimation of the degree of LPO and inaccuracy. In addition, MDA is a reactive and toxic compound with the ability to cross-link with lipids and proteins (cysteine and tryptophan residues). The production of TBARS may influence this process and therefore its measurement is just a general estimation of LPO but not an accurate quantification of LPO in vivo.

In the last decade, fluorometric techniques became widely available to measure cellular peroxidation. One excellent example for detection of LPO is the use of the fluorescent C_{11}-Bodipy$^{581/591}$ probe. In this fatty acid analogue a phenol group is in resonance with the BODIPY group causing a red fluorescence emission under blue light excitation. Disruption of the resonant structure by radicals switches the emission properties under the same excitation wavelength into green. The fluorescent change of the probe can be visualized and localized in situ with fluorescence microscopy or quantitatively assessed per cell or organelle using flow cytometry[99,100,101]. Mass spectrometry has also become a valid tool, alone or combined with high performance liquid chromatography (HPLC) to detect oxidation in endogenous lipids (currently done in lipidomic platforms)[99,44].

I.4.3 Protein oxidation

Protein oxidation may be scrutinized via chromatographic techniques (HPLC, GC-MS) where different forms of modified tyrosine residues (dityrosine, 3-nitrotyrosine, chlorotyrosine) can be analyzed[102,103,104]. Recently a fluorescent method was developed to detect formation of dityrosine, by using a fluorescein-labeled tyrosine analogue (tyramine) which upon ROS attack is converted into tyrosyl radical that can form cross-links with oxidized tyrosine residues in target proteins[105]. Another assay widely used is detection of carbonyl groups in the protein side chains. These groups are forming 2,4-dinitrophenylhydrazone (DNP-hydrazone) derivatives after reacting with 2,4-dinitrophenylhydrazine (DNPH). This adduct can then be

measured either via spectrophotometry[106] or by western blot, using an antibody against dinitrophenyl groups[107].
Likewise RNS are important factors that induce post-translational modification of proteins by cellular reduction and oxidation mechanisms; cysteinyl-nitrosylation or tyrosine nitration may induce pathogenesis. Antibodies that recognize damaged poly L-tyrosine and 3-nitrotyrosine can be used to quantify or localize RNS attack to proteins. In fact patients with systemic lupus erythematosus (SLE) produce autoantibodies against such post-translational protein modifications[108]. The ROS and RNS mediated protein modifications can also be detected by using structural proteomics methodology using oxidative foot printing and mass spectrometry[109].

I.4.4 DNA oxidation

DNA oxidation has equally been quantified and visualized using fluorometric tools in livestock and human sperm assessing damage during storage conditions and ART[110,111,112,113,114]. Double stranded DNA (intact) or single stranded DNA (breaks and nicks) can be detected with several assays. (i) Incorporation of fluorescent nucleotides using the terminal deoxynucleotidyl transferase mediated nick end labeling (TUNEL)[115]. An alternative way to detect nicks is to detect in situ nick translation (ii) Fluorescent labeling of chromatin structure either with acridine orange or with chromomycin A [116]. In sperm the acridine orange assay is called sperm chromatin structure assessment (SCSA). Chromomycin A_3 (CMA_3) has been used to detects defaults in chromatin packaging (protamination)[117,118] in sperm from subjects undergoing infertility treatments. (iii) Single-cell gel electrophoresis of DNA from demembranated cells. This assay is commonly known as the COMET assay as decondensed DNA migrates from nuclear matrix in the form of a comet, which can be detected under UV light or after fluorescent DNA labeling. Another method currently available is estimation of DNA damage, translated by the presence of 8-oxoguanine, as a result of free radical attack[119]. The assay is based on addition of a fluorescent labeled 8-oxoguanine binding protein to fixed cells and the yellow fluorescence is reflex of 8-oxoguanine presence. Recently, Kemeleva et al. used monoclonal antibodies against the common pre-mutagenic base lesion 8-oxoguanine (8-oxoG) in combination with indirect immunofluorescence microscopy and image analysis to follow the relative age-dependent amounts and distribution of 8-oxoG in rat liver cells[120].

II. Male Reproductive System

During the several stages involved in sperm maturation, there are different levels of susceptibility to oxidative stress. In the first differentiation stages where

cell divisions are actively taking place, DNA is the target of mutations and ROS, the presence of cytoplasm has an active protective role, and different checkpoints are present to repair possible anomalies. Later in the maturation phase the cytoplasm is mostly discarded rendering the cells less protected against ROS. Finally in the DNA condensing phase the sperm the plasma membrane becomes enriched in phospholipids with highly unsaturated fatty acid moieties. Such fatty acid residues are suitable to scavenge ROS from attacking alternative substrates such as DNA. The major morphological and chemical involved in the male reproductive system with special emphasis on the production of sperm are described in this chapter.

II.1 The testis and spermatogenesis

Spermatogenesis is a process of germ cell proliferation and differentiation within the testicular seminiferous tubules where haploid spermatozoa is formed[121]. Germ cells have intimate contact with Sertoli cells that besides paracrine interactions nourish them directly by gap junctional contact, providing them with energy substrates[122]. Cytokines (IL-1 and IL-6) released by the Sertoli cells probably influence germ cell DNA replication, and thus exert a control role on the progress of spermatogenesis[123].

Spermatogenesis can be divided into three stages: (i) A mitotic division stage where male spermatogonial cells proliferate (ii) a meiotic division stage where the spermatocytes become haploid cells and (iii) a stage where spermatids are formed, this phase is characterized by cyto-differentiation and condensing of chromatin and ends in the release of spermatozoa into the lumen of the seminiferous tubules[124]. During embryo development the mitosis of spermatogonia stops and resumes a few days after birth leading to differentiation of spermatogonia type-A[124]. During the mitotic phase the nuclear division (*karyokinesis*) is complete but cytoplasmatic division (*cytokinesis*) is not[125], primary spermatocytes derived from one type spermatogonium are linked together by thin cytoplasmic bridges, constituting a large syncitium. These cytoplasmic bridges persist until final spermiation and serve to synchronize the spermatogenesis in the surrounding of Sertoli cells. During the meiosis stage the chromosome number is halved (contributing to genetic diversity) and the secondary spermatocyte differentiates into the round spermatid. During the prolonged meiotic prophase, the spermatocytes are particularly sensitive to damage and widespread degeneration can occur[125]. Y bearing spermatogonia can be target of mutations in the euchromatic Y region (Yq11), known as azoospermia factor, resulting in infertile males[126].

Finally, in the cyto-differentiation stage, also designated by *spermiogenesis*, the round shape of the spermatids shifts to a more elongated form. Four stages can be described at spermatid differentiation namely the Golgi stage, the cap stage, the

acrosomal stage and maturation stage[127]. Glycoprotein-granules bud-off from the trans Golgi apparatus of round spermatid that is curled upside down and faces towards the the nucleus of the spermatid. These granules merge to form a single acrosomal granule, that grows over the nuclear surface, forming a cap structure[125]. Chromatin condensation starts beneath the acrosomal cap, generating a nuclear cap, and superfluous nuclear membrane and nucleoplasm is lost. The Golgi apparatus moves posterior as the acrosome starts to change its shape. At a later stage, the mitochondria migrate to the anterior part of the flagellum condensing and forming spirals rods. At the end of spermiogenesis sperm are released from the Sertioli cells into the lumen of the seminferous tubules of the testis. This is achieved by disconnecting the cytoplasmic bridges between the developed spermatozoa. The released sperm cells contain a cytoplasmic droplet, which is a remainder of those bridges. This superfluous cytoplasm is shed from the maturing spermatozoa after they entered the epididymis[125]. During all these stages, there are checkpoints where possible aberrations may be repaired or where improper developed germ cells can be subjected to apoptosis (the latter with a concomitant burst in formation of radicals). Studies on spermatogenesis in rats, rabbits and cats showed that differentiating mitotic spermatogonia frequently underwent spontaneous apoptosis, and that the incidences could be related with the seminiferous epithelial checkpoints[128]. Although apoptosis is most likely to occur during the mitotic stage of spermatogenesis, it has been shown that spermatocytes and spermatids (pachytene spermatocytes, round and elongate spermatids) are able to generate low level of ROS (in particular, superoxide). The ROS formation is stimulated by NADPH, that is formed by metabolic pathways that are inhibiting the cyto-differentiation stage and the greatest ROS formation was monitored in pachytene spermatocytes[129]. Inadequate redox control of gonadal function can later trigger reproductive pathologies, which often involve oxidative stress. Recently a sperm glutathione S-transferase (GST) has been described as a potent detoxifying enzyme[130] and involved in improved germ cell survival and fertility. For instance under selenium deficient conditions GPx and PHGPx activities (see Table 4) are reduced, then GST plays a primordial role in lowering the level of MDA and 4-HNE accumulation in the cell[130].

II.2 Transit through the epididymis: sperm maturation

The immature sperm that was released from the testis now faces new challenges during its transit through the epididymis. It will face a constantly changing environment each with specific effects on peroxidation rates of sperm oxidation. The sperm cell with its highly reduced and specialized cytoplasm faces

a new challenge during transit in the epididymis: modifications at a molecular level with substantial further DNA condensation when entering the *caput* epididymidis[127]. Progressive dehydration, alterations in deoxyribonucleoproteins and lipids, and changes in metabolic patterns are phenomena occurring during transit in the epididymis and during these maturational changes sperm acquire motility[127]. The functions of the epididymis is fourfold: (i) finalize sperm maturation; (ii) intermix new with old mature sperm in the *cauda* epididymidis providing a heterogeneous sperm cell population; (iii) storage of sperm for approximately 2 weeks; (iv) provision of a different biochemical environment that allows mixing with additional genital fluids secreted by the accessory sex glands during ejaculation[127]. In general, maturing sperm spontaneously generate hydrogen peroxide as they progress through the epididymis and maximal production rates are reported for the caudal epididymal region[129]. Superoxide generation is also detected in suspensions of epididymal spermatozoa, but do not correlate with the maturation status of sperm and dramatically increase after addition of exogenous NADPH[129]. This phenomenon closely correlates with the stage of epididymal development and is maximal for immature cells recovered from the *caput* epididymidis in all species tested so far[129]. The phospholipid composition of maturing sperm cells changes during their epididymal transit. A decrease in phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and cholesterol levels was observed in maturing porcine sperm, whereas, levels of sphingomyelin (SM), phosphatidylcholine (PC), polyphosphoinositides and desmosterol content increased[131]. The relative phospholipid content is higher (70% of total lipids) in the *caput* and *cauda* epididymidis when compared to the *corpus*, but there is a general tendency for increasing levels of two specific polyunsaturated fatty acids namely docosapentaenoic acid (22:5), docosanohexaenoic acid (22:6, DHA), and a concomitant decrease in oleic acid (18:1) and arachidonic acid (20:4) from *caput* to *cauda*[131]. The predominance of 22:5, 22:6 as acyl chains in PC[132], was observed for ram, bull, boar, stallion and rooster species[48]. Note that these acyl chains are particularly suitable substrates for radical mediated peroxidation. Immature human sperm cells that faced incomplete cytoplasmatic extrusion contained high levels of DHA in the cytoplasmatic droplet. These cells showed more susceptibility to LPO than normal matured sperm with lower DHA levels[133]. DHA has been shown to be 5 times more susceptible to peroxidation *in vitro* than linoleic acid (18:2)[8]. Thus, by regulating levels of DHA in the sperm plasma membrane, the sperm can adapt to the varying oxidative stress imposed during maturation along the transit through the epididymis[134]. Sperm proteins are subjected to post-translational processing during the sperm transit through the epididymis. Partly, these changes enable the sperm cell to acquire motility and are prerequisites for post-ejaculatory processes leading to proper fertilization[135].

For example the phosphorylation/dephosphorylation cascades regulating tyrosin phosphorylation of tail proteins are supposed to play an essential role in the acquisition of sperm motility[136]. Mild ROS generation by sperm cells positively correlates with proper sperm motility and with increased tyrosine phosphorylation[137]. Low levels of pro-oxidants promote tyrosine phosphorylation, sperm capacitation and egg penetration whilst higher levels inhibit or block these processes[137]. During transit in the epididymis thiol groups in sperm proteins become oxidized between *caput* and *cauda* epididymidis and this coincides with increased levels protein tyrosine phosphorylation[138]. GSH depletion at the *cauda* affects its capacity to protect spermatozoa from oxidative damage[139].

As mentioned before chromatin condensation in the sperm nucleus continues during transit in the epididymis. The condensation of DNA is accompanied by increasing number of disulphide bonds upon oxidation of protamine thiols[140] and intermolecular cross linkage which may constitute an antioxidant defense. This is an important feature for sperm: Sperm cells lack DNA repair mechanisms and cannot synthesize DNA, RNA or translate proteins (such as repair enzymes). Therefore, in normal mature sperm cells the nuclear DNA is highly condensed and the majority of histones have been replaced by protamines[141]. ROS damage to such condensed nuclear DNA is more difficult as the tight DNA packing is protecting sperm DNA for ROS attack. This protamine protection of DNA is relevant since sperm cells as a terminally differentiated and non-proliferative cell are probably more susceptible to oxidative stress than somatic cells[61].

With all these changes that accompany the maturing sperm cell along the epididymis there should be a network of antioxidants that cooperatively protect spermatozoa from oxidative stress[139]. Induced oxidation in human sperm with xanthine/xanthine oxidase system in presence or absence of antioxidants (catalase, GSH, N-acetylcystein and hypotaurine) showed reduced DNA damage for incubations with antioxidants[111]. The enzyme PHGPx is implicated in the higher thiol-oxidase activity of *caput* epididymidal sperm. The enzyme contributes to sperm maturation as it metabolizes hydroperoxides (both from lipid and protein origin). PHGPx takes part in protamine thiol oxidation and thus to sperm nuclear condensation in the *caput* epididymidis, it also acts as an antioxidant[142]. Expression of PHGPx is affected by apoliprotein E receptor-2 (apoER2) which is highly expressed at the initial epididymal segment which is the part where sperm acquire motility and normally release their cytoplasmatic droplets[143]. Other enzymatic antioxidant defenses (see also Table 4) such as catalase, SOD and indolamine dioxygenase (IDO) also serve to protect the maturing spermatozoa from ROS that is generated in the epididymal lumen[134]. Cu/Zn SOD is produced only in human, rabbit and mouse sperm, and these species it is secreted over the entire length of the epididymal tubule. The same is the case for PHGPx along the

entire epididymal tubule[134]. Catalase is not present in mammalian sperm (except for human and rat sperm) but is expressed in the epididymal epithelia and in other male genital tract tissues[144]. Finally, IDO catalyses the oxygenation of L-tryptophan by superoxide into kynurenine, which can be rapidly removed by the cells[134].

II.3 Accessory Glands and Seminal plasma

After completion of their passage through the epididymis the matured sperm are conducted through de *vas deferens* where they will be mixed with the secretions from accessory glands located along the male tract. The highly concentrated epididymal sperm will be diluted approximately by a factor 10 by this blend of secretions during ejaculation and this results in semen in which the seminal plasma will become the transport fluid for sperm from man to the woman. The proportion of secretions from the particular accessory glands that are contributing to seminal plasma vary between species. Also within one species variations of individual males as well as variations on a day-to-day basis are registered both with respect to volume secreted as well as the molecular composition. In mammals the common accessory organs are the, ampulla, prostate, seminal vesicle, bulbo-urethral or Cowper's gland and urethral or Littré's gland. Some species like hamsters also contain coagulating glands[127]. Besides the dilution effect on ejaculated sperm these glands secretions can activate sperm[127] either by catalytic activity which has been reported for the forward motility protein – FMP or in the form of energy as seminal plasma is a rich source in fructose.
Seminal plasma contains high levels of nucleases, nucleotidases and lysosomal enzymes, such as proteinases, phosphatases, glycosidases, mucolytic enzymes especially hyaluronidase[127]. Seminal plasma also contains vesicle-like structures secreted by the prostate (called prostasomes). Their role on the oxidative physiology of sperm is not yet esteablished. One study reports that prostasomes, in presence of nitric oxide and extremely high levels of progesterone, can fuse with sperm and then induce increased cytosolic calcium levels and the acrosome reaction[328] . However, the calcium and acrosome reaction effects are also mediated by progesterone alone (for review see[329]), moreover, it has not been demonstrated that prostasomes produce pro-oxidants.
In addition, seminal plasma has an important antioxidant role, protecting the spermatozoa from the ROS produced by the sperm cells and leukocytes through non-enzymatic scavengers (Table 5) present in the semen. Examples are taurine and hypotaurine[42], α-tocopherol (vitamin E) and ascorbic acid (vitamin C)[145], glutathione[146], uric acid and pyruvate[15]. Likewise enzymatic anti-oxidants (Table 4) are functional in seminal plasma examples are SOD, catalase and glutathione peroxidase (GPx)[147,15]. The epididymis contributes to this by secreting taurine and

hypotaurine and is the only source for these components in seminal plasma. These components compete as oxidisable substrates with cellular lipids and thus can scavenge radicals such as OHCl and $OH^{\bullet}$[8]. In human[96] and rabbit sperm[148] seminal plasma antioxidants greatly reduce lipid peroxidation. Another antioxidant present in human seminal plasma is citrate (up to 20 mM). Citrate can act as chelator for iron ions[149], but in presence of copper ions it seems unable to prevent ascorbic acid degradation to ascorbyl radical[149]. Ascorbic acid at levels above 1 mM can act as a pro-oxidant[150,151]. Ascorbic acid improves sperm characteristics such as motility, viability and decrease MDA levels at a concentration range of 100-800 μM[151].

Additionally, Thérond *et al.* (1996) found that the concentration of α-tocopherol in sperm positively correlated with SOD and GPx activities and was associated to improved motility, viability and morphology characteristics of spermatozoa. The presence of leukocytes is associated with lower α-tocopherol content in seminal plasma and in most cases associates with reduced seminal volumes, impaired sperm motility and morphology[152]. Human sperm motility and viability improves and MDA levels drops when sperm media are supplemented with 1-2 mM vitamin E was added to the human sperm media[153]. Vitamin E (α-tocopherol) supplements to the boar's diet also leads to increased numbers of spermatozoa per ml of ejaculate[92]. The positive cooperation of α-tocopherol with reduced glutathione (GSH) (see figure 1) lead to a decrease in lipid peroxidation levels[92]. In fact this drop is mediated by PHGPx since it reduces the tocopheryl radical lowering the chances of rising of alkoxyl radicals[154]. Similar results were found in cockerel sperm: diets supplemented with vitamin E and selenium improve GPx activity and decrease lipid peroxidation in the sperm membranes[155]. The selenium dependent GPx activity in seminal plasma is variable between species in bull (containing the highest selenium levels) it plays an active role in protecting sperm from ROS attack[156]. Selenium levels in human seminal plasma are substantially lower[157] compared to bull seminal plasma. GPx activity is relatively low in human and ram seminal plasma compared to bull and absent in seminal plasma from boar and stallion[157]. Sperm and seminal plasma from avian species contain SOD and GPx and their enzyme activities increase in case of increased PUFA levels. Seminal plasma enzymes provide a powerful antioxidant buffer against lipid peroxidation in avian sperm[155,158,159]

Human seminal plasma contains retinol (vitamin A_1), which acts as an antioxidant (table 5). The retinol levels are relatively high in men with normozoospermia and lower in men suffering oligozoospermia, azoospermia or asthenozoospermia[160]. The α-tocopherol/retinol ratio has been suggested to be an important clinical index or biomarker for proper sperm function[160].

DNA damage from oxidative stress can be inhibited by seminal plasma or by the separate fluids from accessory glands that contribute to seminal plasma fluids. This has been demonstrated in male hamsters[140] in which either the ampullary glands or ventral prostate glands were removed. These males still showed adequate prevention DNA damage whereas males in which both the ampullary and the ventral prostrate glands were removed showed extensive DNA damage which became manifest in single and double-stranded DNA breaks after incubation with NADPH. NADPH addition also markedly reduces the capacity of sperm to bind to the oocyte in hamsters without both accessory glands[140]. Higher DNA damage is reported for hamster sperm that is tapped from from the *caput* when compared to the *cauda* epididymidis. This difference may reflect the differences in chromatin condensation of sperm at the two regions of the epididymis[140].

II.4 Ejaculate

Subsets of spermatozoa at different stages of maturation have different ROS production. Oxidative stress in ejaculated spermatozoa may be caused by ROS-producing immature spermatozoa or by contaminating ROS-producing cells (like leukocytes) in combination with poor anti-oxidant components in seminal plasma. This may be an important cause of male infertility[161].
Jones *et al.*[162,163] reported the first studies on lipid peroxidation in human sperm. Regeneration of the plasma membrane is not possible in the sperm cell, therefore any advanced damage to this cell will render in a fertilizing handicap. Mature mammalian sperm characteristically contains a high content of polyunsaturated fatty acids linked to phospholipids[164,165,166] (PUFA) (see also section II.2). The main acyl chains attached to the sn-1 position are palmitic acid (16:0) and palmitoleic acid (16:1), whilst for the sn-2 position chains are longer with higher number of double bonds linoleic acid (18:2), α-linolenic acid (18:3), docosanopentaenoic acid (22:5), docosanohexaenoic acid (22:6). The latter two are predominant on phospholipids in ejaculated sperm. The phospholipids are asymmetrically distributed over the plasma membrane's lipid bilayer. The outer leaflet (exoplasmic site) of the plasma membrane is enriched in choline phospholipids: sphingomyelin (SM) and phosphatidylcholine (PC) and in glycolipids. The inner leaflet (cytoplasmic site) is enriched in aminophospholipids such as phosphatidylethanolamine (PE) and phosphatidylserine (PS)[167,168]. As a consequence of LPO, membrane fluidity decreases and membrane rearrangements of phospholipids can induce altered membrane permeability and so prelude leakage and compartment loss[169]. Key membrane enzymes (e.g.: Ca^{2+}-Mg^{2+} ATPases) may lose their function and activity, as well as, the ability to regulate ion flux which are implicated in sperm motility[165]. Likewise, the integrity of

the acrosome and the axoneme can be deeply affected by lipid peroxidation which may lead to loss of fertilizing capacity in human sperm and a negative correlation with *in vitro* fertilization rates[96]. However, the cell has defense mechanisms such as superoxide dismutase (SOD) and glutathione (GSH) peroxidase against ROS (Table 4) as well as proteolytic enzymes that can remove and degrade oxidized proteins[169]. N-nitrotyrosine[8] can be detected as a marker for protein peroxidative damage (see chapter 1, protein oxidation) and may constitute a to very interesting topic in sperm cells due to relation with protein tyrosine phosphorylation that becomes important for hyperactivated motility of sperm during capacitation (see also section II-2).

II.5 Sperm

After ejaculation, sperm cells are *in vivo* introduced into the female genital tract where, in the isthmus region of the oviduct, they will become competent to associate and fertilize the oocyte (sperm capacitation). *In vitro* sperm activation is induced by the use of an IVF (or capacitation) medium that contains capacitating factors such as bicarbonate, calcium and albumin-like proteins. To this end sperm must first be washed through a density gradient to successfully remove decapacitation factors present in seminal plasma. As mentioned before[15,85,170,171,172] ROS generation in sperm can have two different effects: (a) a positive role during capacitation and acrosome reaction, preparing the cell for binding to and fertilizing the oocyte[15,173] and (b) a negative role which threats the cell viability[85,86,165,166,174]. Work in the last decade has shown that ROS interphere in signal transduction mechanisms influencing tyrosine phosphorylation (see section II-2) resulting in hyperactivated motility (one of the results of sperm capacitation)[15,167,175,176]

II.5.1 *Capacitation and acrosome reaction*

Capacitation-factors (especially Ca^{2+}, bicarbonate and albumin) are involved in the removal of cholesterol from the sperm plasma membrane. This leads to increased membrane fluidity: Bicarbonate mediates directly the activation of a soluble adenylyl cyclase and so causes an increase of cAMP levels. This on turn switches on a protein kinase A dependent protein phosphorylation, and indirectly also activates tyrosine phosphorylation (by a sperm specific signaling cross-talk) coinciding with a rise in intracellular Ca^{2+}. These responses together lead to the hyperactive motility characteristic for capacitated sperm cells but also to a more fusogenic acrosome that is ready to start secreting when in contact with the zona pellucida[177,132]. Calcium dependent enzymes such as phospholipase A2 (PLA2) play a role on regulation of LPO as they can cleave lipid peroxides acid residues (primarily present on the sn-2 position of the glycerol backbone of phospholipids)

during lipid peroxidation cascade[178]. Addition of PLA2 inhibitors together with pro-oxidants in the incubation medium of human sperm inhibited in 4-HNE and MDA production[179]. Similarly glutathione peroxidase (GPx) (see section II-3) reduced 60% of the lipids hydroperoxide levels[180]. Glutathione-S-transferase (same section) activity suppression results in impairment on sperm motility, induction of acrosome reaction and fertilization, due to membrane damage[181].

Superoxide dismutases (see Table 4), Mn-SOD and Cu/Zn-SOD are highly enriched in the mid-piece compared to the sperm head and tail[182]. High levels of SOD (balance between H_2O_2 and $O_2^{-\bullet}$) are associated with defective sperm function. SOD is a marker for cytoplasm and high levels probably indicate a defective removal of the cytoplasmic droplet from the sperm (immature sperm). High levels of SOD induce peroxidative damage through high generation of H_2O_2 and removal of $O_2^{-\bullet}$. The latter acts as a capacitation and fertilization promoter[174]. Results from de Lamirande *et al.*[172] showed that human spermatozoa exposed to sustained levels of $O_2^{-\bullet}$ had a better progressive development of hyperactivation and capacitation than samples where SOD was added. In fact, the ability to scavenge $O_2^{-\bullet}$ from the medium by capacitation inducing fluids was inversely correlated with rate of sperm capacitation[97]. In human sperm low concentrations of H_2O_2 (50 μM) speeds up the sperm hyperactivation and capacitation which is caused by the dismutation of $O_2^{-\bullet}$, however, it does not stimulate or inhibit the acrosome reaction[183]. Increasing concentrations of H_2O_2 inhibit sperm capacitation but cause increased acrosome deterioration at concentrations between 10 and 25 μM[173]. Catalase plays a direct role in the removal of H_2O_2 thus maintaining the sperm functions, and further can inhibit the increased expression of phosphotyrosyl proteins[184]. ROS have many protein targets (chapter 1, section protein oxidation) and some sulfhydryl containing proteins are more sensitive for sulfhydryl group attack by $O_2^{-\bullet}$ while others by H_2O_2. Thus some proteins are more sensitive for SOD and others for catalase[185]. Capacitation induced alterations in the protein sulfhydryl content can be prevented by addition of SOD and/or catalase[185].

Oxidizing conditions ($O_2^{-\bullet}$ and H_2O_2) induce tyrosine phosphorylation of proteins (222 KDa, 200 KDa, 159 KDa, 133 KDa, 116 KDa and 82 KDa) in human sperm (see section II-3). This process is associated with increased rates of sperm-oocyte fusion but also affected sperm motility [137]. In addition, capacitation and tyrosine phosphorylation of two specific proteins (p81 and p105) was abolished when SOD and catalase were added to the medium, suggesting that $O_2^{-\bullet}$ and H_2O_2 are regulating these processes[186]. The enzymatic mechanisms involved in the production of $O_2^{-\bullet}$ during sperm activation (capacitation and the initiation of the acrosome reaction) are still not well understood. Molecular mechanisms involved in sperm activation (for instance PKA and protein kinase C (PKC) with cAMP acting as second messenger[176,187,188], may be sensitive for ROS. Oxidation can lead to

activation or inactivation of key enzymes (PKA, PKC) and result in a decrease of acrosome reacted spermatozoa[173]. In such a way ROS may play a regulatory role in controlling sperm function through redox regulation of tyrosine phosphorylation[137]. Especially increased phosphorylation of the protein p80 is observed at high concentrations of H_2O_2 even after long incubation periods that may take place under stress situations[189].

Higher levels of H_2O_2 (≥0.5 mM) inhibit sperm viability, acrosome reaction, and the ability of sperm to bind to and penetrate the oocyte[190]. This is due to perturbations of redox sensitive metabolism "upstream" of the mitochondrial membrane dependent oxidative phosphorylation[89]. Bovine sperm capacitation is facilitated when treated with the xanthine-xanthine oxidase system and catalase. When SOD was added (0.5 mg/ml) a significant decrease in capacitation occurred but no effects were observed on the acrosome reaction or sperm motility[173].

As mentioned previously (section II-4) $NO^{\bullet}$ promotes capacitation. The quantitation of levels of this radical in tissues or cell suspensions is not easy, mainly due to the lack of suitable methodologies[191]. Recently, Herrero *et al*. (2000)[192] demonstrated that generation of $NO^{\bullet}$ was directly detectable with Electron Paramagnetic Resonance (EPR). Alternatively, more indirect measures have been developed to detect NOS activity[191]. One applicable example is the use of NADPH-diaphoretic activity as a potential marker for nitric oxide synthase (NOS). $NO^{\bullet}$ could be identified with this method in cytoplasm, but the main place of production is within mitochondria[191,193]. Stimulation of intramitochondrial NOS resulted in increased permeability for Ca^{2+} and phosphates while NOS inhibitors prevented this[10,193]. Brookes and Darley-Usmar[194] hypothesized that binding of $NO^{\bullet}$ to the mitochondrial cytochrome c oxidase regulates the H_2O_2 production. Cytochrome c oxidase is one of the enzymes of complex IV. This complex is involved in the oxidative electron transfer that is coupled to ATP production in mitochondria (Fig 1) and may be the major signaling $NO^{\bullet}$ pathway[194]. MnSOD is located in mitochondria and regulates the levels of ROS generated as it scavenges the respiratory chain derived $O_2^{-\bullet}$, by converting it to H_2O_2 generating a certain "redox tone"[194]. Interestingly, Zini *et al.*[195] observed that human sperm preparations incubated with nitric oxide-releasing agents with or without either SOD or catalase only showed reduced or abolished capacitation in presence of catalase. This suggests synergism between $NO^{\bullet}$ and H_2O_2. Endothelial NOS is produced in several tissues of the male reproductive tract (testis, epididymis and vas deferens) suggesting a possible role for $NO^{\bullet}$ in spermatogenesis, sperm maturation and programmed cell death[13].

In one study neuronal nitric oxide synthase has been detected by immunofluorescence in the acrosome and tail of sperm[196]. On the other hand, Zini *et al.*[195] did not detect NOS activity in human. There are indications that NOS

activity may produce RNS inside the female's tract[15] which on turn may have an impact on sperm binding to the oocyte and consequent fertilization. Like ROS, $NO^{\bullet}$ may be involved sperm capacitation and protein tyrosine phosphorylation[197,192]. Again the "redox tone" is important in the regulation of adenylyl cyclase activity. Double phosphorylation of the threonine-glutamine-tyrosine motif (P-Thr-Glu-Tyr-P) in p81 and p105 KDa sperm proteins has been demonstrated under capcitation conditions but also after addition of $NO^{\bullet}$[198]. Low concentrations of $NO^{\bullet}$ proved to be advantageous for capacitation[195] and sperm-zona binding[199], but do not induce the acrosome reaction nor increase fertilization rates. When a NOS inhibitor, such as L-NAME (NG-nitro-L-arginine methyl ester) is added to sperm, a dose dependent inhibition of acrosome reaction is detected which can be counteracted by L-arginine supplementation (the substrate for NOS)[11]. However, L-NAME does not affect motility and viability properties of washed human semen[200]. In contrast, Herrero et al.[192] found that $NO^{\bullet}$ levels dropped to zero when L-NAME was added to the capacitation medium. Sperm motility also positively correlated with $NO^{\bullet}$ production[12,201]. The increase in motility and viability is beneficial to sperm as it coincides with reduced rates of lipid peroxidation[202]. This suggests that there is an interaction between $NO^{\bullet}$ and H_2O_2 and that both are involved in triggering LPO (Fig. 2). Again the literature is not consistent as $NO^{\bullet}$ has been reported to inhibit human sperm motility by inhibiting sperm respiration[203]. Males with elevated concentrations of this radical in seminal plasma showed lower sperm motility and were infertile[204]. This result makes sense as most infertile males have also higher leukocyte contamination, which can induce ROS production.

Apoptosis in sperm is an event under debate. Fully mature sperm probably fail to initiate apoptosis[110], but immature ejaculated sperm that may have escaped apoptotic fate in the testis show variable degrees of DNA fragmentation[111,128] (see also sections II-1-3). Note that DNA damage detected in ejaculated sperm may originate from abnormal sperm production in the testis whilst presence of cytoplasm can be a result of defective remodeling during late spermatogenesis and/or transit in the epididymis[113]. The additional cytoplasmic structure will produce additional ROS generation deplete antioxidants[205]. In fact, ejaculated sperm cells with apoptotic markers are reported to have escaped programmed cell death in the testis and express apoptotic markers (e.g. p53 and Fas). This phenomenon is called "abortive apoptosis"[113]. Nuclear and mitochondrial DNA and mtDNA are subjected to considerable radical stress already at the testis level[206]. In fact negative correlations were observed between DNA fragmentation and semen concentration, motility and ROS generation[113,207,208]. DNA damage in parental germ line may also be associated with childhood cancer and infertility[205]. Impaired functioning of the mitochondria affect the degree of mtDNA damage, sperm motility as well as sperm integrity. This is caused by a depletion of ATP and NAD^{+}

depletion and a decrease in glutathione/glutathione disulfide (GSH/GSSH) ratio[209] which causes an increased calcium influx[8].

III. Female Reproductive System

When compared to the male reproductive system relatively limited knowledge is obtained about oxidative processes originating from the female reproductive system involved in gamete. Probably this relates to the fact that in the female only a low amount of oocytes are produced and this takes place in a relatively lengthy cyclic manner while the sperm production is massive and relatively continuous. Moreover, large parts of the male reproductive system are easier to surgically manipulate for obtaining materials. Nevertheless, we believe that a better understanding of the physiology of especially the oviduct is of crucial importance to understand and even manipulate fertilization in vivo. It also will help us to better understand infertility and to approach in vitro fertilization in a more sensible way (for these topics see chapters IV and V)
Spermatozoa are deposited in the female tract either at the vaginal vestibule (cow, sheep, rabbit and primates) or directly in the uterus (horse, pig, dog and many rodents)[210]. In the former situation sperm capacitation only occurs when sperm passed the cervical mucus, whereas, in the latter capacitation is probably initiated when sperm enters the oviduct[210]. The sperm can be introduced via natural mating or artificial insemination, in both cases the sperm cells face a complete new environmental challenge. Part 1 of this chapter deals with oxidative processes to sperm in the female reproductive tract. Part 2 of this chapter will mention oxidative processes related to oocyte development and maturation as well as effects on the ovaries and oviduct physiology.

III.1 Sperm in the female genital tract.

The cervical mucus is enriched in leukocytes, and these leukocytes produce ROS that may react with substrates on sperm that are busy entering this part of the female genital tract. There are two types of effects elicited on sperm mild ROS induce sperm capacitation in normal functional sperm. However, local leukocyte invasions may bombard sperm and excessive LPO and renders sperm dysfunctional[190,211]. After passing through the cervix sperm are transported across the uterus by peristaltic contractions. The journey of the sperm to meet the oocyte is related to the female genital tract fluids that are secreted from the epithelial cells that line up the lumen of the uterus and oviduct. Only a few hundred of sperm are allowed to pass the utero tubal junction. At the end one of them will be successful

to meet and fertilize the oocyte. The supportive role of the oviduct to the final processing of sperm towards interacting with the oocyte is largely *terra incognita* especially with regards to oxidative processes involved here. Human fallopian tubes show a radical dependent relaxation and the radical $NO^{\bullet}$ is involved in tubal contractibility[212]. In extension, by paracrine regulation, $NO^{\bullet}$ also excerts contractions in the uterus guiding sperm towards the fallopian tube[213]. Both processes are believed to facilitate sperm transport to the oocyte.
In the uterus and especially the oviduct several secretory factors contribute to rearrangement of the plasma membrane with sterol removal, increased calcium influx and expression of receptors and binding of stimulatory ligands as part of the capacitation process before entering the oviduct (De Jonge[214] and references therein). Cytokines secreted by epithelial cells (such as IL-6) have been reported to play a role in activating the capacitation machinery via the tyrosine kinase JAK1 phosphorylation mediated pathway which is present in human spermatozoa[215]. Precocious capacitation and hyperactivated motility is accomplished by presence of certain antioxidant enzymes, such as catalase, in the female tract[190]. Recently it has become clear that the mammalian oviduct secretes sufficient amounts of anti-oxidant enzymes to protect the oocyte against free oxygen radicals. Enzymes detected at substantial levels were gamma-glutamylcysteine synthetase (GCS), glutathione peroxidase (GPx), Cu-Zn-superoxide dismutase (Cu-Zn-SOD), Mn-superoxide dismutase (Mn-SOD) and catalase[216]. We suspect (although not yet experimentally tested) that sperm cells in the oviduct are similarly protected against ROS/RNS by these enzymes. We may note here that this antioxidant defenses become functionally disordered after heat stress[217].

III.2 Oocyte development, maturation and release in the female genital tract

Females at birth are already equipped with a large number of primordial follicles in which the primordial oocyte is arrested at the diplotene phase (dictyate stage) of the first meiotic division. Those premature oocytes contain the distinctive germinal vesicle (GV) which can be detected easily under a light microscope[218]. Most of these primordial follicles will not mature but will become atretic[219]. The meiotic I arrest lasts until the female reaches puberty when the hypothalamus-pituitary-ovary endocrine axis becomes fully operational[220]. Shortly the release of pituitary gonadotrophins FSH and LH induces the production of estrogens and progesterone by the follicular granulosa en theca cells, respectively. By this action the primordial oocyte can pass the meiotic I arrest and the GV breaks down. This is followed by chromosome rearrangement recognized as anaphase I to telophase I stages[221]. The completion of the first meiotic division is accompanied with the

extrusion of the first polar body[125]. The same hormones induce the maturation of the follicle to become at the antral stage (sometimes called Graaffian follicle), which is followed by ovulation.
Contrary to seasonal species (mare; ewe; goat), in non-seasonal species the female has a regular cycle that can vary (for the cow and sow 21 days for women 28-30 days). Before ovulation the follicle serves to accommodate the growth and maturation of oocytes. Three types of oocyte populations exist in mammalian ovaries: 1) growing oocytes, which have not yet attained full size or complete maturation, and that do not do so after release from the oocyte or cultured *in vitro*; 2) medium-sized oocytes arrested at metaphase I (MI); and 3) fully grown oocytes that resume maturation after stimuli from gonadotrophins or later release from follicles or cultured *in vitro*[220]. Those cells develop until they become arrested in the metaphase stage of meiose II. This arrest is maintained until fertilization by sperm which introduces the signals to pass the meiosis II arrest[222].

III.3 Effects of ROS on the ovary

In respect to female reproduction system ROS has a positive effect on follicular maturation but also on apoptosis of the theca and granulosa cells of the ovulatory follicle[223]. Apoptosis itself has been reported to be involved in oogenesis, folliculogenesis and oocyte selection or atresia[219]. At birth more than 50 % of the oocytes have died from apoptosis, the latter is essential for good ovarian function and development and makes the female clock tick[219,222]
Follicles may contain NADPH/NADH oxidase activity, since phorbol ester-stimulated ROS generation by follicular cells was completely suppressed by SOD and diphenylene iodonium bisulfate (NADPH/NADH oxidase inhibitor)[224]. In follicular fluid it is possible to assess presence of both ROS but also antioxidant capacity. Follicular fluid ROS, at low concentrations showed to have a positive effect luteolysis (regression of the *corpus luteum*)[225]. ROS up regulation initiates apoptotic cascades in bovine luteal cells by the production of p53, COX-2 and Bax mRNAs[225] driving to luteolysis and new ovulation.
In follicular maturation there is a higher incidence of apoptosis, which can be attributable to $NO^{\bullet}$. This may explain the atresia of smaller follicles compared to larger and possibly later ovulatory follicles[223]. Additionally, in pre-ovulatory follicles there is a NADH/NADPH dependent production of $O_2^{-\bullet}$, which is hormonally regulated and activated by a PKC dependent pathway[224]. The regulation of superoxide production may, therefore, indicate the role of ROS in follicle maturation until ovulation.
In analogy with the sperm cell being a subject of ROS attack, it is legitimate to assume that a similar attacks are operating on the follicle/oocyte system. ROS can

be produced in the *corpus luteum* and in the ovulatory oocyte itself but mainly originate from infiltrated leukocytes (neutrophils to be precise)[226]. ROS play an important role in the maturation of the oocytes. Again, like in sperm, ROS may be beneficial to oocytes and follicles but over a certain threshold ROS adversely influence the oocyte developmental competence (for recent reviews see[227,228]. Follicles that are recruited from the resting pool have ended the Meiosis I and extrude the first polar body. This allows them to escape from atresia and allow further maturation possibly to antral follicles culminating with ovulation. Prior to an ovarian cycle, pre-ovulatory follicles show aromatization of the theca derived androgens through the granulosa cells (for review see[222]). Theca cells seem to synthesize high quantities of estrogen. This increased synthesis is independent from alterations in granulosa cells, since there is a higher expression of enzymes such as P450 aromatase[229]. Antioxidants are known to block the resumption of meiosis as well as gonadotrophin-induced oocyte maturation in immature follicles[226]. ROS probably contribute to the resumption of meiosis by influencing the pre-ovulatory LH peak surge[226]. Normal developing follicles are known to increase the amount of LH receptors during maturation. Interaction between ROS and hormone levels in the estrous cycle is notorious and the leukocyte infiltration causes increased superoxide production in luteal cells within 2 hours of $PGF_{2\alpha}$ pretreatment[226]. Furthermore, H_2O_2 mediated $OH^{\bullet}$ radical formation, induced DNA damage, ATP depletion and inhibition of protein synthesis[226] will affect the use of cholesterol by mitochondrial P450 for steroid genesis causing an uncoupling of the adenylyl cyclase activity and LH receptors sensitivity by the decreased levels of cAMP. The cumulative effect is *corpus luteum* regression[226].

IV. ROS and Fertilization

Fertilization, either *in vivo* or *in vitro*, is a process which involves not only the union of two gametes but also the reproductive health of both the donor and recipient individuals. After the union of the two gametes the fertilized oocyte is activated and forms a zygote that after the first cleavages develops into a morula stage where the embryonic genome (mostly at 4-8 cell stage) becomes transcribed and translated. At the blastula stage the embryo will hatch and become recognized by the endometrium of the uterus. This chapter deals with the oxidative processes involved in fertilization and early embryonic development until the blastocyst stage. It will also deal with oxidative processes related to infertility and the aging of reproductive organs. Effects of artificial fertilization techniques and gamete as well as embryo processing in vitro will be mentioned in chapter 5.

IV.1 Fertilization

The mammalian zona pellucida is a three dimensional matrix that is formed by three glycoproteins, ZP1, ZP2 and ZP3[230,231]. The oligosaccharide chains of the ZP proteins can bind to sperm ligands and thus anchor capacitated sperm. This is most likely a membrane protein complex containing a receptor for the zona pellucida as well as a signalling protein that triggers the acrosome reaction. The hyperactivated motile sperm now efficiently penetrates the zona and reaches oolemma where it will bind and fuse with the oolemma (oocyte fertilization). Sperm binding to the zona pellucida is enhanced in the presence of low concentrations of $NO^{\bullet}$ partly as a consequence of improved motility: Under such conditions some LPO is demonstrated but sperm sulfhydryl groups remain unmodified and sperm functions remain normal[95,192,199]. Sperm cells that are fully capacitated acquire enhanced fusogenicity at the apical area of the sperm head due to reduced cholesterol levels and a concomitant activation of phospholipase A2 and C. When primed sperm binds to the zona pellucida a massive Ca^{2+} influx into the sperm's interior causes instant and multiple fusions of the sperm's plasma membrane with the outer acrosomal membrane (AR).[132,232,233]. Tyrosin phosphorylation of membrane proteins has been suggested to be implicated in zona binding and the AR, thus playing a role in fertilization[168,176,233] (see section II.5).

The fertilizing ability of sperm seems also to relate with the quality of the cumulus oophorus cells that surround the zona pellucida. The cumulus cells cells can trap sperm but its function is yet not understood[234]. Potential candidates for mediating the fertilization fusion between the oocyte and the sperm cell are the ADAM (desintegrin and a metalloprotease domain) family (examples are fertilin α and β and cyritestin) and CRISP (cystein-rich secretory proteins) family (example: epididymal DE protein). ADAM's are supposed to interact with integrins present at the egg plasma membrane while CRISP's may play a role in the arrangement of proteins into an active fusion protein complex between the sperm and oolemma surfaces[235]. Recently, a new antigen named Izumo has been reported to be involved in the sperm-egg fusion. Izumo was found in mouse and human sperm and fertilization could be blocked completely with an anti-Izumo monoclonal antibody [236]. After sperm-oocyte fusion the oocyte initiates a pancellular exocytotic secretion event. The so-called cortical reaction involves a release of the content of secretory granules into the perivitellin space and serves to alter the ZP structure and to prevent polyspermy[237].

Sperm delivers more to the oocyte than just its haploid genome, at fertilization paternal messenger RNAs are also delivered to the egg[238]. These RNA sequences may be required for pronuclear formation and are hypothesized to play part in pathways involved in oocyte activation and in the transition from maternal to

embryonic activation and establishment of imprints in early embryos[238]. It is known that sperm mitochondrial DNA is not inherited. Sperm mitochondria are eliminated before the embryo achieves blastocyst stage. Sperm mitochondrial proteins are ubiquitinized and subjected to proteosome mediated degradation when entering the ooplasma[239]. The active and maternal inherited embryonic mtDNA can be damaged as a result of radical stress in the embryo or somewhere in history of the oocyte[206]. Even though spermatozoa may suffer DNA damage, the embryos normally develop as the the oocyte posesses a high DNA repair capacity[140].
After fusion of the sperm and oocyte the male and female pronucleus will develop. However, preliminary acrosome reaction that can be induced by oxidative stress (H_2O_2), will decrease the chance of oocyte fertilization. Under IVF conditions normal fertilization rates can be obtained with pro-oxidant treated sperm when zona free oocytes are used[240,241]. In contrast to an adverse effect on fertilization, low levels of ROS proved advantageous to fertilization. Low levels of $NO^{\bullet}$ improved the capacity of sperm to fuse with the oocyte. Furthermore, inhibition of NOS significantly reduced fertilization without affecting ZP binding[242,243]. Low levels of ROS on follicular fluid showed equally to have a positive role in IVF[244].

IV.2 Embryo development

During embryo development the intrinsic metabolism generates ROS in its extracellular surroundings[43]. As seen with other cellular entities extreme effects of ROS on embryo development can block or retard normal development of the embryo. Apoptotic events occur[245] and increasing concentrations of H_2O_2 reflects in higher apoptosis incidence by presence of apoptotic bodies and cytoplasmatic condensation in the fragmented blastomers[246]. IGF-1 may play a survival role during pre-implantation its receptor expression is related to resistance to oxidative stress and longer life span in adult mice[247]. Likewise the number of apoptotic cells in human and bovine blastocysts was lower but the number of embryos increased[248,249] in presence of growth factor I (IGF-1) in the culture media. Oxygen tension and presence of antioxidants are important for the development of the blastocyst inner cell mass (ICM)[250]. Low oxygen tension (5% O_2), EDTA free media, catalase and SOD and GSH showed beneficial effects towards normal blastocyst development[251,252]. Nitric oxide has a high toxic effect on the electron transporting chain (attack to cytochrome c) and thus limits oxygen consumption of for instance the pre-implanted blastocyst. Although this inhibits ATP production and mitochondrial respiration it results in better developmental competence of such blastocysts[253].
Sperm DNA damage can jeopardize early embryo development and therefore it makes sense to pay attention, along with classic semen analysis, to detect sperm

DNA damage to predict the possible fertility status and sperm competence[117]. In some reports is has been shown that sperm with DNA damage can fertilize and normal pronucleus formation rates when compared to DNA intact sperm can be obbtained[254]. On the other hand negative influences of DNA damage on fertilization competence of sperm have also been reported[208,255] Detection methods for DNA deterioration in sperm are reviewed elsewhere[256]. Of interest is a study that demonstrated that sperm from infertile human males (with obstructive azoospermia) contained fewer mutations and fragmentations on both mtDNA and nDNA in testicular sperm when compared with than epididymal sperm[206], indicating that the epididymal environment could be harmful for azoospermic men (see also section II.2).

IV.3 ROS and implantation

ROS may play a positive role by preparing the endometrium for a possible implantation. Enzymes such as SOD are thought to play an important role in regulating the endometrial function during the menstrual cycle and pregnancy[257]. ROS in follicular fluids showed at low concentrations to have a positive effect on pregnancy rates in humans[244]. Oxidative stress and protection against ROS in the pre-implantation embryo and its surroundings is a complex and largely neglected research area[43]. It is now common to add antioxidant compounds to culture media. Nevertheless, maintaining the pro-oxidant/anti-oxidant equilibrium in embryos through such supplementation is a complex problem. Further studies are necessary to limit oxidative stress during embryo culture.

IV.4 ROS related infertility

ROS related male infertility can have at least three main causes:

1- External ROS generation by leukocyte and endothelial cells through a NADPH dependent pathway[16,31,174,85,86,89,152,94,179,184,258,259,260,261];

2- Lack of antioxidants for instance low levels of selenium, GSH, SOD, CAT[146,262];

3- Excessive generation of ROS by spermatozoa themselves[85,174], for instance by immature sperm.

Ad 1) Leukocyte contamination in the ejaculate could be due to pathogenic infection in the epididymis.
Ad 3) Such sperm often is seriously damaged (loss of motility and increased DNA damage)[263] .

When leukocytes are mixed to sperm at the time of ejaculation (for instance because they are secreted at that time by the prostate or seminal vesicles) the contact time is minimal and ROS effects are modest due to the anti-oxidant power of seminal plasma (see section II.3 and 4)[261]. Morphological abnormal and immature spermatozoa seems be more susceptible to NADPH induced oxidative damage[264], therefore, defective sperm function is associated with low cell count, impaired motility and abnormal morphology. Leukocytospermic semen has increased DNA damage and higher amounts of immature germ cells, which may be related with alterations in sperm maturation regulation[265]. The ability of sperm cells to maintain motility in presence of ROS production is highly dependent and attributable to the anti-oxidant capacity and protective role of the epididymal fluids and seminal plasma (see chapter II). Remarkably varicocelectomy treatment of infertile men with varicocele, reduced levels of MDA, H_2O_2 and $NO^{\bullet}$ while the anti-oxidant activity of SOD, CAT, GPx and vitamin C in seminal plasma increased[266].
Recent findings suggest that PHGPx-defective individuals have abnormal sperm mitochondria. Defective expression of this enzyme could result in human oligoasthenozoospermia[267]. In agreement with this is the observation that restored PHGPx activities resulted in improved structural integrity, motility and viability of human spermatozoa[268]. Deficient PHGPx expression are not related to blood selenium deficiencies as the level of enzyme expression in blood leukocytes from infertile and fertile men are identical[267]. One other postulated function of PHGPx is to protect DNA protection against oxidative damage[269]. Besides this function (also described for somatic cells) PHGPx is believed to be important for the maintenance of sperm structure[270]. It is believed that sperm PHGPx is the raw material to build up the mitochondrial capsule during spermatogenesis and peroxides may play a role as co-reactants to assure appropriate sperm maturation[271]. To this end PHGPx seems to use GSH and thiol groups as substrates through a hypothetical thiol oxidase role. Thiol oxidation of specific sperm proteins occur during sperm maturation and transit in the epididymis (see chapter II). Most significantly the DNA/chromatin condensation depends on this process[269] (see sections II-1 and 2). In line with this PHGPx is localized in rat sperm heads and in the mid-piece where it surrounds the mitochondria[142]. Moreover, PHGPx exists as a soluble peroxidase in spermatids with high thiol oxidase activity towards in isolated epididymal *caput* protamines. In the last stages of maturation however, PHGPx is not enzymatically active, and is oxidatively cross-linked and thus insoluble in *cauda* epididymidis where sperm acquired its last stage of maturation[142,272].
Anti-oxidants such as Mn-SOD and Cu/Zn-SOD have an important role in detoxification of $O_2^{\bullet-}$ in the cell[273]. Zinc has a positive effect in human sperm by inhibiting the $O_2^{\bullet-}$ production and decreasing the iron-induced LPO[94]. Increased sperm longevity has indeed been linked to increased cellular SOD activities[174]. The

SOD activity probably originates from the cytosol of elongating spermatids[129]. Its activity can be used as a marker for cytoplasmic droplets (see section II-2)[174]. The amount of cytosolic SOD varies considerable and may very well correlate with the sensitivity of sperm suspensions for ROS attack as high SOD activities reflect higher levels of sperm with cytoplasmatic droplets[174]. But low SOD activity are associated with proper sperm function and are required in order to have sufficient amounts of $O_2^{\bullet-}$ and H_2O_2 to form $OH^{\bullet}$[174].

In the sperm cytoplasm a NADPH dependent generation of $O_2^{\bullet-}$ can induce oxidative cascades[81]. In line with this sperm cells have been shown to produce superoxide anion radicals after activation by extracellular NADPH[31,86]. Specific flavoprotein inhibitors could inhibit the $O_2^{\bullet-}$ production[85,86] and the effect was independent from other $O_2^{\bullet-}$ sources as inhibitors for respectively mitochondrial electron transport chain, diaphorase, xanthine oxidase and lactic acid dehydrogenase did not show the same effect. In contrast recent data suggest that morphologically normal sperm can not produce $O_2^{\bullet-}$ via NADPH oxidase. More likely the cytoplasmatic droplets in sperm are a source for NADPH mediated ROS production and sperm DNA damage[264]. Richer and co-workers (2001) were unable to identify the enzyme in human sperm. Nonetheless, generation of ROS has been recently found to depend on a NADPH oxidase (NOX5) related to the neutrophil phosphoprotein gp91-phox[274]. It is of course possible that NOX5 presence in semen is due to leukocyte contamination. At any rate the amount superoxide production via NOX5 was much lower than the ROS produced by white blood cells in human semen[275]. The reduced amount of cytoplasm in mature sperm minimizes $O_2^{-\bullet}$ production. However, immature sperm cells with cytoplasmatic droplets show high levels of cytosolic enzymes, including lactic acid dehydrogenase, creatine phosphokinase (CK) and glucose-6-phosphate dehydrogenase (G6PDH)[184,276] and the activity of these enzymes correlate to sperm dysfunction[174,276]. In fact, when sperm cells are washed over a 40 %/ 80 % Percoll gradient, almost all $O_2^{\bullet-}$ producing cells resides in the interface of the two Percoll layers. Although, immature sperm cells that were recovered from the interface indeed produced $O_2^{\bullet-}$ in addition to impaired function, the majority of the $O_2^{\bullet-}$ production was generated by neutrophils and macrophages from this interface cell suspension. This was demonstrated after magnetic isolation of these cells from spermatozoa with anti-CD45 antibodies coupled to paramagnetic beads[174]. Only very limited $O_2^{\bullet-}$ production (approx. 0.1 % of total semen production) was recovered from the mature sperm cells that pelleted to the bottom of the 80 % Percoll cushion[172].

In contrast to these adverse effects, NADPH also plays part in the sperm antioxidant capacity[1]. It is involved in the reduction of tocopheryl radical (α-Toc$^{\bullet}$) to α-tocopherol (vitamine E) (see Table 5). The NADPH can be produced by the pentose phosphate pathway, through action of glucose-6-phosphate

dehydrogenase (G6PDH). It supplies the reducing equivalents to regenerate GSH and the ability of GPx/GR to reduce lipids hydroperoxides (see figure 1). If the availability of NADPH is limited infertility could result from excessive premature LPO inside the female tract[31]. Another feature of immature sperm is that they may have escaped apoptotic germ waves in the testis (see section II.1), or still did not complete maturation in the epididymis (section II.2). Such cells may contain incompletely condensed DNA which aside the cytoplasmatic droplets may contain remnant tools for activation of apoptosis during ART[277]. Immature sperm expose higher levels of LPO[278,279] with random DNA fragmentation and caspase-3 activated sperm showed chromatin aberrations that resemble apoptotic patterns in somatic cells[280].
DNA damage and presence of apoptotic markers are related to a deficient maturation process either at the nuclear level (protamination) or due to cytosolic remodeling[113]. A positive correlation between apoptotic markers and poor sperm parameters has been shown[281]. The establishment of full mature and immature sperm populations is, therefore, important to understand different chances for reproductive success for similar sperm concentrations[280].

IV.5 Aging

We are all aware that at a certain age oocyte production in females is retarded[282]. During aging ovaries fail to develop follicles and also fail to mature oocytes up to a fertile state. In humans the fertility curve drops drastically after 30 years of age[283,284]. Likewise the chances for blastocyst implantation into the endometrium reduces[285]. The most prominent defects in aged oocytes are: (i) the impaired capacity to start meiosis at metaphase I and to undergo ovulation (meiotic competence) (see section III-2) and (ii) the disability to activate embryo development after fertilization (developmental competence)[283]. The latter requires cytoplasmic factors present in the antral follicle. The number of antral follicles enormously reduce at the end of the reproductive age of women [286]. Chromosomal aneuploidy is one of the major causes of age-related decline of oocyte quality become manifest in highly increased incidences of trisomy 21 and spontaneous abortions[62,284]. Aneuploidy is a consequence of spindle malformations, and is reported to relate with mitochondrial dysfunction in a yet not completely understood manner[287]. It was shown that aged mouse oocytes after fertilization failed to induce ATP production required for proper development and calcium oscillations[288]. Further to this aged mouse oocytes, probably due to oxidative stress, show alterations in the regulation of intracellular calcium concentration and consequently affects calcium oscillations in fertilized oocytes[289] .

V. ROS and Assisted Reproductive Technologies

In modern time a number of assisted reproductive technologies (ART) have emerged that involve invasive in vitro handling of gametes, and embryos. ART significantly contributed to help women in infertile couples to achieve pregnancies. However, with respect to the topic of this review it is important to discuss the consequences of processing gametes and embryos to this end since intervention may alter the impact of oxidative processes towards gametes and embryos. We should realize that storing, washing and processing gametes or embryos are part of the procedures involved in ART. They are applied to animal and human reproductive research and medicine to overcome infertility problems, genetic value spread and germplasm units.

V.1 ROS during IVF and ICSI.

Artificial reproductive technology (ART) procedures are intimately related with the inability of couples to generate a child via normal intercourse. For breeding companies and/or farmers it is a method that can be used for speeding up the spread of genetic valuable genes into herds. Male infertility is one of the major causes for couples to look for ART solutions[290]. Prior to ART sperm quality selection routinely is performed in the fertility clinic. One of the parameters tested is the degree of response of sperm to a Ca^{2+} ionophore challenge, since the degree of induced acrosome reactions positively correlates with human pregnancy rates[291].

Oocytes must undergo *in vitro* maturation (IVM) to a stage where they can be used for IVF purposes. Successful IVM can only be achieved when oocytes are collected from large follicles. They cannot be collected from too small follicles. Human follicles ranging between 2-8 mm are preferably aspirated[292]. This limitation implies that an immense pool of primordial follicles (the most abundant ones in the ovary) cannot be used. In the future novel approaches may make it possible to culture oocytes from such material[222]. The IVM properties of oocytes from premature cows is lower than that for adult cows and this is also reflected in the corresponding IVF and embryo development rates[293]. Classical IVF give better mouse embryos when compared to ICSI[294]. Equally, ICSI derived children had a higher incidence for major congenital malformations than IVF children, although both ICSI and IVF children had a higher probability to need health care than naturally conceived children[295].

During *in vitro* procedures is important to assess the extent of oxidative damage throughout oocyte maturation and the involvement in this process of anti-oxidant systems. During *in vitro* maturation oocytes are known to produce ROS[296,297,298].

Importantly, cumulus cells have been shown to play a major role in anti-oxidant defense by administering SOD, GPx and catalase activities to the oocyte's direct environment[296]. These enzymes are more active in the oocyte in a later maturation phase and are then diminished in the cumulus cells[296]. At the blastocyst stage cell apoptosis can be assessed and linked to oxidative stress (in most cases this reflects to the redox history of the oocyte). In fact apoptotic rates was lower in blastocysts made from cumulus surrounded oocytes compared to blastocysts from denuded oocytes[297]. Metabolites such as glucose can stimulate ROS production and decrease the GSH content[298]. Finally a compromised blastocyst development can be the result of altered metabolism of arachidonic acid[299]. Thus it appears that oocytes may obtain the capacity to control ROS production during maturation by adjusting their own antioxidant system.

V.2 Sperm washing and ART

Mature sperm has a minimal cytoplasmic compartment and lacks the mechanisms of plasma membrane turnover and de novo synthesis of lipids (see section II.5). Its limited intracellular scavenging enzymatic consortium only poorly protects this cell against intracellularly produced ROS or extracellularly ROS produced by for instance leukocytes[89,165,258]. As a result, the tendency is that cells that are separated from seminal plasma (and its anti-oxidant activity) become more susceptible to oxidative stress[16]. After washing through a discontinuous density, the pelleted human sperm appear to be more competent with this respect, and with such sperm preparations also better fertilization rates can be obtained[183]. Those cells have better nuclear integrity[117] than centrifugation swim-up which induce per se lipid peroxidation[183]. Swim up fractions from samples of poor, teratozoospermia-related, morphology positively correlate with caspase-3 activity. However, swim-up and gradient centrifugation manages to significantly deplete caspase-3-positive sperm when compared to semen fractions[300]. Spontaneous DNA fragmentation in swim-up selected spermatozoa correlates with basal levels of DNA fragmentation in teratozoospermic but not normospermic semen, and oxidative stress may be the cause for this DNA fragmentation which is aggravated by in vitro incubation[301]. The differences again relate to nuclear condensation and shedding of the cytoplasmic droplet from the mature sperm (those cells pellet during washing and have the best swim up properties).

V.3 Sperm storage

To overcome male infertility, sperm storage becomes an important technique in order to improve the existent limitations for natural mating. In animal

production, sperm storage is certainly one manner of spreading genes of high genetic value animals across the world and to preserve equally endangered species. During sperm processing -either for liquid storage or cryopreservation- there is always to some extend damage introduced to the semen samples. Sperm should be stored for liquid preservation in a nutritive media that allows survival under reduced metabolic activity, moreover, precocious capacitation should be avoided as well as cell death[302] . The deteriorated sperm will contribute for ROS production by the release of polyunsaturated fatty acids to the storage medium which is a good source for initiation of peroxidation chain reactions[303]. ROS produced by mitochondria could impair sperm DNA if a weakened anti-oxidant network is not able balance ROS production. In fact, addition of antioxidants[304,305,92,306,145] and chelating agents (EDTA)[307] to the storage media can minimize these problems and are routinely added in semen diluents[308,303].

Liquid storage for farm animal species should aim to reduce the degree of endogenous protein phosphorylation, in order to prevent precocious capacitation[302]. Inappropriate sperm responses may render the cell sensitive for ROS attack in situ produced[303]. Studies conducted in ram sperm showed that addition of antioxidants improved survival, acrosome integrity and fertilization potential of spermatozoa during liquid storage[309]. These beneficial effects were also confirmed with addition ascorbic acid in chilled extender stallion semen[304].

Freeze-thawing cycles cause a change in the sperm plasma membrane's architecture that may affect the functionality of certain membrane proteins. Depletion of these same components may compromise adequate progression of sperm in the female tract and fertilizing ability of thawed sperm cells. Studies have shown that cryopreservation caused a decrease in heat-shock protein 90 levels in human sperm which was due to protein degradation[310]. Similarly this proteolytic change rendered equine sperm more sensitive to capacitation inducers limited the sperm's life span[311] when compared to fresh sperm. The distribution of sulfhydryl groups in plasma membrane proteins is affected by cryopreservation and thus cryopreservation influences the activities of physiologically important sperm proteins. This problem could be prevented by suppleting the cryoprotectants reduced (GSH) or oxidized (GSSG) glutathione[312]. Freeze-thawing may alter nuclear protein relationships that are coupled with chromatin condensation and may impair the sperm's fertilizing ability[313] and lower IVF conception rates. Cryopreservation makes use of cryoprotectants such as, glycerol and egg yolk. The latter has a low lipoprotein fraction (high density molecular weight component) which can only interact with the sperm cell surface. In a yet unknown way these lipid particles prevent membrane disruption during cooling process and help to stabilize the lipid bilayer structure[303,314]. In addition egg-yolk is a rich source of vitamin E, contributing to the anti-oxidant power during the cryopreservation

procedure (see Table 5)[315]. Recently, it has been shown that increased cholesterol levels (after loading with cholesterol-saturated cyclodextrins) in bull sperm membranes resulted in improved cryosurvival without affecting the ability of sperm to undergo capacitation and acrosome reaction after thawing[316]. This shows that, that besides additional oxidative stress as a result of cryopreservation, the stability of the sperm membranes is also of importance for the success of thawed samples in AI.

Cryopreservation and thawing are associated with apoptotic-like events in human[317,318] and bovine sperm[112,319]. Those cells that show these apoptotic like responses can be selected from the healthy non-responsive sperm population and the latter can subsequently be used for AI, IVF or ICSI[317,319,318]. This can either be achieved by using flow cytometric sorting or by magnetic sorting of cells after labeling with paramagnetic beads.

The most commonly used anti-oxidants in sperm storage are vitamin E, GSH[306,305], and some enzymes such as CAT[190] and SOD[306]. GSH has showed to be a very effective protective agent against ROS derived motility loss in bull sperm[320], as well as, addition of thiols to the cryoprotectant extender[305].

V.4 Oocyte and Embryo Storage

Multiple ovulation and embryo transfer (MOET) has been worldwide used in embryo production. The number of transferable embryos stagnated the past 20 years. MOET has to some extent been replaced by embryo *in vitro* production (IVP) which combines IVM and IVF and requires more specific equipment and expertise. At any rate IVM remains the bottle neck for acquiring more efficiently competent oocytes whilst the embryo culture system affects mainly the quality and viability of the embryos to be transferred[321]. Cryopreservation has been shown to disrupt cytoskeletal and mitochondrial structures[322]. Bovine embryos are more susceptable to this cryodamage than sheep and goat embryos. The latter not only gave better results but did differ much from cryopreserved *in vivo* produced embryos[322]. In equines IVP is still very difficult and unsuccessful. Partly, this is due to the fact that only small numbers of embryos are available for this use, partly because it is difficult to induce super ovulation in mares. Furthermore these embryos are poorly resistant to cryopreservation. Attempts to stabilize the cytoskeleton by addition of substances (for instance cytochalasin-B) to the blastocysts reduces cell death during cryopreservation have been successful but long-term effects of such cytostatic drugs remain to be tested[323]. Pig embryos are enriched with high levels of intracellular lipids (in the form of lipid droplets). The embryos are very sensitive to chilling and temperatures below 15 °C severly decrease the cahnce to establish pregnancies after embryo transfer[324]. Delipidation

of 2 to 8 cells pig embryos improved their cryosurvival [325,326]. In addition, cryopreservation and vitrification of morulae/early blastocysts enhanced the developmental competence of embryos after their transfer[322]. Despite this kind of ART being available for 20 years only recently the impact of oxidative stress on embryo storage is taken into consideration and especially effects at the membrane level are considered to be important for cryosurvival.

VI. Conclusions

In summary, oxidative stress has not allways adverse and deleterious effects, it shows beneficial influences on sperm motility, capacitation and oocyte fusion[85]. Changes in membrane architecture (lipids and proteins) are driven by ROS during capacitation. Such effects on membrane molecules during the acrosome reaction culminates in a more fluid membrane with increasing fusogenic properties[15,173]. Similarly, $O_2^{-\bullet}$, H_2O_2 are involved in the maturation of follicles in which steroid genesis and ovulation is taken place. $NO^{\bullet}$ is more involved in contractibility and vascularization of the uterus walls creating a soft "nest" for implantation of a possible fertilized oocyte. $NO^{\bullet}$ also has a role in blastocyst pre-implantation development by limiting oxygen consumption at a mitochondrial level. Extreme levels of LPO ironically are functionally produced by ROS during apoptosis. Peroxidation can be used as therapy tools in women undergoing chemotherapy and radiotherapy treatments. More specifically ceramide and sphingosine-1-phosphate have been put forward as putative lipid therapy to protect oocytes from undergoing apoptosis as a result of cancer treatment[327]. Like this, many other therapies will in the future make use of ROS as inhibitors of deleterious processes or stimulators of others that are impaired.

Finally the state of the art of ROS in reproduction is evolving rapidly, but in livestock fertility much more research is needed to overcome problems still present during liquid storage of sperm and cryopreservation of gametes and embryos, where much is still unknown.

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CHAPTER 2

New assays for detection and localization of endogenous lipid peroxidation products in living boar sperm after BTS dilution or after freeze-thawing

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Abstract

Reactive oxygen species have been implicated in sperm aberrations causing multiple pathologies including sub- and infertility. Freeze/thawing of sperm samples is routinely performed in the cattle breeding industries for semen storage prior to artificial insemination but unusual in porcine breeding industries as semen dilution and storage at 17°C is sufficient for artificial insemination within 2–3 days. However, longer semen storage requires cryopreservation of boar semen. Freeze/thawing procedures induce sperm damage and induce reactive oxygen species in mammalian sperm and boar sperm seems to be more vulnerable for this than bull sperm. We developed a new method to detect reactive oxygen species induced damage at the level of the sperm plasma membrane in bull sperm. Lipid peroxidation in freshly stored and frozen/thawed sperm cells was assessed by mass spectrometric analysis of the main endogenous lipid classes, phosphatidylcholine and cholesterol and by fluorescence techniques using the lipid peroxidation reporter probe C_{11}-Bodipy$^{581/591}$. Peroxidation as reported by the fluorescent probe, clearly corresponded with the presence of hydroxy- and hydroperoxyphosphatidylcholine in the sperm membranes, which are early stage products of lipid peroxidation. This allowed us, for the first time, to correlate endogenous lipid peroxidation with localization of this process in the living sperm cells. Cytoplasmatic droplets in incompletely matured sperm cells were intensely peroxidized. Furthermore, lipidperoxidation was particularly strong in the mid-piece and tail of frozen/thawed spermatozoa and significantly less intense in the sperm head. Induction of peroxidation in fresh sperm cells with the lipid soluble reactive oxygen species tert-butylhydroperoxide gave an even more pronounced effect, demonstrating antioxidant activity in the head of fresh sperm cells. Furthermore, we were able to show using the flow cytometer that spontaneous peroxidation was not a result of cell death, as only a pronounced subpopulation of living cells showed peroxidation after freeze–thawing. Although the method was established on bovine sperm, we discuss the importance of these assays for detecting lipid peroxidation in boar sperm cells.

Keywords: Lipid peroxidation; Sperm membrane damage; Cholesterol; Phospholipid; Fluorescent lipid probe; Confocal microscopy; Flow cytometry; Mass spectrometry

1. Introduction

All living cells produce under aerobic conditions reactive oxygen species which mostly originates from normal cellular metabolic activity. Mammalian sperm cells do not form an exception and are able to produce superoxide anions ($O_2^{-\bullet}$) [1,2], which spontaneously or enzymatically dismutates into hydrogen peroxide (H_2O_2) [3]. Due to the low reactivity (half life of 1 ms), $O_2^{-\bullet}$ is not very harmful, although chemical reactions with oxidizable substrates can produce more toxic radicals such as thiyl residues ($RS^{\bullet}$). Although present at very low concentrations, iron is sufficiently present in almost all biological fluids to catalyse the formation of hydroxyl radicals ($OH^{\bullet}$) from H_2O_2 or other ROS (lipid hydroperoxides). Hydroxyl radicals are extremely reactive (half life of 1 ns) and can react with virtually any cell component. Polyunsaturated fatty acid moieties of phospholipids and cholesterol are preferred substrates for $OH^{\bullet}$ generated in membranes. Sperm cells and seminal plasma, like other cells and body fluids, contain a battery of ROS scavengers, including enzymes such as super oxide dismutase [2,4], catalase [5] and the glutathion peroxidase/reductase system [6,7]. Furthermore, they also contain numerous types of small molecules with SOD- or catalase-like activities [8] such as albumin [9], alpha-tocopherol [10], pyruvate [11], glutathione [12], vitamin C [13], taurine and hypotaurine [9]. The balance between ROS generation and scavenging as well as the moment and location where sperm cells come into contact with ROS determines the effects imposed on these cells. In vitro manipulation of ejaculated semen affects ROS formation and scavenging in several ways: (1) sperm cells are diluted or washed from seminal plasma removing a rich source of ROS scavengers. (2) For porcine AI practice aliquots of approximately 80 ml containing diluted boar semen (approximately 10 times in BTS) are stored in principle under aerobic condition for up to 3 days prior to insemination [14,15]. Obviously the extended exposure to oxygen results in extra peroxidative stress to the processed sperm sample when compared to ejaculates that are deposited directly in the sow after natural mating. (3) Washed sperm cells can be diluted into specific buffers for cryopreservation. During cryopreservation, water crystal formation [16] and related dehydration of and lateral phase segregation of phospholipids [17] induce irreversible sperm damage. Boar sperm is notoriously low tolerant for cold temperatures (cold-shock sensitive). However, cryopreservation buffers have been prepared with cryoprotectants such as glycerol which indeed, at the optimal cooling temperature curves (see Woelders et al. [57]), maximally protect sperm from cryodamage. However, a draw back of glycerol is that it causes a post-thaw osmotic pressure on the sperm cell which has become hypertonic to its environment and specific thawing procedures have evolved for boar sperm [18]. This has resulted in optimized cryopreservation protocols that nowadays can be used for cryopreservation of boar

sperm [57]. (4) BTS diluted fresh stored sperm of frozen/thawed sperm will at a certain moment be inseminated in the sow. The cells that have faced extra stress by in vitro manipulation will now face new challenging extracellular environments in the female genital tract each with different ROS and scavenging systems [19]. Therefore, in general, we assume that manipulated sperm cells are less capable to cope with oxidative stress than unmanipulated cells. This may explain that low (physiological) ROS generation in a physiological scavenging environment supports sperm capacitation in the female genital tract [20] whereas pathological high ROS generation, for instance in infertile males with inflammatory responses in the genital tract [21] or in in vitro manipulated sperm [19], causes sperm deterioration.

2. Cholesterol and phospholipids are sperm plasma membrane substrates for peroxidation

In this manuscript we focus on particular substrates for ROS in the sperm plasma membrane: phospholipids and cholesterol (not shown before). Together with seminolipid (for review, see [22]) these lipids structurally form the lipid bilayer of sperm membranes.

The phospholipids of boar sperm cells are extremely rich in polyunsaturated phospholipids with either five or six unsaturations [23]. ROS radicals preferentially attack polyunsaturated acyl chains by abstracting a hydrogen atom from a bisallylicmethylene ($–CH_2–$) group [24,25]. The carbon carrying the unpaired electron ($–^{\bullet}CH–$; alkylradical) that is left behind, most of the time rearranges into a conjugate diene that easily reacts with molecular oxygen to from a peroxyl radical ($–ROO^{\bullet}–$). Peroxyl radicals can propagate the oxidation by abstracting a hydrogen atom (thus becoming a lipid hydroperoxide –ROOH–) from a neighboring unsaturated fatty acid, which in turn, becomes an alkylradical. Under the influence of transition metals such as iron or copper, lipid hydroperoxides can generate either peroxyl- ($–ROO^{\bullet}$) or alkoxyl- ($–RO^{\bullet}$) radicals, accelerating the process of peroxidation even further. This chain reaction can proceed until a scavenging agent will react with the propagating radicals (termination reaction). As mentioned above hydroxyl radicals are the ROS usually recognized as the initiator of lipid peroxidation, $O_2^{-\bullet}$ and H_2O_2 being not sufficient reactive. However, $O_2^{-\bullet}$ can reduce the ferric (Fe^{3+}) into the ferrous (Fe^{2+}) ion or react with SH-groups forming thyil radicals ($RS^{\bullet}$). Fe^{2+} is a catalyst of oxidation and $RS^{\bullet}$ can initiate lipid peroxidation [3]. Lipid hydroperoxides are susceptible to cyclization and decomposition into reactive alkenals [3], and can, like their degradation products 4-hydroxynonenal [26,27] and malondialdehyde [28,29,30], be highly toxic for sperm. Eutherian sperm cells contain a specific type of glycolipid called seminolipid (a sulfogalactoglycerolipid)

and are virtually devoid of normal somatic cell glycolipids (containing a ceramide backbone instead of a alkylacylglycerol backbone). Seminolipid is believed to play an important role in the ordering of the sperm head plasma membrane into functionally heterogeneous regions (involved in different adhesion, membrane fusion and cell signaling events [22,31]). However, only one, completely saturated species is known for boar sperm: 1-O-hexadecyl-2-O-hexadecanoyl-3-β-(3-sulfogalactosyl)-glycerol (seminolipid), which is not a suitable substrate for lipid peroxidation due to the absence of bis-allylic carbon atoms.

Cholesterol is also an important lipid component of the sperm plasma membrane. High sterol levels stabilize the sperm cells resulting in better cryopreservation. This is reflected in the success of cryopreservation of semen from different mammals. Human, canine and bovine sperm cells contain high levels of cholesterol and are known to have better cryoresistant properties than species with lower sterol levels like porcine and caprine sperm [22,32]. Porcine sperm has been shown to be particularly sensitive for irreversible induced lateral phase segregation of phospholipids during cryopreservation when compared to bovine sperm [17]. From biophysical studies, it is well established that cholesterol prevents lateral phase segregation of phospholipids [33]. Furthermore, cholesterol is depleted from the sperm plasma membrane upon capacitation [23]. Species with relatively high sterol levels (human and bovine) require lengthy in vitro capacitation (8–24 h); whereas species with low sterol levels (porcine, caprine) are capacitated in vitro within 2 h [31]. Depletion of cholesterol occurs after a specific bicarbonate-induced bilayer rearrangement of phospholipids (see Harrison and Gadella [8]) and a concomitant lateral rearrangement of glycolipids [34,35] and cholesterol [23] in the sperm head. Despite the crucial role of cholesterol in protecting the sperm membranes during cryopreservation and in destabilizing the membrane during capacitation nothing is known about its oxidation during these processes. Nevertheless, it is well established that cholesterol can be readily oxidized in all types of biological samples, causing problems ranging from decreased food quality to impaired cell function [36,37].

3. Effects of phospholipid and cholesterol peroxidation in sperm cells

Indeed ROS has been shown to cause membrane deterioration, resulting in ATP depletion [11,38], DNA damage [39], decreased sperm movement [38], and blocked sperm–egg fusion [40]. However, knowledge about where lipids are oxidized, what types of lipids are preferential substrates for ROS and the physiological consequences of this molecular damage is limited. Therefore, in our lab, new lipid peroxidation assays have been developed that can be used to detect endogenous

phospholipid and cholesterol oxidation products generated during sperm processing for cryostorage BTS dilution and in vitro sperm capacitation. Besides this we have developed a very sensitive ratiometric fluorescence assay that can be used to detect phospholipid peroxidation in individual sperm cells using a flow cytometer and to localize this process at the subcellular structural level in sperm cells. The principles of the assays will be explained and the promising features that are now being discovered will be discussed.

4. Detection of lipid peroxides formed in sperm cells

4.1. Detection of endogenous phospholipid peroxidation

Sperm cells that are manipulated in vitro either during cryopreservation, fresh dilution or in vitro capacitation are subjected to extra oxidative stress. After such treatments, total lipid extracts of Percoll washed samples (removal of extracellular lipids) can be made following the extraction protocol of Bligh and Dyer [41]. The dried lipid extract redissolved in methanol/chloroform (2:1, v/v) will be injected into a mass spectrometer to analyze phosphatidylcholine (PC) and sphingomyelin (SM) phospholipids. This is done by recording all parent positive molecular ions $\{M+H\}^+$ that loose the phosphocholine ion ($(HO)_2PO_2CH_2CH_2N+(CH_3)_3$), mass to charge ratio (m/z) 184 as a fragment ion [42,43] (Fig. 1). One of the major advances of this new method is that the oxidation of sperm PC species can be followed. Instead of the 'classic' end products of lipid oxidation (malondialdehyde and 4-hydroxynonenal), initial, intermediate and end products of oxidation are identified: Identified peroxidation products originating from the abundant PC species 16:0–22:6 PlasCho and the mass spectrometric (collision induced) fragmentation reaction leading to identification of the oxidation products, are summarized in Fig. 2.

Simultaneous detection of peroxidation of endogenous PC and SM species can be done on the same precursor scan (both lipids contain a phosphocholine headgroup). Classical techniques such as malondialdehyde and 4-hydroxynonenal determination can only be used for rough quantitation of lipid peroxidation rates. Besides the abovementioned precursor scan that is specific for phosphocholine, similar scans can be made corresponding to other phospholipid classes (see [44] for a summary of headgroup specific fragmentation of phospholipids), enabling phospholipid class specific assessment of lipid peroxidation. Taken together the method of headgroup specific scanning of phospholipids in general is useful to determine the phospholipid species composition and the fate of these molecular species during physiological and artificial oxidative stress. The oxidation and

metabolic conversions of arachidonic (20:4) acid has been reported to be of importance for many biological activities [45,46,47]. In analogy, docosahexaenoic (22:6) acid peroxidation may well induce sperm events important for activating [48] or deteriorating [49] sperm cells prior to fertilization [50,51].

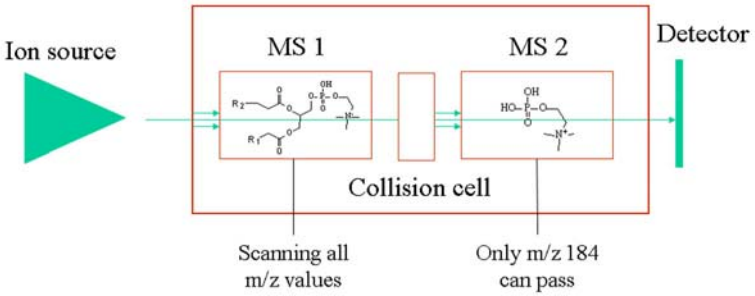

Fig. 1. Schematic setup of a tandem mass spectrometer to identify the cholinergic phospholipids (PC and SM). Ions are typically made by electrospray ionisation. MS1 is operated in a scanning mode, allowing only one m/z value to pass at a time. These ions are fragmented in the collision cell and fragment ions subsequently enter MS2, set (fixed) to the m/z of phosphocholine headgroup (m/z 184). The resulting spectrum reflects the detector signal at different m/z value of MS1.

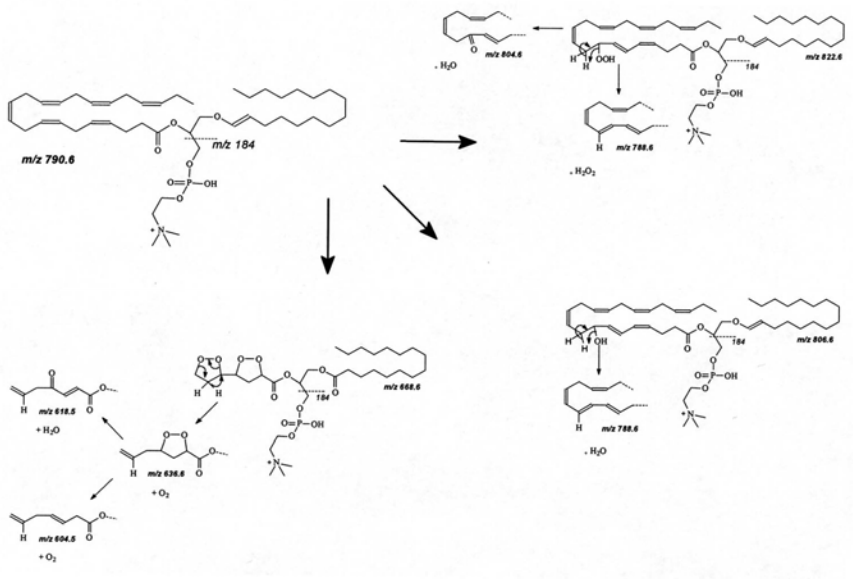

Fig. 2. Identified oxidation products of 16:0–22:6 PlasCho by mass spectrometry. Bold arrows indicate tentative chemical reactions leading to the indicated products. Observed fragmentation reactions in the collision cell, supporting the given structures, are indicated by normal arrows. Corresponding m/z values are given in italics (Adapted from [52]).

4.2. Detection of cholesterol oxidation

As mentioned above, sterols form an important fraction of the sperm plasma membrane both for membrane stability (important for cryopreservation) and for the regulation of lateral mobility of membrane proteins (which enhances during sperm activation). The peroxidation of endogenous cholesterol in sperm cells can easily be followed by using a similar approach as described for the detection of phospholipid peroxidation products. The neutral fraction of a total lipid extract can be isolated over a generic silica column by elution with acetone. The dry neutral lipid fraction is reconstituted into methanol/ acetonitrile (2:3, v/v) and injected onto a reversed phase HPLC column. After separation of cholesterol and its oxidation products (commonly referred to as oxysterols), the column effluent is introduced into a MS via an atmospheric pressure chemical ionization (APCI) source. Identification of individual oxysterols can be achieved either by multiple reaction mode (MRM) analyses which requires a tandem-MS setup, or by multiple ion monitoring, in which case a single stage MS suffices. Multiple ion monitoring simply samples the intensity of m/z values of known (oxy)sterols, and identification of oxysterols is done based on retention time and molecular mass. MRM analysis on the other hand, includes oxysterol fragmentation as an additional confirmation step (Fig. 3).

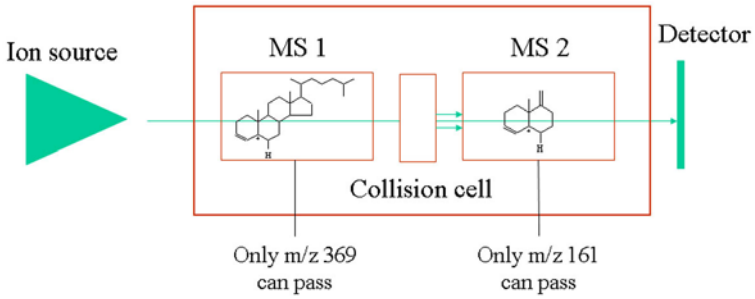

Fig. 3. Schematic setup of a tandem mass spectrometer to monitor for a known set of oxysterols by multiple reaction monitoring (MRM). Ions are typically made by atmospheric pressure chemical ionisation. Both MS1 and MS2 are operated at fixed m/z values. When monitoring for a given (oxy-)sterol, only a signal will be detected when the corresponding m/z value enters MS-1, and subsequently produces the fragment ion with the m/z value that MS-2 is set to. By switching between different m/z values for MS1 and MS2 (typically every 100 ms), multiple (oxy-) sterols can be monitored in a single HPLC run.

Remarkably, sperm cells contain a number of cholesterol oxidation products known to exert physiological and toxicological activities [36,53,54,55] (for structures detected, see Fig. 4). Preliminary work on boar sperm indicates that in vitro capacitation,

particularly after freeze–thawing, accelerates the formation of these oxysterols. The relevance for sperm physiology and fertilization efficiency needs to be elucidated in the future.

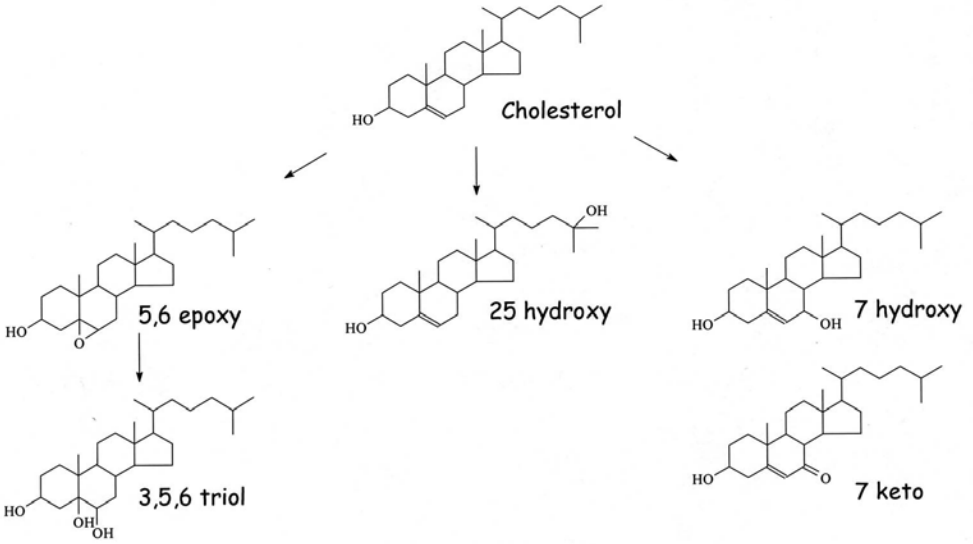

Fig. 4. Major oxysterols identified in porcine sperm cells. Oxidation preferably occurs at either (i) the double bond between carbon atoms 5 and 6 resulting in both the alpha and beta isomers of 5,6-epoxycholesterol (5,6-epoxy), and 3,5,6-triol cholesterol. (ii) Carbon 25, resulting in 25-hydroxycholesterol, or (iii) carbon atom 7, resulting in both the alpha and beta isomers of 7-hydroxycholesterol (7-hydroxy) or in 7-ketocholesterol (7-keto).

4.3. Localization of lipid peroxidation

Although we have described accurate methods to follow phospholipid and sterol oxidation, the problem with these analytical methods is that they must be performed on lipid extracts of washed sperm suspensions. Thus these techniques will only measure the amount of lipid peroxidation without knowing where this oxidation takes place (localization in the sperm cell) and on which subpopulation of sperm cells this actually happens (for instance in activated cells, or in deteriorating cells). Recently, a fluorescent fatty acid analog, C_{11}-Bodipy$^{581/591}$, has been described that changes its fluorescent properties after peroxidation. The intact probe fluoresces red when intercalated into the membrane and its fluorescence shifts towards orange (540 nm) emission after oxidation by peroxynitrite and to green (520 nm) emission after attack with other oxidative radicals [56]. This probe is very suitable for sensitive quantitation of lipid peroxidation and can even be used

as a single ratiometric probe to simultaneously quantitatively distinguish peroxynitrite mediated lipid peroxidation from other lipid peroxidation mediated by other ROS (Fig. 5). The amount of C_{11}-Bodipy$^{581/591}$ peroxidation correlated well with the formation of phospholipid peroxides as well as cholesterol oxidation products [52]. This made us confident that C_{11}-Bodipy$^{581/591}$ can be used to localize lipid peroxidation in single sperm cells (using confocal laser scanning microscopy) and to detect subpopulations of sperm cells with varying sensitivity for lipid peroxidation using flow cytometry (published for bovine sperm; [52]). From this study, we learned that: (i) lipid peroxidation is most prominent mid-piece and to a lesser extent in the posterior parts of the spermtail but virtually absent from the sperm head; (ii) induction of lipid peroxidation showed that the sperm head is quite resistant to this treatment whereas the tail and mid-piece are not; (iii) only a subpopulation of the life sperm cells shows lipid peroxidation whereas this is negligible for deteriorated sperm cells; (iv) lipid peroxidation is dramatically increased after freeze–thawing.

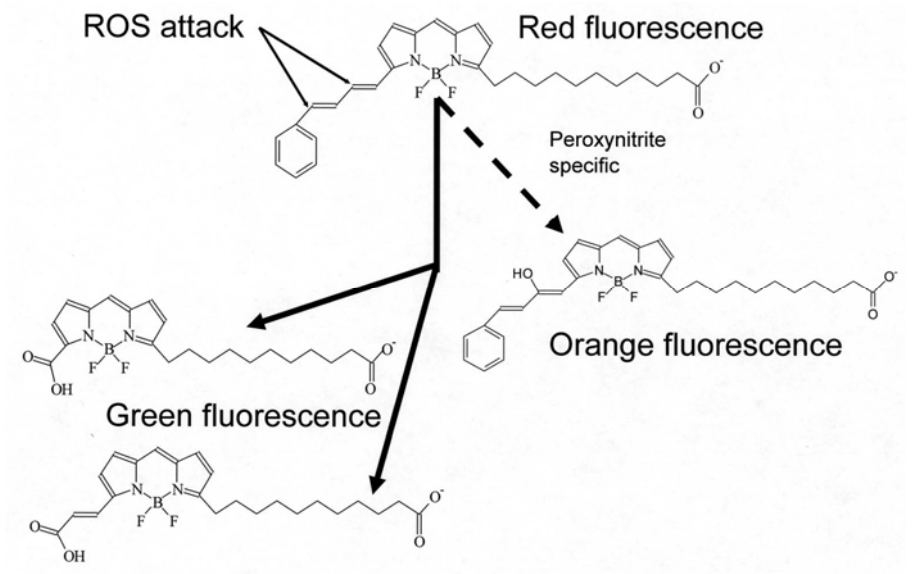

Fig. 5. Fluorescent properties of C_{11}-Bodipy$^{581/591}$ and its oxidation products. Note the specific formation of one particular oxidation with distinct fluorescent properties product after oxidation by peroxynitrite. Adapted from [56].

We have tested this probe on porcine sperm diluted in Beltsville Thawing Solution at 17°C (standard fresh semen dilution storage conditions for AI in The Netherlands) and in cryopreserved boar sperm [57]. Most sperm cells showed no

green formation of C_{11}-Bodipy$^{581/591}$. However, some of the cells showed green fluorescent mid-pieces (especially after freeze–thawing) while others show clear fluorescence on cytoplasmic droplets (Fig. 6). Taken together labelling sperm cells with C_{11}-Bodipy$^{581/591}$ is a useful approach both to quantify lipid peroxidation and to qualify lipid peroxidation (localization and subpopulation of sperm cells).

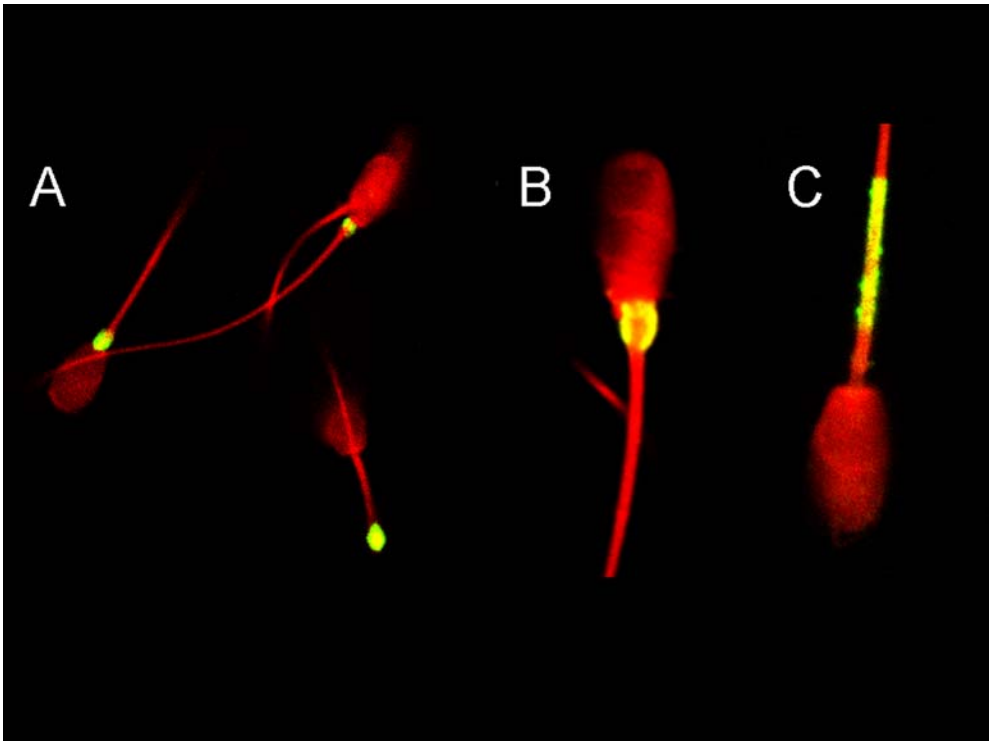

Fig. 6. Confocal fluorescent microscopic images of sperm cells labeled with C_{11}-BODIPY$^{581/591}$. In panel A, cytoplasmatic droplets in (freshly ejaculated) immature porcine sperm cells show intense lipid peroxidation. Panels B and C show extensive peroxidation in the midpiece and cytoplasmic droplets of frozen/thawed/washed porcine sperm cells.

5. Conclusions

This article describes new assays to detect endogenous lipid peroxides in spermcells, on one hand, and a very sensitive fluorescent fatty acid labelling method to quantify and localize this process per sperm cell. The relevance of the new information obtained with these new techniques and their implementation in male fertility research remains matter for the future.

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CHAPTER 3

Detection of damage in mammalian sperm cells

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Abstract

Ejaculated semen is washed for in vitro fertilization or diluted and processed to allow optimal and long-term low temperature liquid-and cryo-preservation. However, sperm are vulnerable to the washing, dilution, temperature and osmotic changes involved in sperm storage. In this review, a number of techniques are considered for detecting damaged spermatozoa. Staining protocols have been developed to detect the membrane and organelle integrity of mammalian sperm cells. Plasma membrane integrity is usually assessed after staining cells with membrane-impermeable dyes or alternatively with acetylated membrane (AM) permeable probes that are selectively de-esterified and become membrane impermeable and thus entrapped into viable cells only (AM ester loading). Organelle-specific dyes are commonly used to detect functionality of mitochondria or the acrosome. A distortion in the lateral and bilayer organization of lipids as well as the peroxidation of fatty acid moieties can be quantified and localized in living sperm. The relation of a disordering in the sperm membrane's lipid architecture and sperm deterioration versus capacitation is discussed. Finally, the integrity of sperm DNA can be measured at three different levels by assessing the degree of DNA-protamine condensation, the incidence of breaks and nicks in the DNA and the frequency of fragmentation of the nuclei into sub-haploid apoptotic bodies. The relevance of detecting DNA aberrations and especially the putative link to the incidence of apoptosis is critically considered.

Keywords: Sperm deterioration; DNA; Acrosome; Plasma membrane; Mitochondria; Lipid peroxidation; Sperm storage; Cryo-preservation; Capacitation; Fertilization

1. Introduction

Ejaculated sperm is in the middle of its journey towards the oocyte. Sperm is formed in the testis[1,2] and matured in the epididymis[3,4]. After ejaculation sperm should reach the isthmus part of the oviduct at the ipsilateral side where ovulation takes place[5,6,7]. At this side the sperm that have been activated (capacitation, for review, see[8,9,10]) may bind to the extracellular vestment of the oocyte (the zona pellucida[10,11]). This zona binding induces the acrosome reaction and the hyper-active motile sperm can drill through the zona pellucida (see [9]). Finally, sperm cells will bind to and fuse with the oolemma[12]. After fertilization the condensed DNA of the sperm is unfolded and a male pronucleus is formed that fuses with the female pronucleus so that a diploid genome unique to the new individual is formed[13,14]. Thus, for a successful conception the fertilizing sperm should have functional competent membranes, organelles and an intact haploid genome. In assisted reproductive techniques (ART), such as artificial insemination (AI)and in vitro fertilization (IVF), sperm is not directly introduced in the female genital tract at ejaculation but first collected in a bottle or tube either by the use of an artificial vagina, by manual stimulation or by electro-ejaculation (for overviews of sperm collection techniques, see[15,16]). The collected sperm is washed, diluted and, for storage purposes, eventually cooled and stored for extended time prior to (i) its introduction in the female genital tract (AI)[15,16], (ii) addition to a fertilization well with a mature oocyte (conventional IVF)[16] and (iii) selection of one morphologically perfect motile sperm for injection into the oocyte (intracytoplasmic sperm injection ICSI)[17,18]. All sperm processing steps may introduce damage to sperm DNA, membranes and organelles. In order to be able to obtain better fertilization results it is necessary to assess the quality of sperm just before their use in ART. This review provides an update of assays developed to monitor deterioration in sperm membranes, organelles and DNA. We have focused on protocols that make use of fluorescent probes measurable in a flow cytometer for high-throughput, objective and repeatable multi-parametric assessment of sperm deterioration. The working principles of the stains used for assessing certain sperm deterioration are reviewed. Finally, the relevance of the fluorescent staining protocols for predicting the quality of semen is discussed.

2. Membrane and organelle integrity

2.1. Plasma membrane

The plasma membrane surrounds the entire sperm cell holding together its organelles and intracellular components and by its semi-permeable features maintains the chemical gradient of ions and other soluble components. Specific plasma membrane proteins facilitate transport of glucose and fructose from the extracellular environment into the sperm[19,20,21]. These transporters are indispensable energy source substrates: in the mature sperm approximately 90%

of ATP is produced by glycolysis (anaerobic) indicating the importance of monosaccharides as substrates for ATP production[22,23]. If the sperm plasma membrane is not functionally intact the sperm is considered deteriorated (dead) and in vivo is not capable to fertilize. The latter is not valid for ICSI in oocytes where freeze-dried and rehydrated sperm (thus without a functional plasma membrane) can been used to produce off-spring[24,25,26]. However, for other ART like conventional IVF and AI it is essential to assess plasma membrane integrity of sperm. Plasma membrane integrity is usually assessed after staining cells with membrane-impermeable dyes. Those cells that are capable to exclude these dyes can be considered to be alive. An array of membrane-impermeable fluorescent probes with affinity for DNA is currently used for this purpose. Different probes that work on this principle have been tested for sperm cells each with distinct excitation (ex) and emission (em) properties: Hoechst 33258 (ex/em of 358/488 nm wavelength[27,28]); YoPro-1 (ex/em of 488/515 nm wavelength [29]); propidium iodide[30,31] or ethidium homodimer-1[32] (both have an ex/em of 488 and 568/ >620 nm wavelength); ToPro-3 and TOTO (ex/em of 647/670 nm wavelength[33]). An alternative (or simultaneous) way to study membrane integrity is the use of acylated membrane dyes (AM loading). By virtue of their acetyl moieties these membrane probes are amphipathic and thus can pass the intact membrane and enter the living sperm. Entered probes are immediately deacylated by intracellular esterases leaving the probe membrane impermeable. Thus, living sperm cells will get loaded with probes that become entrapped in the cell, whereas, the entered probes easily leak out of deteriorated cells with damaged membranes. Fluorescein diacetate and carboxy(methyl) derivatives have been used as AM loading probes to stain viable sperm[31,34]. Recently, a new AM loading dye (SYBR-14) has been introduced into spermatology[35,36]. Once entered the living cell, SYBR-14 deacylation also results in binding of this probe to DNA rendering this probe with fluorescent properties (ex/em 488/515 nm wavelength). For principles of each of the above-mentioned DNA binding sperm viability probes, see Fig. 1. It is possible to color living cells with SYBR-14 (green fluorescent) and deteriorated cells with ethidium homodimer or propidium iodide (red fluorescent)cells simultaneously. In fact, such combination of stains is available as the LIVE/DEAD Sperm Viability Kit. This double staining kit for sperm plasma membrane integrity is handy when working with frozen/thawed sperm. The combination of dyes enables flow cytometric staining immediately after freeze-thawing without any laborious processing step (like diluting and centrifuging) that may affect sperm integrity in between[37]. Another neat positive effect is that non-sperm particles, deliberately added to get better cryopreservation media (like egg-yolk vesicles or lipid particles from milk), do not have to be separated from sperm before flow cytometric assessment of sperm plasma membrane integrity. Those added particles normally

interfere with flow cytometric analysis of sperm cells, but since theydo not contain DNA they will remain unlabeled for both dyes. In contrast, both living and deteriorated sperm always will pick up one of the two DNA binding probes and the detected fluorescent emission predicts their viability status (see Nagy et al.[37]). An alternative way to test the functionality of sperm membranes is to subject them to a hypo-osmotic environment. For an overview of this so-called hypo-osmotic swelling (HOS) test, see Refs.[38,39,40,41,42].

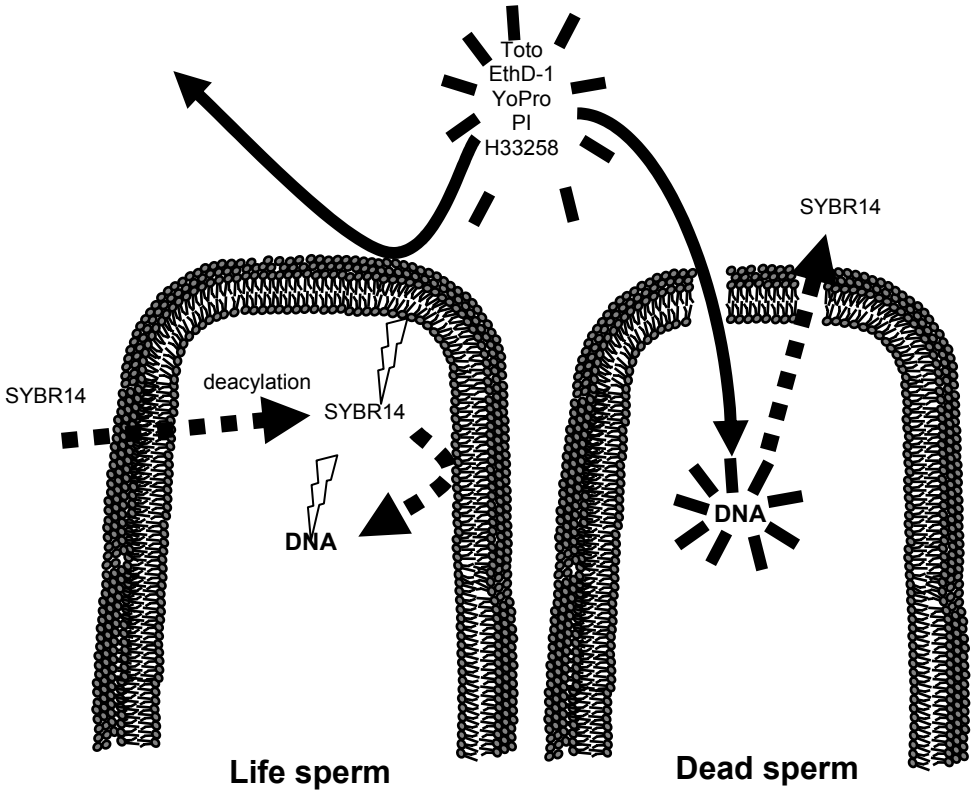

Fig. 1. Discrimination between intact and plasma membrane damaged sperm using DNA affinity probes. DNA of life sperm can be stained with the AM probe SYBR-14. This membrane permeable DNA binding probe is deacylated by intracellular esterases. The deacylated probe cannot diffuse back over the plasma membrane out of sperm. The entrapped fluorescent probe stains the sperm nucleus as it has weak affinity for DNA. In sperm with deteriorated plasma membranes SYBR-14 will leave the cell. DNA of deteriorated sperm can be stained with membrane-impermeable fluorescent DNA staining probes. These probes are not entering life cells but diffuse into the sperm nucleus to bind DNA once the plasma membrane has become deteriorated.

2.2. Acrosome

The acrosome is a large Golgi/ER derived acidic secretory organelle. It is filled with hydrolytic enzymes that are organized in a kind of enzyme matrix and most enzymes are heavily glycosylated[43]. Initial sperm-zona binding will trigger the acrosome reaction resulting in the release and activation of acrosomal enzymes. This together with the acquired hyper-activated motility will help the sperm to penetrate the zona pellucida[44]. The acrosome must remain intact before and during the transit of the sperm to the isthmus until zona binding has been accomplished. Early acrosome reactions render sperm infertile, and therefore, it makes sense to assess acrosome integrity before ART. Acrosome integrity is commonly measured with fluorescent conjugated lectins. The lectin conjugates bind to specific carbohydrate moieties of glycoproteins that are exclusively localized in the acrosome. Depending on the mammalian species the most commonly used lectin conjugates used are Pisum sativum (green pea; PSA), or of Arachis hypogaea (peanut; PNA) although for human sperm concanavalin A lectins (conA) can be used as well[45]. The ultra-structural localization of lectin conjugates can be followed after immuno-gold labeling of ultra-thin cryo-sections of sperm. For porcine, equine and canine sperm PNA labeling was specific for the outer acrosomal membrane[46,47,48], whereas, PSA was labeling acrosomal matrix glycoproteins [48]. Both conA and PSA also bound to some extent to the sperm plasma membrane [48,49]. Acrosome-specific lectins can be conjugated with an array of fluorescent groups. For sperm the use of PNA-FITC (ex/em 488/515 nm wavelength[467]), PNA-TRITC (ex/em 568/590 nm wavelength[50]) and PNA-RPE (ex/em 488/620[51,52]) wavelength are described in literature. Detection can be done on living sperm: the absence of fluorescence is indicative for an intact acrosome, and fluorescence is indicative for acrosome disruption or acrosome reaction. This technique is commonly used in flow cytometry[53] or in life cell imaging microscopy[52]. When acrosome staining is carried out on fixed and permeabilized sperm, full fluorescent acrosomes are considered to be intact[32], whereas, acrosomes with lower, patchy or equatorial band fluorescent staining show signs of acrosome disruption or reaction. With the latter technique it is possible to show the degree of acrosome reactions of sperm that have bound to the zona pellucida at a given time[54]. Similar to the lysosome, the acrosome is an acidified organelle with an internal pH of 5. Therefore, specific probes normally used to stain lysosomes, such as Lysotracker Green TM (ex/em 488/515 nm wavelength), can be used to specifically stain the sperm acrosome[55,56]. A variety of other Lysotracker dyes (ex/em Orange TM488/550, Red TM 568/590, Deep Red TM 628/650 nm wavelengths) are available with different absorption and emission spectra enabling combinations with other dyes for multi-parametric fluorescent assays (see Section 2.4). Antibodies raised against specific intra-acrosomal proteins can also be used for acrosome integrity assessments. The immunolabeling of the inner acrosomal membrane protein CD46 has been described for this purpose for a number of different mammals (for references, see Fig. 2). The principles of acrosome staining methods are summarized in Fig. 2.

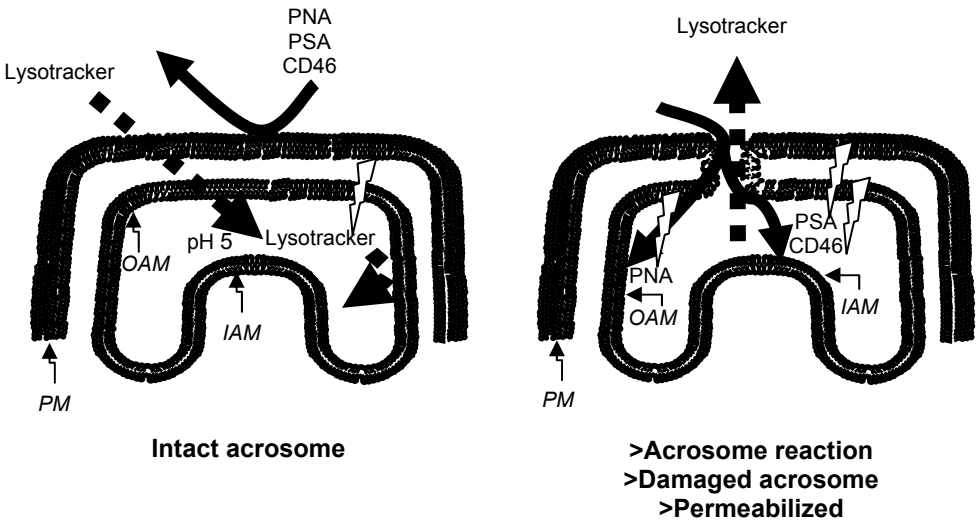

Fig. 2. Discrimination between intact and reacted (or deteriorated) acrosomes. Intact acrosomes can be stained with Lysotracker™ dyes. These dyes fluoresce at pH 5.0 and accumulate in acidic organelles, such as the acrosome. Once the acrosome in not anymore intact the pH becomes neutral and the Lysotracker dye can diffuse out of the acrosome. Lectins (PNA, PSA and others) and antibodies (antiCD46 indicated as CD46) raised against intra-acrosomal (glyco)proteins can be used to discriminate intact and deteriorated/reacted acrosomes either using unfixed sperm or in permeabilized sperm (see Section 2.2). Intact are impermeable for lectins and antibodies, whereas, acrsome reaction or disruption allows entry of those macromolecules. PNA binds to a membrane glycoprotein of the outer acrosomal membrane (OAM), PSA binds to a component of the enzyme matrix in the lumen of the acrosome while antiCD46 binds to a membrane protein of the inner mitochondrial membrane [134,135]. In unfixed cells lectin and antibody staining is indicative for acrosome disruption/reaction. In permeabilized sperm only full fluorescence of the acrosome reflects an intact acrosome and diminished signal indicates the loss of acrosomal content.

2.3. Mitochondria

Sperm mitochondria are localized in the mid-piece area enrolled over the principal part of the flagellum. Mitochondria produce ATP by oxidative phosphorylation. However, the importance of mitochondria for sperm motility (an ATP consuming process) is recently reconsidered.

(i) Freshly ejaculated sperm largely produce ATP by glycolysis (>90%) even in buffers that are in contact with air (16% oxygen)[23].

(ii) The ATP-dependent tubulin sliding, responsible for sperm motility, takes place at the distal part of the flagellum in the tail. Thus, ATP consumption is rather far away from the site of aerobic ATP production.

(iii) The fibrous sheath of the tail contains enzymes involved in glycolysis (anaerobic ATP production) and knock out mice that fail to express one of these enzymes are immotile[57].
(iv) Sperm subjected to chemical drugs that uncouple oxidative phosphorylation from the electron carrier chain in the inner mitochondrial membrane (IMM) remain motile and alive[22].
(v) Paternal mitochondria are not involved in any event after fertilization and are degraded by the fertilized oocyte[58,59]. Nevertheless, mitochondria are thought to provide the mid-piece and the sperm head with ATP required for housekeeping processes of membranes. Among them one of the most important and ATP consuming housekeeping process is to maintain the Na^+/K^+-gradient over the plasma membrane. The Na^+/K^+-ATPase involved also indirectly drives other transporters and thus regulates the chemical and electric gradient over the plasma membrane. The functional integrity of mitochondria may thus be important for sperm survival in the female genital tract or during ART. Mitochondria can be stained with specific dyes for these organelles (for an overview, see Ref.[60]). Originally, Rhodamine 123 was used to selectively stain functional mitochondria. The principle of this probe is that it only fluoresces red when the proton gradient over the IMM is built up (Rhodamine 123 is a potentiometric membrane dye). This proton gradient is a result of an active electron carrier chain and is coupled to oxidative phosphorylation: the protons flowing back through the ATP synthase to the matrix of the mitochondrium are driving the aerobic ATP production. In unstained mitochondria the proton gradient has collapsed and consequently aerobic ATP production has ceased. Thus, unstained sperm do not contain functional mitochondria, whereas, positive stained cells are aerobically functional[61]. Remarkably, an individual sperm either has a fully fluorescent mid-piece or is not fluorescent indicating that depolarization of the IMM is an orchestrated event occurring at once over the entire mid-piece[22]. The newly developed Mitotracker Deep Red TM (628/650), Mitotracker Red TM (568/595), Mitotracker Orange TM (488/550) and Mitotracker Green TM (488/515) (ex/em in nm wavelengths, respectively) also selectively label the respirating mitochondria and can be used for multi-parametric sperm assessments (see Section 2.4). Mitotracker Orange CM-H_2TMROS (551/576) and Mitotracker X-Rosamine CM-H_2XROS (578/599) (ex/em in nm wavelengths, respectively) become fluorescent after oxidation, a process that only takes place under oxidative respiration. Since this process is only relevant in functional mitochondria these probes are suitable to discriminate sperm with deteriorated mitochondria from aerobically capable sperm [52,62]. For mitochondrial staining principles, see Fig. 3.

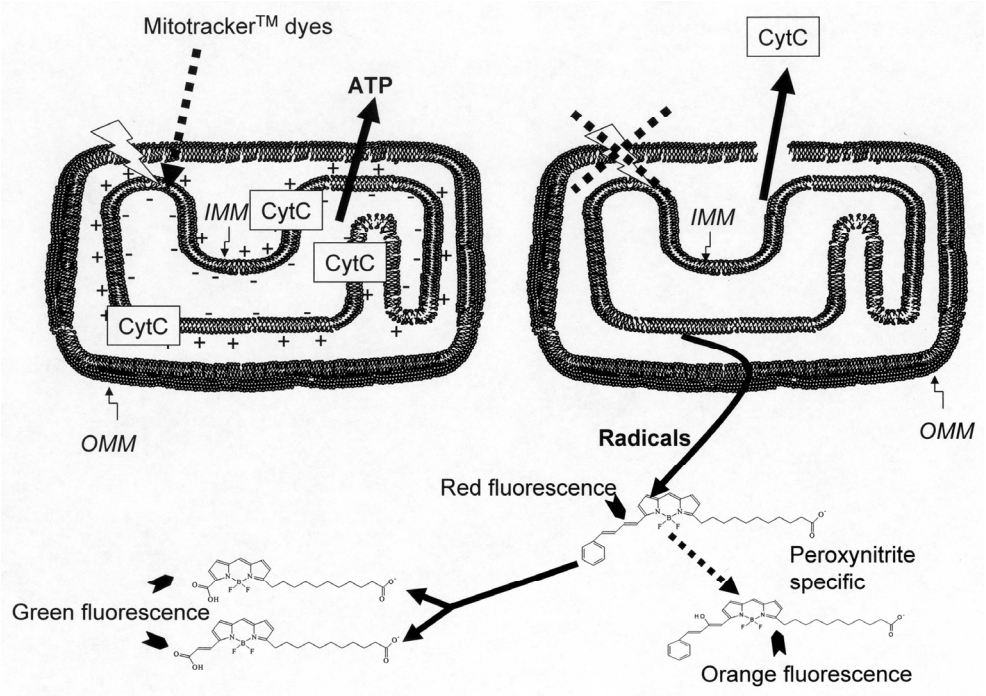

Fig. 3. Detection of respiratory status of sperm mitochondria. As detailed in Section 2.3, aerobe metabolism uses the build up of a proton gradient to couple the electron transport to oxygen (to form water) with the production of ATP by the ATP synthase. The potential of the inner mitochondrial membrane (IMM) can be sensed by potentiometric dyes (Mitotracker™ dyes) that diffuse over the plasma membrane and the outer mitochondrial membrane (OMM) and accumulate in the IMM. Some of the Mitotracker dyes only fluoresce when intercalated in the IMM's potential gradient. Others only fluoresce after interacting with radicals that convert them to fluorescence. For principle of JC-1 staining, see Section 2.3. Once the mitochondria loose their integrity the membrane proton gradient is lost and thus ATP production. Furthermore, cytochrome C (CytC; one of the electron transport chain components) is lost and diffuses out of the mitochondrion. As a consequence respiration is blocked and Mitotracker dyes do not sense the IMM potential. Radicals are no longer kept within the matrix of the mitochondria but can oxidize environmental molecules. C_{11}BODIPY$^{581/591}$ (see Section 2.6) can be used to detect lipid peroxidation in the sperm plasma membrane. The red fluorescent probe can peroxidize into the three structures indicated resulting in a green shift in the probe's emission properties.

Recent developments have resulted in new applications for the so called Mitotracker dyes. (i) After staining sperm, the suspensions can be fixed and remain stained specifically on mitochondria[63]. (ii) Some of the Mitotracker dyes like JC-1 change their fluorescent properties due to changes in the potential of the IMM (JC-1 switches from orange fluorescence in the aerobic functional mid-piece towards green fluorescent after IMM depolarization)[64,65]. JC-1 can be used to report depolarization of the IMM and thus to report on mitochondrial

functionality. The wide spectral range of available fluorescent Mitotracker dyes make these probes applicable for multi-parametric fluorescent assays (see Section 2.4). An alternative way to detect proper ATP production is to observe sperm motility characteristics by computer assisted sperm motility analysis (CASA) (for a recent review, see Ref.[66]).

2.4. Multiple sperm parameter staining

Obviously, the color palette available for preparation of fluorescent conjugates of proteins (for instance, lectins and antibodies) and for each of the mentioned types of fluorescent sperm integrity probes allows multi-parametric sperm staining[37]. For example, it is possible to stain sperm cells simultaneously with four dyes to discriminate:
(i) viable sperm from plasma membrane deteriorated sperm (using PI and SYBR-14), (ii) acrosome integrity using PNA-PE, (iii) functional status of mitochondria using Mitotracker Deep Red TM and (iv) non-sperm events[37]. The wide range of spectral variations of each class of membrane and organelle probes makes it possible to design assays to detect multi-parametric features of sperm deterioration. The sperm properties can be measured after staining frozen/thawed sperm immediately after thawing. No washing and fixation steps are required and very accurate data can be read within 10–20 s when using a flow cytometer equipped with a blue (argon 488 nm wavelength) and a red (helium/neon 638 nm wavelength) laser at a speed of approximately 10,000 sperm/s.

2.5. Lipid organization

The capacitation status of sperm usually is assessed with the fluorescent antibiotic chlortetracycline (CTC). This membrane probe binds to the sperm plasma membrane in a divalent cation-dependent manner thus forming highly fluorescent complexes of CTC with Ca^{2+} and Mg^{2+} ions bound to membranes[21]. In somatic cells the probe can be intracellularly transferred by endocytosis and consequent vesicle mediated membrane transport towards membranes of organelles. Therefore, CTC is used to detect Ca^{2+} ordering to certain organelle membranes[21,22]. Viable sperm cells become labeled with CTC at those parts of the surface membranes where Ca^{2+} is present above a certain threshold concentration to allow CTC immobilization. In intact non-capacitated sperm this results in a plasma membrane confined F-pattern, an overall staining of the sperm head. This pattern changes into a B-pattern for capacitated sperm, a more prominent staining of the apical area of the sperm head and decreased staining at the posterior area of the sperm head[69]. Finally, the AR pattern emerges indicating that sperm has induced the acrosome reaction[70]. Those cells have a characteristic loss of CTC staining at the apical head area probably by the removal of the mixed acrosome plasma membrane vesicles generated during the acrosome reaction. Nowadays, CTC is routinely used to simultaneously assess the incidence of sperm capacitation and acrosome reactions[71] and the staining protocol has been recently adapted for flow cytometric analysis.

The flow cytometer can only discriminate different amounts of fluorescence but to a lower degree can monitor the distribution of that fluorescence per sperm particle. It is not clear whether the amount of CTC fluorescence of pattern B differs from pattern F or that only AR patterns show a decrease in CTC staining[72]. We should note that the Ca^{2+}-dependency of CTC staining makes the probe useless for discriminating between the calcium dependent and independent pathways leading to capacitation[8,52]. Another interesting phenomenon with respect to the topic of this review is that the capacitation-specific B staining pattern for CTC has also been observed in frozen-thawed sperm[73,74,75,76,77]. Consequently, some authors proclaimed from this finding that freeze-thawing induces a capacitation-like response to sperm. The phenomenon speculatively was termed cryo-capacitation [78]. Recent published data place this hypothesis under debate[79,80]. Finally, it is noteworthy to mention that sperm can only very briefly be stained with CTC and need to be fixed immediately after this procedure. It remains unclear how the CTC-sensing of Ca^{2+} attachment to sperm membranes relate to the increase in intracellular free calcium supposed to take place during sperm capacitation enabling the acrosome reaction. An alternative method for assessment of capacitation is to stain sperm with the hydrophobic dye Merocyanine 540 (M540). M540 monitors a decreased packing order of phospholipids in the outer leaflet of the plasma membrane lipid bilayer[81,82], which allows its intercalation into the hydrophobic core of the membrane, this believed to occur in capacitated spermatozoa[8,83]. In the pig, capacitation brings about modifications in the surface of the spermatozoa[5], a collapse in the plasma membrane phospholipid asymmetry[29,51,52] and an increase in the ability of live acrosome-intact cells to bind zona pellucida components[85]. Under these conditions, M540 staining also increased[51] and the membrane fluidity changes detected by M540 preceded the changes picked up by CTC. This together with the fact that M540 stains sperm Ca^{2+} -independently makes M540 a more valuable stain for evaluating the early and Ca^{2+}-dependent events of capacitation[83]. Incorporation techniques that make use of labeled phospholipids analogs or labeled aminophospholipid-binding proteins can also be used to investigate the phospholipid changes occurring in the plasma membrane of spermatozoa. For example, 6-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)amino-caproyl (C6NBD)-phospholipids can be used to assess the degree of phospholipid asymmetry in the plasma membrane of the spermatozoon. C6NBD-phospholipids are used to show that boar spermatozoa maintain lipid asymmetry in their plasma membranes after ejaculation. The reported asymmetry is similar to that reported for other species of mammalian spermatozoa, as well as for other cell types[86]. Annexin V is a calcium-dependent phosphatidylserine (PS)-binding protein. When conjugated to a fluorochrome, such as FITC, annexin V permits the recognition of cells with exposed PS. In ejaculated sperm, or sperm that does not respond to in vitro capacitation treatments, PS is confined to the cytoplasmic side of the sperm plasma membrane[86]. Similarly, labeled Ro-09-0198 can be used to detect surface exposure of phosphatidylethanolamine (PE). Gadella and co-workers[52,62] used FITC-annexin V and labeled Ro-09-0198 to demonstrate that bicarbonate induces the exposure of PE and PS on the surface of boar stallion and human

spermatozoa. The findings were in agreement to those with the C6NBD-phospholipid analogs[51]. Thus, an array of probes can be used to monitor more disordered phospholipid arrangements in the plasma membrane bilayer during sperm capacitation. The principle signaling pathway behind the membrane alterations detected by the above-mentioned membrane probes is summarized in Fig. 4.

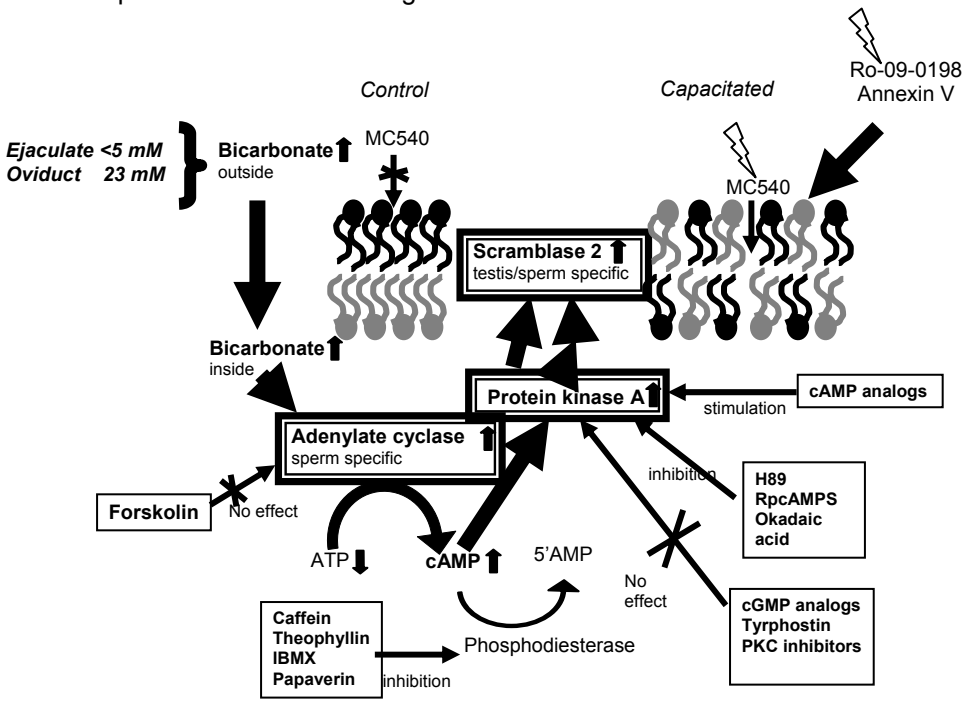

Fig. 4. Sperm signaling resulting in bicarbonate dependent lipid rearrangements in the apical sperm plasma membrane. Elevated bicarbonate levels of the oviduct fluids cause an influx of bicarbonate into the sperm cell. The protein kinase A dependent activation of phospholipids scrambling results in a change in lipid ordering in the sperm plasma membrane. The altered (capacitated) sperm lipid ordering allows MC540 intercalation. Therefore, MC540 can be used to discriminate non-responsive to bicarbonate responsive cells. The exposed aminophospholipids can be stained with Ro-09-0198 (specific for PE) or annexin V (specific for PS). The reordering of the sperm surface can also be assessed with the fluorescent antibiotic chlortetracycline although the principle of this staining is not completely understood. The pathway for lipid scrambling indicated is evidenced by the pharmacological effects of the drugs indicated on this process.

As mentioned above these changes might equally be linked to initial membrane damage during cryo-preservation[87,88,89] or to early capacitation-specific membrane alterations[8,51,52]. We should note here that the capacitation-related CTC, M540 and annexin V staining patterns predominantly show up on viable sperm cells if physiological conditions were maintained during experimentation. To guarantee this sperm suspension should be at 38.5 ^{0}C under humidified atmosphere and constantly in equilibrium with 5% CO_2 in air. Adaptations should

be made to allow equilibration and maintenance of these experimental conditions in the tubing of the flow cytometer and for the slide chamber of the inverted confocal microscopy equipment to get appropriate set ups to do life sperm imaging experiments. Under such conditions capacitating cells show only marginal deterioration within a period of 4 h incubation in IVF media[52]. In contrast, cooling to even 30 ^{0}C will cause membrane disruption in the capacitating sperm leading to cell death[8]. When working with frozen-thawed sperm the "capacitation-like" staining is almost exclusively for deteriorated sperm[88]. These artificial deteriorated cells remain stained and look like capacitated sperm. We have a possible explanation for these phenomena: both incubations cause disordered lipid packing in the sperm membrane. In capacitating cells these changes are required for preparation of the acrosome reaction and the higher fluidity of the sperm membrane at this temperature allows bilayer integrity. Freeze-thawing (and cooling of capacitated sperm) results in a further distortion and collapse of the sperm membranes as the lipid bilayer has become instable by the lowered temperature.

In retrospective we should note here that, as long as, the sperm remains viable every response to an IVF incubation has been considered as a sign of sperm capacitation. However, the relation of these changes to capacitation has not yet been functionally tested.

Do the changes relate: (i) to the ability of sperm to bind to the zona pellucida? (ii) To their priming for the acrosome reaction after this binding? (iii) To acquire hyper-active motility and thus to enable zona penetration? A relation between the detected changes and fertilization characteristics is required. The same is more or less also valid for the tyrosine phosphorylation response occurring in capacitated sperm as well as in frozen-thawed sperm [78,80,90]. As discussed above, membrane changes may alternatively indicate early signs of sperm membrane disruption in capacitating viable cells. Although the same membrane changes and tyrosine phosphorylation patterns have been observed for capacitated and frozen-thawed sperm, the deduced cryo-capacitation concept is under debate and we believe that this theory needs to be experimentally confirmed.

2.6. Lipid peroxidation

In our laboratory, new methods have been developed to assess lipid peroxidation in fresh and frozen stored sperm. Rather than measuring end products of lipid peroxidation (like the thiobarbituric acid reaction, where the formation of malondialdehyde can be detected) our new methods are designed to detect endogenous phospholipid and cholesterol peroxidation in freshly stored and frozen/thawed sperm. Assessments on mass spectrometry allow accurate quantitative and qualitative analysis of the composition of the main phospholipids classes (phosphatidylethanolamine, phosphatidylcholine, sphingomyelin and cholesterol[91]). Furthermore, lipid peroxidation can be followed, quantified and localized after labeling sperm with a lipid peroxidation reporter probe C_{11}BODIPY$^{581/591}$ which is a fluorescent analog for unsaturated fatty acids, the main targets for reactive oxygen species (ROS).

Essentially, C_{11}BODIPY$^{581/591}$ changes its fluorescent properties after peroxidation. The intact probe is red fluorescent but turns into green fluorescence when peroxidized by ROS and into orange when peroxidized by peroxynitrite[92]. This green and orange emission shift indicates the presence of reactive oxygen and nitrogen species in the hydrophobic part of lipid bilayers of sperm membranes. The ratio of green + orange fluorescence versus total fluorescence (an indication of the degree of probe peroxidation) correlates well with the degree of endogenous phospholipid peroxidation as measured with mass spectrometry[93]. The degree of probe peroxidation can be followed in particular sperm subpopulations using flow cytometry or localized in individual sperm using life cell imaging microscopy. With the aid of an inverted spectral confocal laser scanning microscope we showed that C_{11}BODIPY$^{581/591}$ is preferentially peroxidized in the mid-piece and (in immature sperm) at cytoplasmic droplets [91,93]. This is probably due to dysfunctions in mitochondrial respiration (see Fig. 3). Furthermore, in our laboratory it is demonstrated that washed frozen-thawed sperm is much less resistant to ROS stress than freshly diluted semen[94]. The relevance of the new lipid peroxidation assays and their implementation in male fertility research remains matter for the future.

3. DNA damage assessments

The integrity of the paternal DNA is of crucial importance for the further development of an embryo. The degree of DNA damage is clearly correlated with the impairment of embryo development and severe DNA damage cause male infertility[95]. However, IVF experiments with gamma irradiated sperm showed that sperm with severe DNA damage but remained functionally intact at the level of membrane and organelle and motility parameters. In fact, the DNA damaged sperm showed normal ZP binding characteristics and even the fertilization and cleavage rates of the fertilized oocytes remained normal. However, about all four to eight cell embryo stages initiated apoptosis[96,97,98]. Thus, the reproductive failure, caused by DNAaberrations, appears not at the level of fertilization but at the onset of embryonic DNA expression. DNA damage can be measured at different levels and for sperm DNA mainly three different approaches are currently used.

3.1. DNA condensation

Firstly, the DNA of matured sperm cells (probably the fertilization competent subpopulation) is extremely highly condensed on protamines in a toroid structure. The DNA loops around 500 times around the DNA/protamine toroid structure and extends to approximately 50,000 base pairs per toroid [99]. The sperm's head contains about 50,000 of such structures (for arrangement of DNA-protamine toroids in the sperm head, see Refs. [99,100,101,102,103]). Condensation takes place during spermatid development where histones are

removed from nucleosomes by transition nuclear proteins[104,105,106]. The stripped DNA is coated with protamines and repacked in late-step spermatids in two transition phases [100,107,108,109]. Proper condensation probably stabilizes the DNA and makes it less vulnerable for oxidative damage. However, repair of DNA damage is not possible in the mature sperm [110]. The condensation status of individual sperm cells can be assessed using transmission electron microscopy (TEM). Condensed nuclei appear homogeneously black in contrast to non-condensed nuclei[111,112] and this appearance in TEM is achieved only in late spermatids during the last steps prior to spermiation. Using a single-cell DNA gel electrophoresis assay (COMET) discrimination can be made between fluorescently labeled DNA of normally condensed sperm nuclei (minimal migration) and more loosely packed DNA (tailing of DNA) after allowing DNA migration on an agarose gel under an electric field. This tailing of DNAwas noted for mice with low expression of protamine 2 showing the protamine dependency for late spermatid DNA condensation[113]. Protamines are the key proteins involved in final condensation of sperm DNA[101,102,103,104,105,114,115]. Chromomycin A3 (ex/em of 440 and 470 nm wavelength) can be used to follow the last compaction steps of DNA to protamines. This fluorescent dye binds to deprotaminated DNA (at GC specific regions) but fails to do this after protamination of DNA[116]. The probe is used to detect protamination defects in sperm[117]. The incidence of chromomycin A3 staining on human sperm appears to correlate with the incidence of sperm nicks[118] (see Section 3.2).

3.2. DNA breaks and nicks

The second level is to detect whether sperm DNA is double stranded (intact) or whether single stranded DNA (damaged, for example, in nicks) are formed. Acridine orange can be used to stain single stranded DNA (red fluorescent) and double stranded DNA (green fluorescent). The degree of red acridine orange staining of sperm DNA has been showed to correlate with male infertility in the sperm chromatin structure assay (SCSA, see the contribution in this volume of Dr. Evenson, for further literature, see Refs[119,120]). Another method to detect DNA damage is to allow enzymatic incorporation of fluorescent nucleotide analogs by a terminal nucleotide transferase into single stranded DNA areas. TUNEL: fluorescent sperm cells contain single stranded DNA that were labeled by dUTP nick-end labeling (TUNEL) at the 3-OH termini[86,95,121,122]. The proportion of $TUNEL^+$ cells appears to correlate well with decreased pregnancy rates using ART[122]. The proportion of $TUNEL^+$ cells may increase after cryo-preservation[4] although this is contradicted by Duru et al.[123]. Interestingly, the mature and motile human sperm fraction resulting from a swim-up procedure only contained <0.5% $TUNEL^+$ cells[110] indicating that DNA damage is limited in functional spermatozoa. Long-term intensive exposure of sperm to UV light does induce DNA strands and breaks (see Fig. 5). Irrespective of DNA damage, sperm will remain capable to fertilize and activate the oocyte followed by the first cleavages. However, embryo development is blocked after reaching the four to eight cell stages[97,98]. In situ translation of

fluorescent nucleotide to nicks with a template dependent DNA polymerase (ISNT) has not been used as alternative to TUNEL to detect DNA damage in sperm cells.

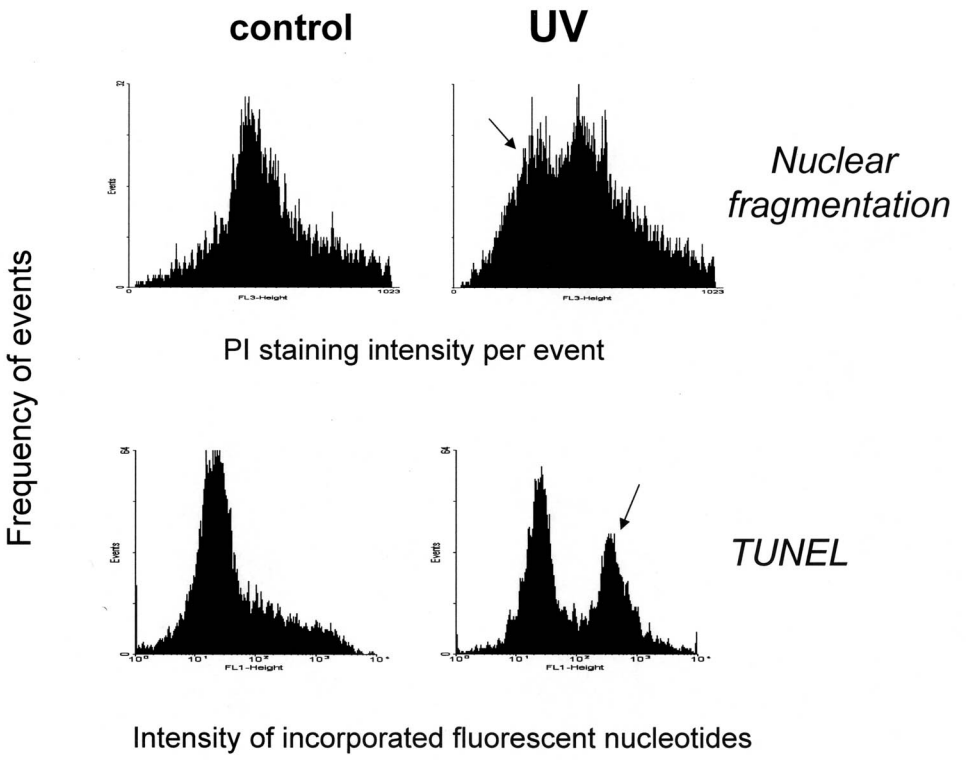

Fig. 5. Detection of DNA damage in human sperm by an hour UV light illumination. Histograms showing the relative distribution of the amount of PI staining (nuclear fragmentation) or amount of fluorescent nucleotide incorporation (TUNEL) per sperm are indicated. Left panels represent control sperm whereas right panels are the sperm stained after UV exposure. The arrow indicates the fragmented sperm nuclei (upper panel) or incorporation of fluorescent nucleotides (lower panel) both resulting from the UV light induced DNA damage.

Another method currently available is estimation of DNA damage, translated by the presence of 8-oxoguanine, as a result of free radical attack[125,126,127]. The assay is based on addition of a fluorescent labeled 8-oxoguanine binding protein to fixed cells. The yellow fluorescence indicates the presence of 8-oxoguanine. Although this assay is not yet widely used in sperm it may prove a useful tool in ART. COMET (see Section 3.1) and TUNEL assays correlate well with the amount of DNA oxidation and all three responses can be induced by reactive oxygen species[126,128].

3.3. Nuclear fragmentation

The third level to detect DNA damage is to look at nuclear/DNA fragmentation (the latter is defined as a result from double strand breaks in DNA. DNA fragmentation is (unlike the so far mentioned DNA damage) supposed to be specific for apoptosis. DNA fragmentation in apoptotic cells is elicited by effector caspases 3, 6 and 7 that become activated by the caspase cascade during the execution phase of this process (for reviews [129,130]). Briefly, the apoptotic nucleus condenses and falls apart into apoptotic bodies due to caspase 6 mediated breakdown ofthe nuclear lamina (proteolysis oflamin)andto other effector capsases involved in degrading PARP and in activating domain nucleases and DNases. The resulting nuclear fragments can be imaged by TEM or a fluorescent microscope after staining the sperm with a DNA probe. The double strand breaks generated during cell apoptosis by caspase activated DNases (CAD)are situated between the histones at the so-called interhistone DNA regions. CAD is activated by the effector caspases 3 and 7 that become activated by the apoptotic machinery. DNA from apoptotic cells will after loading and electrophoretic separation appear as a typical DNA ladder on an agarose gel when illuminated with UV light. The discrete DNA bands represent the amount of nucleosomes that were holding the DNA fragment. This DNA ladder is specific for apoptotic somatic cells. We may note that TUNEL staining is not specific for cell apoptosis and can also be detected during cell necrosis. In somatic cells the chromatin breakdown is accompanied with the chromatin condensation into apoptotic bodies representing nuclear fragments and this process can be visualized under EM or by fluorescent DNA staining. However, the super-compression of DNA in mature sperm makes further condensation spatially impossible (see Section 3.1) and caspase-mediated breakdown of the sperm DNA has so far not been described. In presence of Triton X 100 (a detergent that disrupts sperm membranes) and 20 mM $MgCl_2$ endogenous sperm DNases actively breakdown the proportion of human sperm DNA that links the DNA-protamine toroids and this process can be stimulated by the addition of DNase I[9]. However, the DNA-protamine toroids remain intact during such experiments. Thus, DNase treated sperm nuclei fall apart into multimeres of 50 kbp DNA-protamine toroids rather than the apoptosis-specific histone-DNA multimeres. It is questionable whether this inter-toroid DNA breakdown takes place during sperm deterioration. When instant (freeze-dried) sperm is rehydrated it will have severe membrane damage but can successfully be used for ICSI[24,25,131,132,133]. Nevertheless, nuclear fragmentation has been observed in ejaculated human sperm and can be induced by UV light illumination of sperm suspensions (see Fig. 5 or Ref. [62]). We believe that either the non-condensed DNA in aberrant (immature) sperm is selectively destructed by this UV irradiation or that irradiation selectively disrupts DNA segments that are connecting the DNA-protamine toroids.

4. Conclusions

Sperm deterioration can be measured at the membrane and organelle level and an increase of sperm with compromised membranes/organelles will lead to reduced fertility rates. Nevertheless, freeze-dried and dehydrated sperm (i.e. without functional membranes) can still be used for ICSI as DNA remains condensed and intact during such treatment. Sperm deterioration at the DNA level may not affect fertilization rates as long as sperm membranes and organelles remain functionally intact. However, the DNA damage leads to a reduced embryo development after the onset of the embryonic genome. Thus, on one hand, intact functional sperm membranes are essential to achieve fertilization in vivo, but do not essentially contribute to later processes after conception. On the other hand, sperm DNA has no function in achieving fertilization but becomes importantly involved in embryonic development from the onset of embryonic DNA expression (i.e. after the first cleavages). Thus, both deterioration of sperm membranes and DNA are probably important factors involved in male subfertility or infertility. The hypothetical concepts of sperm apoptosis and sperm cryo-capacitation should be reconsidered and experimentally further tested. In this light, special attention should be paid to the unique molecular organization and functioning of sperm membranes and DNA condensation, respectively. Finally, objective high-throughput multi-parameter sperm assessments (preferably with the aid of flow cytometric detection of fluorescent staining) provide statistically stronger data, which will be required for future studies to be able to get correlations between sperm quality parameters and fertility results. This may lead to industrial applications, such as selecting top male animals with respect to high quality sperm for breeding programs.

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CHAPTER 4

Cholesterol oxidation in sperm cell membranes

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Submitted

Abstract

We report on the presence and formation of cholesterol oxidation products (oxysterols) in mammalian sperm. Although cholesterol is the most abundant molecule in the membrane of mammalian cells and is easily oxidized, this is the first report on cholesterol oxidation in membranes of living cells as investigated by state of the art liquid chromatographic and mass spectrometric methods. It could be demonstrated that oxysterols are already present in fresh semen samples that are commonly considered to be unperoxidized, showing that lipid peroxidation is part of normal sperm physiology. After chromatographic separation (by HPLC), detected oxysterols were identified with atmospheric pressure chemical ionization mass spectrometry (APCI-MS). Secondly, we show that freeze/thaw procedures, routinely performed in animal reproductive biology, do not result in significant elevation of oxysterol levels. Thirdly, it was found that exposure of sperm cells to oxidative stress does not result in the same oxysterol profile as obtained after exposure to oxidative stress of reconstituted, protein-free membranes from sperm cell lipids, suggesting that living sperm cells do metabolize oxysterols or control oxysterol formation in another way. We hypothesize on the biological consequences of the occurrence of these (toxic) oxysterols in sperm cells.

Keywords: Oxysterols, sperm, cholesterol, mass spectrometry, lipid peroxidation.

Introduction

Lipid peroxidation in sperm cells has received much attention over the past years and beneficial as well as detrimental effects have been attributed to lipid peroxidation, resulting in the idea that pro- and anti-oxidant systems need to be carefully balanced for optimal sperm functioning [1,2,3]. It is evident that extensive peroxidation damages sperm cells, as lipid radicals involved in propagation of the lipid peroxidation chain reaction (see [4] for an overview of the radical mechanisms involved) are rather indiscriminate towards the biomolecules they target. This extensive peroxidation will ultimately lead to functional impairment of proteins and DNA. One of the conditions that may induce extensive lipid peroxidation is the freeze/thawing process that is routinely performed in livestock breeding industries and human reproductive medicine. In a recent study, we confirmed that a freeze/thawing procedure induces peroxidation and we were able to visualize this process in bovine sperm. In the same study we demonstrated the existence of oxidatively modified phospholipids [5]. However, the fate of the most abundant lipid molecule in sperm membranes, cholesterol, has remained unresolved.

In the food industries the oxidation of lipids is also of great importance, as the quality of lipid-rich foods diminishes drastically after the onset of lipid peroxidation. Analysis of cholesterol and its oxidation products is an attractive way to assess the quality of these products, as i) cholesterol is abundantly present in lipid rich foods from animal origin, ii) cholesterol is prone to auto-oxidation as well as radical induced oxidation [6,7,8,9], thereby forming an excellent sensor of lipid peroxidation and iii) several oxysterols species are toxic [10,11,12,13]. Although there are over 30 different oxysterols known, only a few are quantitatively important [7,14]. Oxysterols can be sensitively analysed and quantified by liquid chromatography (LC) combined with atmospheric pressure chemical ionisation (APCI) mass spectrometry (MS) [9,15].

Cholesterol is the most abundant molecule in sperm cell membranes [16], and it stands to reason to apply the analysis of oxysterols to determine the extent of lipid peroxidation in sperm cells. Sterols have a function in sperm membrane stability and play an important role in sperm membrane organisation and physiology [16,17,18]. Therefore, assessment of its oxidation status is also of physiological significance. This approach enables the investigation of the intriguing question whether or not sperm cells do metabolize oxysterols, in an attempt to counteract the toxic actions of these components. Here, we show that sperm cells do indeed contain oxysterols, even directly after ejaculation. We investigated whether the freeze/thaw procedure alters the sensitivity of sperm cells to induced lipid peroxidation (mimicking the exposure of sperm cells to ROS after insemination). Furthermore, we demonstrate that there is an apparent metabolism or control of formation of

oxysterols by the sperm cell, as the relative content of the different oxysterols clearly differs from that of a cell- and protein free lipid extract exposed to oxidative stress.

Materials and Methods

Chemicals

All chemicals, including reference lipids, were obtained from Sigma Inc. (St Louis, MA) and of the highest purity available unless stated otherwise. Solvents (acetonitrile, chloroform, methanol and hexane) were of HPLC grade and obtained from Labscan (Dublin, Ireland).

Sample preparation

Bovine ejaculates were collected using an artificial vagina and immediately processed to generate either fresh sperm samples or frozen/thawed sperm samples. For fresh sperm samples, ejaculates were washed over an discontinuous Percoll(tm) gradient as described previously [19]. Pelleted cells were subsequently resuspended in Hepes buffered Tyrodes (HBT: 120 mM NaCl, 21.7 mM lactate, 20 mM Hepes, 5 mM glucose, 3.1 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM pyruvate, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, and 100 µg/ml kanamycin; 300 mOsm/kg, pH 7.4, supplemented with 0.5 mg/ml polyvinylalcohol and 0.5 mg/ml polyvinylpyrrolidone), spun down at 750x g to remove any remaining Percoll(tm) and finally resuspended in HBT at a concentration of approximately 50 million cells per ml. These cells are further referred to as 'fresh cells'.

Frozen/thawed sperm samples were generated by resuspension of ejaculated cells in a freezing buffer and frozen in 0,25 ml straws in liquid nitrogen according to the method of van Wagtendonk-de Leeuw [20]. After thawing for 30 seconds at 38□C, cells were washed over a discontinuous Percoll(tm) gradient and with HBT as described above, and also resuspended at a concentration of 50 million cells/ml. These cells are further referred to as frozen/thawed cells.

Extraction and isolation of oxysterols

The total lipid fraction from sperm cells was extracted according to the method of Bligh and Dyer [21]. Subsequently, cholesterol and oxysterols were separated from phospholipids by solid phase extraction on 200 mg silica columns (Merck, Darmstadt, Germany). To this end, the lipid extract was dissolved in chloroform and applied to a column preconditioned with acetone, and the cholesterol plus oxysterol fraction was eluted with three volumes of acetone [15,22]. Acetone was evaporated under a constant stream of nitrogen gas and cholesterol and oxysterols were stored at -20ºC until use.

Artificial, protein-free membranes were made from extracted sperm cell lipids as described previously [23,24]. In brief, sperm cell lipids were extracted as above and dried under nitrogen in a conical tube to form a lipid film. Lipids were hydrated with 96% (v/v) ethanol, dried again under nitrogen and HBT was added before extensive vortexing. Finally, small unilamellar vesicles were formed by sonication on ice for 5x 5s with the probe of a Soniprep 150 (MSE scientific instruments, Crawly, UK).

HPLC-MS analysis

Extracted oxysterols, cholesterol (and desmosterol) were dissolved in a small volume of acetonitrile/methanol (6/4 v/v), and injected on an Aquasil C18 250 x 4.6 mm 5µm analytical column (Keystone Scientific, Bellefonte, PA, USA) [9]. Elution was performed isocratically with acetonitrile:methanol (6:4 v/v) at a flow rate of 1 ml/min and the column effluent was introduced by an atmospheric pressure chemical ionization (APCI) interface (Sciex, Toronto) into a 4000 QTRAP mass spectrometer (MS). For maximal sensitivity and for linearity of the response, the MS was operated in “multiple reaction monitoring” (MRM) mode at unit mass resolution. Peaks were identified by comparison of retention time and mass spectrum with authentic standards of the (oxy-)sterols described (Merck, Darmstadt, Germany). Ion transitions monitored were m/z 369.2/161.1 (cholesterol), 401.2/175.1 (7-ketocholesterol) and 367.2/159.1 (other oxysterols and desmosterol). When exploring the existence of other oxysterols (such as desmosterol derived oxysterols) the MS was operated in "enhanced" MS (ion trapping) mode in the mass range 200-600 amu. Data were analysed with Analysttm software version 1.4.1 (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

Results

Detection, identification and quantification of oxysterols (Oxy)sterols can readily be ionized by APCI (Fig. 1A). In accordance with earlier reports [9,25], (oxy)sterols were not observed as (quasi-)molecular ions, but had lost any hydroxyl groups (including that on C-3) as a molecule of water (see insert Fig. 1A). Product spectra of the $[M+H-H2O]^+$ ions generated by collisional activation were complex and structural information was hard to obtain from these spectra, as they consisted of numerous fragment ions that were common to product spectra of many (oxy-) sterols (Fig. 1 B-D).

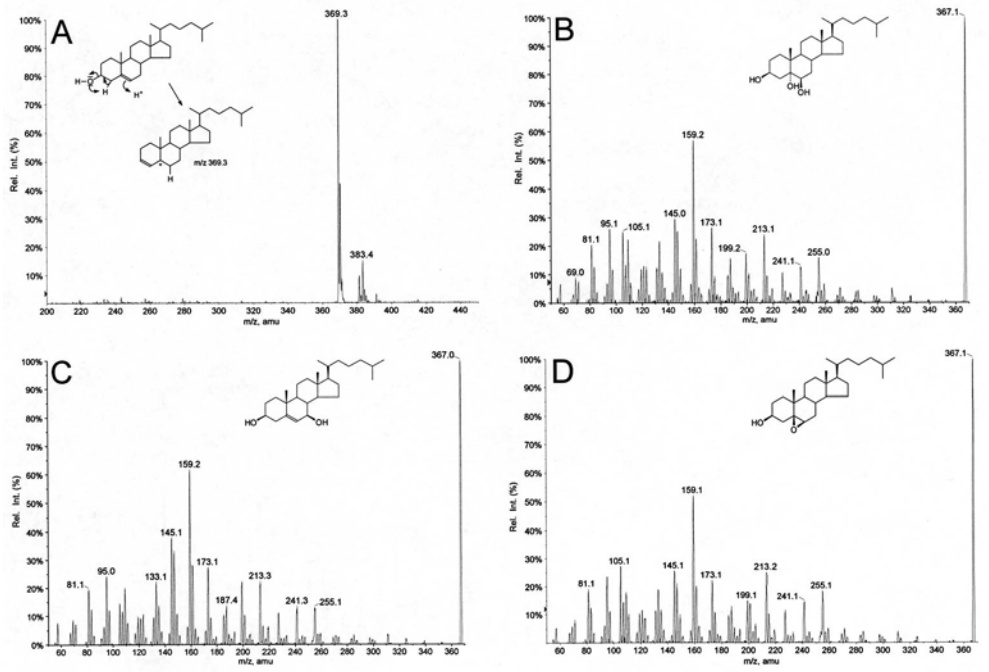

Figure 1. Mass spectrometry of authentic standards of (oxy-)sterols. **A:** Mass spectrum recorded during the elution of cholesterol. The insert shows a proposed mechanism for the formation of the $[M+H-H_2O]^+$ base peak ion during APCI ionization.. **B-D:** Indiscriminate and extensive fragmentation of (oxy-)sterols. Fragmentation spectra of (oxy-) sterols at 35V collision energy. **B:** cholestane-3,5,6-triol **C:** 7ß-hydroxycholesterol, **D:** 5,6ß-epoxycholesterol

The discrimination between (oxy)sterols was therefore based on the different retention times of the oxysterols during reverse phase chromatography as detected by evaporative light scattering detection (Fig. 2A) or mass spectrometry (Fig. 2B). Calibration curves for quantification purposes were constructed in MS/MRM-mode for eight (oxy)sterols that are of physiological importance. All calibration curves were linear up to at least 100 pmole and had correlation coefficients (r2) between 0.99 and 1.00. The (oxy)sterols were detected with small variations in sensitivity due to differences in their ionization- and fragmentation efficiencies, but had limits of detection of around 50 fmoles (oxysterols) to 200 fmoles (cholesterol and desmosterol) (Fig. 3). For assessment of lipid peroxidation, the absolute amounts of cholesterol, desmosterol and individual oxysterols were calculated using the corresponding calibration curves. Oxysterols were expressed as mole-% of cholesterol.

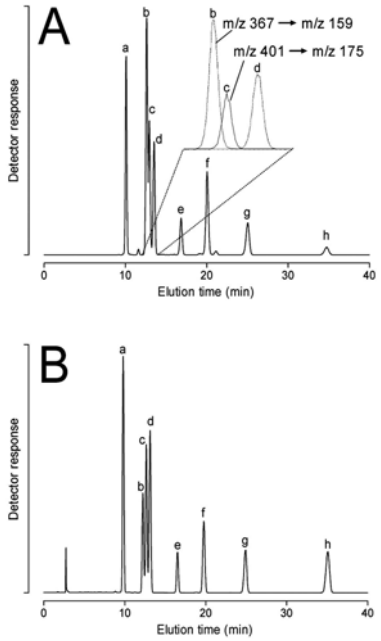

Figure 2. Detection of (oxy-)sterol standards by MRM-MS (**A**) or evaporative light scattering detection (**B**). Standards shown are: **a**: triol; **b**: 7ß-OH; **c**: 7-keto; **d**: 25-OH; **e**: 5,6ß-EP; **f**: 5,6α-EP; **g**: desmosterol; **h**: cholesterol. The insert in A shows distinction between oxysterols based on different mass spectrometric fragmentations.

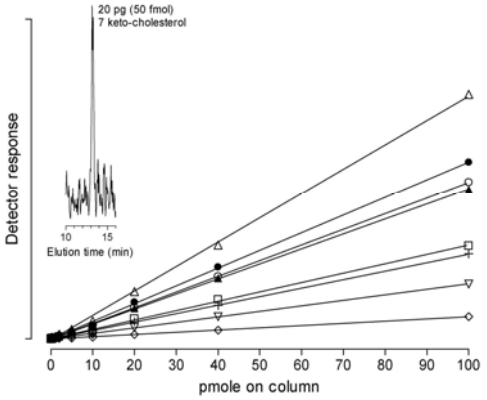

Figure 3. (Oxy-)sterols produce linear calibration curves over a broad dynamic range. Differences in sensitivity result from differences in ionization- and fragmentation efficiencies. 7ß-OH; • triol; ° 5,6α-EP; ▲ 25-OH; □ 7-keto; + desmosterol; ∇ 5,6ß-EP; ◇ cholesterol. The insert shows the signal-to-noise ratio at the lower level of detection of 7-ketocholesterol.

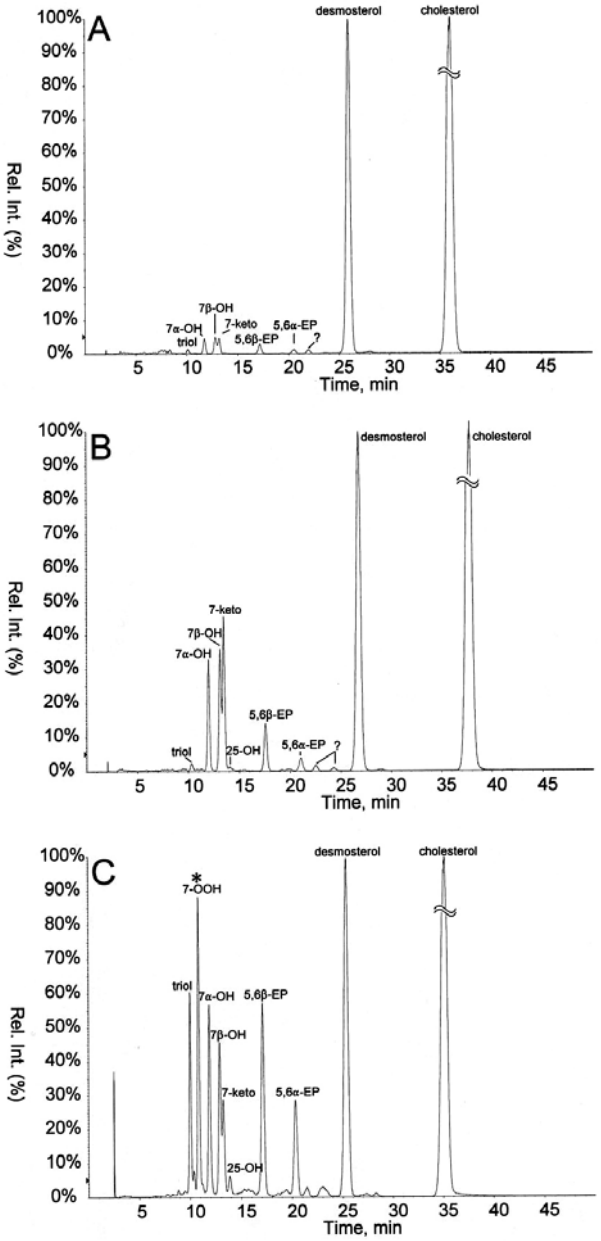

Figure 4. Detection of (oxy-)sterols in Percoll(tm) washed sperm cells by MRM-MS. **A**: bovine sperm cells directly after ejaculation. **B**: Sperm cells incubated in the presence of oxidants. **C**: small unilamellar vesicles constructed from a sperm cell lipid extract incubated in the same presence of oxidants as at B. Figures are normalized to desmosterol for clarity

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	25-OH	3,5,6-triol	5,6α-epox	5,6β-epox	7α-OH	7-keto	7β-OH	Cholesterol	Desmosterol	***Sum oxysterols***
Fresh=	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.13 ± 0.05	0.04 ± 0.01	0.07 ± 0.05	0.09 ± 0.02	100	17.28 ± 2.30	***0.38 ± 0.12***
Frozen=	0.01 ± 0.00	0.02 ± 0.03	0.02 ± 0.00	0.11 ± 0.02	0.06 ± 0.01	0.08 ± 0.03	0.10 ± 0.02	100	17.34 ± 2.53	***0.40 ± 0.10***
Fresh incub	0.02 ± 0.02	0.01 ± 0.00	0.04 ± 0.02	0.32 ± 0.19	0.22 ± 0.19	0.34 ± 0.34	0.35 ± 0.25	100	15.57 ± 2.25	***1.31 ± 1.01***
Frozen incub	0.01 ± 0.00	0.01 ± 0.01	0.03 ± 0.02	0.17 ± 0.13	0.07 ± 0.04	0.11 ± 0.09	0.12 ± 0.07	100	15.15 ± 2.01	***0.53 ± 0.37***
Fresh stressed	0.03 ± 0.02	0.04 ± 0.02	0.24 ± 0.01	2.23 ± 0.10	1.12 ± 0.15	2.15 ± 0.55	1.83 ± 0.13	100	15.79 ± 2.52	***7.64 ± 0.94***
Frozen stressed	0.01 ± 0.00	0.03 ± 0.02	0.23 ± 0.04	2.07 ± 0.50	0.92 ± 0.16	1.74 ±0.07	1.58 ± 0.30	100	14.72 ± 2.62	***6.58 ± 0.94***
Protein free memb	0.20 ± 0.03	1.19 ± 0.08	2.53 ± 0.43	7.54 ± 0.05	0.88 ± 0.07	0.95 ± 0.06	0.77 ± 0.07	100	16.35 ± 0.15	***14.06 ± 0.79***

Table 1
Molar amounts of oxysterols as percentage of cholesterol. Fresh=: sperm cells washed and extracted directly after ejaculation. Frozen=: Ejaculated, washed and frozen/thawed sperm cells. Fresh incub: fresh, washed sperm cells incubated for 16 hours. Frozen incub: Frozen/thawed sperm cells incubated for 16 hours. Fresh stressed: Fresh, washed sperm cells incubated in the presence of oxidants. Frozen stressed: Frozen/thawed sperm cells incubated in the presence of oxidants. Protein free membr: Small unilamellar vesicles from extracted sperm cell membrane lipids incubated in the presence of oxidants.

Oxysterols are present in fresh and frozen/thawed sperm cells. Analysis of Percoll(tm) washed, fresh sperm cells, from which lipids were extracted as soon as feasible after ejaculation, revealed the presence of cholesterol, desmosterol and oxysterols in these cells (Fig. 4A). When these sperm cells underwent the freezing protocol (taking a total time of approximately 3 hours) and were subsequently thawed, the total amount and composition of oxysterols in these cells remained unaltered compared to fresh cells, showing that the freezing procedure itself did not result in elevated oxysterol levels ($p=0.52$, $n=3$, Table 1). Subsequent incubation of fresh or frozen/thawed sperm cells for 16 hours did not change the oxysterol pattern of sperm cells either ($p=0.12$, $n=3$, Table 1). Only when sperm cells were incubated in the presence of pro-oxidants, oxysterol levels increased by approximately one order of magnitude, resulting in 7.64 ± 0.94 mole-% and 6.58 ± 0.94 mole-% oxysterols in fresh and frozen/thawed cells respectively ($p < 0,01$, $n=6$, Table 1). Notably, all oxysterol levels were increased in the stressed sample and their relative contribution to the total amount of oxysterol resembled that of unstressed cells (Fig. 4B).

Induction of lipid peroxidation in protein-free membranes

When the same oxidative stress that was applied to fresh and frozen/thawed cells was applied to sperm cell lipids in small unilamellar vesicles, also extensive formation of oxysterols was observed (Table 1, Fig. 4C). However, this peroxidation of protein-free vesicles led to the formation of different oxysterols, most notably to the reduced abundance of 7-ketocholesterol and the more abundant presence of 3,5,6-cholestane-triol. One additional oxysterol was observed that was not detected in living sperm cells (marked with an asterisk in Fig. 4C). Based on its retention time and its fragmentation spectra in positive and negative mode, it was tentatively identified as 7-hydroperoxycholesterol (data not shown).

Discussion

Sperm cells contain high amounts of polyunsaturated phospholipids which makes them particularly vulnerable to lipid peroxidation [26,27]. Many papers have demonstrated the occurrence of lipid peroxidation during incubation of sperm cells under various conditions [2,5,27,28,29]. However, as these experiments have typically assessed the formation of lipid peroxidation products such as malondialdehyde and 9-hydroxynonenal during incubation, little is known about the amount of lipid peroxidation products (e.g. oxysterols) in sperm cells directly after ejaculation. Additionally, it is important to realize that in the female genital tract, sperm cells are

exposed to exogenous oxidative stress, resulting from the contact between sperm cells and leukocytes in the female genital tract. It is therefore of importance to analyse both the sperm cell's "load" of oxysterols directly after ejaculation, as well as the ability of sperm cells to handle oxysterols resulting from exogenous oxidative stress.

Mass spectrometry

The observed APCI mass spectra of the individual oxysterols were identical to the spectra shown for processed foods by Razzazi Fazeli [9] (Fig. 1A). Many oxysterols are observed at identical mass to charge ratio's, particularly after the loss of hydroxyl groups as molecules of water, a typical event during the APCI ionization process. Therefore, it was investigated whether discrimination between oxysterols could be made on basis of their fragmentation spectra. If so, oxysterol specific fragments could be used for mass spectrometric multiple reaction monitoring during liquid chromatography, a sensitive and specific technique for the detection of components. The resulting fragmentation spectra after collisional activation of cholesterol and oxysterols showed particular extensive and indiscriminate fragmentation (Fig 1 B-D). This clearly implicates that it is not possible to discriminate between (oxy-)sterols based solely on their fragmentation spectra, and that chromatography is an indispensable tool in the characterization of (oxy-)sterols. Moreover, many fragment ions showed similar intensities, meaning that there was no fragmentation pathway yielding high amounts of a particular ion to be used in multiple reaction monitoring. Despite the lack of specific, high-abundance fragment ions, the technical improvements in the latest generation of mass spectrometers results in a detection limit of approximately 50 fmol of oxysterol and a linear dynamic range of more than 3 orders of magnitude (Fig. 3). Assuming that 0.1% of cholesterol has been converted into a particular oxysterol, this would correspond to the amount of oxysterol present in 50,000 sperm cells. This illustrates that commercial cell sorters (able to sort cells at typical rates of up to 10,000 cells per second) can easily provide us with sorted sperm subpopulations for investigation of their lipid peroxidation profiles under variable physiological conditions.

Presence of oxysterols in fresh sperm cells

Oxysterols are still very hydrophobic molecules, and that they will be retained in the cellular membranes. Combined with the low level of detection of oxysterols (typically around 50 fmoles), this makes analysis of oxysterols the technique par excellence for the assessment of the total oxidative stress cells have encountered. In contrast, peroxidation of phospholipids results in the formation of hydrophilic end products such as malondialdehyde and 9-hydroxy-nonenal,

compounds that are water soluble and do therefore not remain associated with the sperm cell. Assessment of lipid peroxidation by fluorescent techniques has also successfully been performed in sperm cells, but this only detects oxidative stress from the moment cells have been fluorescently labelled [5,28], and not (as is the case with oxysterols) the oxidative stress that a cell has encountered so far.
Analysis of sterols in sperm cells directly after ejaculation revealed the abundant presence of cholesterol and desmosterol, as expected from previous data [30,31,32]. Small amounts (0.4 mole-%) of oxysterols were also clearly present (table I), demonstrating that sperm cells have been subjected to peroxidation at the time they are ejaculated.
Based on their retention time and their molecular weights, the observed oxysterols appeared to be derived from cholesterol and not from desmosterol. This is remarkable, considering the fact that around 16% of the sterol in bovine sperm cells is desmosterol, and that the only chemical difference between cholesterol and desmosterol is the double bond between carbons 24 and 25 in desmosterol. Theoretically, desmosterol would be expected to be a better substrate for peroxidation, as unsaturations are preferred sites of oxidation. 25-Hydroxycholesterol is the only oxysterol that could partly be derived from desmosterol, as the double bond between carbon atoms 24 and 25 in desmosterol would have been lost during oxidation. The fact however, that 25-hydroxycholesterol was only a minor oxidation product in our experiments, points towards a different underlying reason for the observed preferred oxidation of cholesterol. One might consider the possibility that desmosterol is localized to membranes that are to a lesser extend exposed to oxidative stress, such as the (intracellular) acrosomal membrane. We are currently performing oxidation assays on sterols in homogeneous solution as well as studies on artificial membranes with various lipid compositions to elucidate this distinct behaviour of cholesterol and desmosterol.

Oxysterol formation during incubation of sperm cells

Incubation of fresh sperm cells for 16 hours in HBT did not result in an elevation of oxysterol levels and the amount of oxidized cholesterol remained below 1 mole-% (Table 1). Although this is only a very modest molar quantity, and dramatic effects on biophysical properties of the sperm cell membranes (e.g. stability and permeability) are not likely to exist, the toxic effects of oxysterols may have profound physiological implications. Many averse effects of oxysterols have been reported. Most relevant to sperm cells (or the fertilized zygote) are mutagenesis, cytotoxicity, and inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase (an enzyme in sterol biosynthesis, reviewed in [14]). Obviously, it is essential to prevent mutagenesis in cells whose DNA will be copied in each and

every cell of the new organism. Although paternal DNA is protected from mutagenesis by heavy condensation inside the sperm cells, after sperm-egg fusion and subsequent decondensation of DNA, the oxysterols formed in sperm cells are likely to be still present. Similarly, interference with sterol biosynthesis via inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase will not interfere with sperm cell functionality directly, as these cells do not synthesize cholesterol, but the averse effects may be postponed until fertilization has occurred and growth commences. Many studies have been performed that suggest that cytotoxicity of oxysterols results from induction of apoptosis [11,33,34,35,36,37,38,39,40,41]. As with the afore mentioned averse effects of oxysterols, sperm cells themselves are well protected from apoptosis due to the absence of cytosol (containing the apoptotic machinery) and the hypercondensation of DNA (preventing fragmentation), but sperm cells loaded with oxysterols would turn out to be poor matches for the egg cell they fertilize.

The effect of the freeze/thawing procedure on oxysterol formation

In animal reproduction, freeze/thawing of sperm is common practice to store and distribute sperm before use. Freeze/thawing of sperm is also frequently performed in human medicine, e.g. when a donor has to undergo chemotherapy which typically has adverse effects on sperm quality. As this freeze/thawing procedure is known to induce lipid peroxidation [5,42,43], we investigated whether the formation of oxysterols was induced in sperm cells that had undergone this treatment. However, both fresh and frozen/thawed sperm cells did not show an increase in oxysterol levels upon incubation. This is different from the observations made with the fluorescent peroxidation-reporter C_{11}-Bodipy$^{581/591}$ in sperm, where an increased peroxidation was observed under these conditions [5]. Several reasons may cause this difference such as a different reactivity of cholesterol and the Bodipy probe towards various ROS. Both molecules are oxidized by hydroxyl- and peroxyl radicals, as well as by peroxynitrite [4,44,45]. However, whereas the reactivity of C_{11}-Bodipy$^{581/591}$ towards ROS is comparable to that of polyunsaturated fatty acids such as arachidonic acid [46], the reactivity of (monoenoic) cholesterol is considerably lower [4]. Furthermore, it is known that lipid peroxidation is not homogeneously distributed over the sperm cell, but that the midpiece is particularly prone to oxidation [5]. Cholesterol however, is enriched in the sperm head cell plasmamembrane where relatively little lipid peroxidation was observed, which offers another explanation for the lower level of peroxidation observed with cholesterol [5,16].

Metabolism of oxysterols by sperm cells

To obtain insight into whether or not sperm cells do metabolize oxysterols, we compared oxysterol formation in incubated sperm cells and oxysterol formation in oxidatively stressed artificial membranes reconstituted from sperm cell lipids. Striking differences in relative amounts of oxysterols were found between these samples although the oxidative stress was generated in an identical fashion (Fig. 4C and TABLE 1). This suggests that the oxysterol profile found in living sperm cells resulted from peroxidation of cholesterol and subsequent enzymatic actions. I.e., cholestane-3,5,6-triol, which is a well known cytotoxic oxysterol, was abundant in stressed reconstituted membranes, but in sperm cells the formation of this oxysterol seemed to be prevented. Also the presence of the tentatively identified 7-hydroperoxycholesterol in protein-free membranes but not in membranes of live sperm cells, points towards an enzymatic reduction of this peroxide to 7-ketocholesterol, which is in line with the elevated concentration of 7-ketocholesterol in living sperm cells. From the apparent oxysterol metabolism by sperm cells it is tempting to speculate that oxysterols have had their influence on the success of fertilization in evolution and that a link may exist between altered oxysterol metabolism and reduced fertility. At this stage, however, it is difficult to pinpoint exactly what averse effects of oxysterols might be prevented by the apparent metabolism of oxysterols by sperm cells, as the mechanism of oxysterol toxicity is cell type specific [33] and we are just beginning to appreciate the role of oxysterols in sperm cell physiology.

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CHAPTER 5

Exposure of bovine sperm to pro-oxidants does not inhibit fertilization but impairs the developmental competence of the zygote

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Submitted

Abstract

The present study describes the effects of incubations of bovine sperm to mild and more intense ROS generating conditions (and a control without ROS stimulating agents). The membrane integrity of the incubated sperm was assessed and the incubated sperm were used for IVF. The percentage of fertilization, cleavage and blastocyst formation was followed for a period of 9 days. The sperm membrane integrity as well as the fertilization rate and first cleavage rates obtained with sperm from mild ROS generating conditions were not different from sperm incubated without pro-oxidants. Meanwhile, the corresponding incubated sperm samples showed significant increased levels of molecular oxidation in the plasma membrane the mitochondria, the cytosol, and to a lesser extent in the sperm's DNA. Moreover, the subsequent zygote development was virtually completely arrested after first cleavage when sperm had been exposed to oxidative stress in contrast to sperm that were not subjected to oxidative stress. From these results we conclude that the impact of oxidative stress to sperm becomes primarily manifest after fertilization and more specifically after the first cleavage of the formed zygote. The degree of lipid peroxidation in the sperm plasma membrane significantly correlates with the degree of blastocyst formation at day 9 when the corresponding sperm is used for in vitro fertilization of oocytes.

Keywords: ROS, RNS, oxidative stress, sperm, oocyte, fertilization, IVF, ICSI, cryopreservation.

Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved in several cellular signalling mechanisms. As by-products of the normal mitochondrial respiration process they can interact with molecular substrates such as lipids, proteins and DNA, triggering cascades that can lead to severe pathologic conditions among which are different types of cancer, atherosclerosis and neurodegenerative diseases [1,2].

Likewise, in reproductive biology, ROS/RNS are supposed to be detrimental to gametes, which is reflected by lipid peroxidation [3,4], impairment of protein function and DNA damage, compromising the gamete's membrane integrity and viability. With this respect the sperm plasma membranes are particularly sensitive to free radicals as they contain high levels of poly-unsaturated fatty acids which form a suitable substrate for oxidation by free radicals. Despite investigations on the effects of free radicals on female and male gametes, little is known about fertilization outcome and embryo developmental aptitude after exposure of gametes to ROS. In humans, infertility affects 5-7% of all male individuals [5] mainly because of leukocyte infiltration in the ejaculate. Leukocytes produce large quantities of ROS and are reported to decrease sperm viability either before [6] or after ejaculation [7]. Sperm cells may also generate their on radicals and this is especially noted for immature sperm [8,9].

Processing sperm for cryopreservation often introduces an additional source for ROS attack on sperm and during this procedure the sperm membranes become more susceptible to lipid peroxidation (LPO) [10]. Sperm DNA damage does however not necessarily affect fertilization but because of DNA repair mechanisms present in the oocyte further development is possible after fertilization with DNA damaged sperm [11,12]. The effects of LPO on the fertilizing ability of sperm cells and subsequent embryo development are largely unknown. It is unclear whether LPO brought to the plasma and acrosome membranes completely block its *zona pellucida* binding properties or inhibits fertilization. Moreover, once fertilization occurs, the effects on developmental competence are unclear.

Although large quantities of free radicals are supposed to impair the reproductive success several research groups have reported that under physiological conditions lower quantities of free radicals may be functional in promote gamete functioning and fertilization. Mild generation of ROS/RNS like superoxide ($O_2^{-\bullet}$) [13], hydrogen peroxide (H_2O_2) [14,15,16,17] and nitric oxide ($NO^{\bullet}$) [18] are supposed to play a role in the ability of sperm cells to undergo capacitation and the acrosome reaction as well as to acquire fertilizing ability [19]. In addition, mild ROS generation can be beneficial for maturation and developmental competence of oocytes [20,21].

We attempted in this study to unravel whether generation of free radicals is supporting or inhibiting sperm in their quest to fertilize the oocyte. To this end we have incubated sperm under mild and more intense ROS generating conditions and compared sperm integrity as well as the degree of molecular oxidation with sperm incubated without ROS generating agentia. The incubated sperm were also used for IVF experiments in which the rate of fertilization, first cleavage of the zygote, as well as blastocyst formation was followed for a period of 9 days. The results reported indicate that the impact of oxidative stress to sperm becomes primarily manifest after the first cleavage of the zygote.

Materials and methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise.

Sperm washing and oxidation treatments prior to in vitro fertilization

Frozen 0,25 ml French-straws (IMV, l'Aigle, France) from 3 different sires containing 50 million sperm cells per ml with proven fertility were thawed in a 37° C water bath for 50 seconds and the motility of non-diluted thawed sperm assessed by placing a drop of semen on a pre-warmed slide (37° C) overlaid with a cover slip and examined under a phase contrast microscope (Olympus, Tokyo, Japan) by viewing 4 random fields at 100x and 400x magnification and percentages of motile spermatozoa determined. Samples with more than 65 % motile sperm cells were used. The sperm 0,5 ml was washed through a discontinuous Percoll® density gradient 1 ml 90% (v/v) under 1 ml of 40% for 30 min at 27°C at 700 x g. Percoll® and remains of egg-yolk were removed by washing pelleted sperm at 27 °C at 300 x g with saline-Hepes medium solution [137 mM NaCl, 24.9 mM KCl, 10 mM Glucose, 17 mM Hepes, 100 μg/ml kanamycin, pH, 7.5, 295-300 mOsm]. The pellet was resuspended in 1 ml Hepes buffered Tyrodes (HBT) solution [116 mM NaCl, 20 mM Hepes, 5 mM glucose, 3.1 mM KCl, 0.4 mM $MgSO_4$, 21.7 mM lactate, 0.3 mM NaH_2PO_4, 100 μg/ml kanamycine, 1 mM pyruvate, 2 mM $CaCl_2$, 1 mg/ml each of polyvinyl alcohol (PVA) and polyvinyl pyrrolidon (PVP), 300 mOsm, pH 7.4], sperm suspensions were finally diluted to a final concentration of 1 x 10^7 cells per ml and incubated for 3 h in HBT without bicarbonate. Four different group treatments were assigned: (i) No addition of pro-oxidants (further referred as *no oxidants*) (ii) addition of *100 μM H_2O_2*, (iii) addition of *500 μM H_2O_2*, or (iv) the addition of 100 μM ascorbic acid, 20 μM $FeSO_4$ and 500 μM H_2O_2 (referred to as *Cocktail*). These conditions were compared to the *IVF control* treatments, where no incubation took place and the sperm was processed as routinely performed for IVF.

Damage to sperm membranes of the incubated sperm samples was detected flow cytometrically using a triple staining method as described by [22]. Briefly sperm plasmam membrane integrity and acrosome intactness were assessed by 100 nM Sybr-14, 12 µM propidium iodide (PI) and 2.5 µg/ml PNA-Alexa Fluor™ 647, added to 1 ml of sperm and incubated at 37 °C for 10 min prior to flow cytometric assessment.

Detection of molecular peroxidation in sperm

I: Detection of sperm DNA oxidation

Aliquots of sperm were processed prior to IVF as described above. Oxidative DNA damage was detected using the OxyDNA kit (Biotrin Ltd, Dublin, Eire), this assay makes use of a FITC-tagged antibody against 8-oxoguanine, a DNA base oxidatively modified by ROS [23]. Samples were processed according to the kit instructions. Briefly, sperm cells were fixed with 2% paraformaldehyde in PBS (Gibco) for 1 h at room temperature (RT). Cells were washed with PBS, resuspended in 0.1 % Triton X-100 in PBS and washed with PBS. Sperm cells were resuspended in blocking solution (according to the manufacturer's instructions) and incubated for 1 h at 39 ºC. Samples were cooled at RT and 15 µl of FITC-conjugated antibody was added to each sample and incubated for 1 hour at RT. Samples were finally washed with PBS prior to flow cytometric analysis.

II: Detection of lipid peroxidation at the sperm plasma membrane

Simultaneous detection of sperm plasma membrane lipid peroxidation in combination with sperm viability were performed after labeling the incubated bovine sperm samples with 2 µM C_{11}-Bodipy$^{581/591}$ and 12 µM PI staining respectively. Sperm cells were labelled with C_{11}-Bodipy$^{581/591}$ for 30 min, and with PI for 10 min at 37 °C and submitted to flow cytometric analysis [10].

III: Detection of sperm oxidation in the cytosolic and mitochondria

Detection of cytosolic oxidation and mitochondrial oxidation on incubated bovine sperm samples was done after labeling with a final concentration of 5 µM CM-H_2DCFDA (chloromethyl probe [24]) and 25 nM Mitotracker® CM-H_2Xros [25], respectively. Cells were labelled with CM-H_2DCFDA for 1 h and with Mitotracker® CM-H_2Xros for 15 min at 37 °C and submitted to flow cytmetric analysis.

Flow cytometry

For each analysis, two replicates per treatment were used. Forward and side-scatter (FSC and SSC respectively) values were recorded on a logarithmic scale and sperm specific events were recognized by the typical L-shaped scattering profile. For each measurement 10,000 double-gated specific events were recorded. Scatter and fluorescent properties were analysed with WinMDI

shareware version 2.8 (http://facs.scripps.edu/). Both flow cytometers, FACScan and FACScalibur (Becton Dickson, Franklin Lakes, NJ, USA), were equipped with an air-cooled, 15 mW output, 488 nm excitation argon laser. All samples were analysed using FACS buffer (BD Biosciences, San Jose, CA, USA) as sheath fluid. The molecular probes used in the flow cytometric analysis were all purchased from Molecular Probes, Leiden, the Netherlands. PI was detected using the FL-3 (670 nm long pass filter) detector, SYBR-14 with FL-1 (530/30 nm band-pass filter) and PNA-Alexa Fluor™ 647 with FL-4 (661/16 nm band-pass filter) on the FACScalibur. Adjustment of compensation values for the three emission detectors followed the guidelines of Roederer (http://www.drmr.com/compensation).
C_{11}-Bodipy$^{581/591}$ and CM-H_2DCFDA were detected with FL-1 (530/30 nm band-pass filter). PI and Mitotracker® CM-H_2Xros were detected with FL-3 (620 nm long pass filter) on the FACScan.

Collection and Culture of Oocytes

Bovine ovaries were collected from a slaughterhouse and transported in a thermo flask to the laboratory. Cumulus oocyte complexes (COCs) were obtained by aspirating follicles of 2-8 mm diameter, and selected on the presence of a multilayered compact cumulus investment. The selected COCs were then rinsed in Hepes-buffered M199 (Gibco BRL, Paisley, UK) with 1 % (v/v) penicillin-streptomycin (Gibco) and assembled randomly in groups of 30 to 35 in each well of a 4-well culture plate (Nunc A/S, Roskilde, Denmark) containing 500 µl M199, 10 % (v/v) fetal calf serum (Gibco), recombinant human FSH (hFSH)-Org 32489 (Organon, Oss, The Netherlands) to a final concentration of 0.05 IU/ml and 100 µM cysteamine. Culture was performed for 22 h at 39 °C in a humidified atmosphere of 5% CO_2 in air [26].

IVF and Embryo Culture

Groups of 30-35 COCs were fertilized *in vitro* according to the procedure described by Parrish et al. [27] with small modifications [28]. Per well, sperm cells were added to a final concentration of 50 x 10^6 cells/ml, in addition to 20 µl heparin (final concentration 10 µg/ml) and 20 µl PHE (containing 20 µM D-penicillamine, 10 µM hypotaurine, 1µM epinephrine). Embryo culture was as previously described [29]. On day 5 cleaved embryos were scored for cell number and transferred to fresh synthetic oviductal fluid (SOF) culture medium. The embryonic development was assessed on days 7, 8 and 9.

DNA, actin and tubulin staining of the developing early embryos

Embryos were washed with PBS and permeabilised for 1 h in a glycerol-based microtubule-stabilizing solution [30] at 37 °C and then fixed with 4% (w/v) of

paraformaldehyde overnight at 4° C. The embryos were washed in PBS with 0.1 % (w/v) PVA (PBS/PVA) and incubated in PBS with 2% (v/v) goat serum (PBS-serum). The embryos were incubated for 1 h in a 1:50 dilution of mouse-anti-α-tubulin antibody (catalog # T-5168; Sigma) in PBS-serum, followed by washing with PBS, 0.1 % (v/v) Tween-20 (PBS-Tween), and secondly incubated for 1 h with a Alexa Fluor™ 633 goat-anti-mouse antibody (Molecular Probes) 1:100 in PBS-serum followed by washes with PBS-Tween. The microfilaments were then stained for 30 min with Alexa Fluor™ 568-phalloidine (Molecular Probes) in 1:25 solution in PBS. After washing in PBS/PVA, DNA was stained for 5 min with Sytox-green (Molecular Probes) diluted 1:100 in PBS.
Stained embryos were mounted on glass slides using anti-fading mounting medium (Vectashield, vector Lab, Burlingame, CA, USA) and the coverslips were supported with a Vaseline-wax mixture and sealed with nail polish.

Confocal Laser Scanning Microscopy
Embryos were examined using a confocal laser scanning microscope (CLSM; Leica TSC-SP, Mannheim, Germany) equipped with a krypton-argon ion laser for simultaneous excitation of Alexa Fluor™ 568 (microfilaments), Alexa Fluor™ 633 (microtubules) and Sytox green (DNA) using 488/568/650-nm excitation/barrier filter combinations.

Statistical analysis
All the experiments consisted of at least 3 independent experimental runs. The number of cleaved embryos at day 5 and blastocyst at day 9 were analysed by logistic regression with lowest Akaike's information criterium (AIC) for best model fit (statistical software R version 2.0.1; http://ww.R-project.org). The number of embryos were analysed by logistic regression and a quasi-likelihood with binomial mean and variance was used. The FACS data was equally analysed using a logistic regression with overdispersion. Correlations between staining properties, cleavage and blastocysts at Day 9 were drawn and linear regression analysis performed using SigmaStat version 3.0 (SPSS, Inc.). In all analysis differences were considered significant when $P<0.05$.

Results

By exposing sperm to different oxidative agents we mimicked the environment that sperm cells can encounter either in vivo or in vitro. To avoid contamination from seminal plasma or other cells that would possibly give rise to unknown levels of oxidative stress, the sperm cells were washed through a Percoll gradient. The sperm cells were subsequently exposed to different ROS inducing

media: (i) No additions of pro-oxidants (referred to as no oxidants) (ii) addition of 100 μM H_2O_2, (iii) addition of 500 μM H_2O_2, or (iv) the addition of 100 μM ascorbic acid, 20 μM $FeSO_4$ and 500 μM H_2O_2 (referred to as Cocktail). Hydrogen peroxide has been reported to induce capacitation at low concentrations by promoting tyrosine phosphorylation but at high concentrations (>500 μM) to block this process [17,31]. The sperm cells were exposed to these oxidative agents for 3 h and sperm cells were incubated for 3 h in normal medium (no-oxidants control) and sperm was processed as routinely performed for IVF (IVF control).

Oxidative stress does not induce significant DNA damage in sperm cells

Oxidative modification of DNA was determined by the detection of 8-oxoguanine formation by flow cytometry [23]. Already in the control situation low levels of oxidised DNA were detected in sperm. The amount of 8-oxoguanine formation slightly (but not significantly) increased during the control incubation (no added pro-oxidants), and similar levels were found under incubation with 100 μM H_2O_2 (Fig. 1). Although marginally higher levels of 8-oxoguanine formation were found under incubations with 500 μM H_2O_2, or with the oxidant cocktail, this increase was significant compared with the IVF control (Fig. 1). Nevertheless the low increase in DNA damage inflicted by oxidative stress shows that the sperm's DNA appears to be well protected against the pro-oxidant treatments tested.

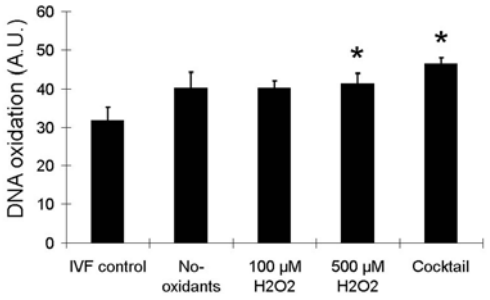

Figure 1
DNA oxidation as measured by 8-oxoguanine formation in sperm cells under various conditions as indicated in the text. Bars represent means ± SEM of three different experiments. [a] indicates levels that are statistically different at $p < 0.05$.

Oxidative stress does not affect sperm viability and acrosome status

To determine the effects of oxidative stress on viability and acrosome damage, sperm cells were analysed by flow cytometry after PNA-Alexa Fluor, SYBR-14 and PI staining, which enabled the discrimination between acrosome-

reacted, live and dead cells [22] Incubation of sperm cells for 3 h led to a decrease in the percentage of sperm cells with an intact acrosome, independent of the incubation with oxidants. On the other hand, an increase in the percentage of dead cells with damaged acrosome was observed when sperm cells were exposed to the cocktail (Fig. 2A).

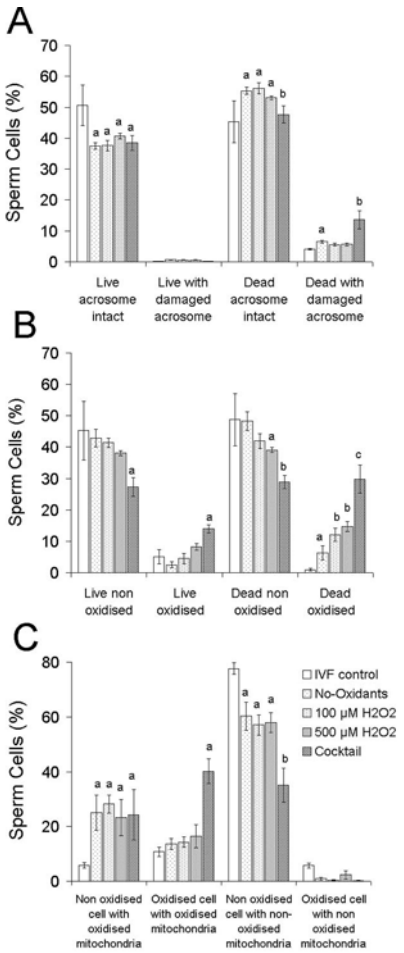

Figure 2
Percentages of various categories of sperm cells (as indicated) after exposure of sperm to different conditions. Sperm cells were analysed for A) viability (Sybr-14 and propidium iodide staining), acrosome reaction (PNA-Alexa Fluor staining), B) viability (Sybr-14 and propidium iodide staining) and lipid peroxidation in the plasma membrane (C_{11} Bodipy$^{581/591}$), and C) cytosolic oxidation (CM-H_2DCFDA) and mitochondrial oxidation (CM-H_2Xros). Bars represent means ± SEM of three different experiments. Letters on bars indicate levels that are statistically different at $p < 0.05$.

ROS induces plasma membrane oxidation

To further determine the consequences of oxidative stress to sperm cells, washed semen samples were labelled with C_{11}-Bodipy$^{581/591}$. This is a fluorescent ratiometric probe that can be used to monitor lipid peroxidation in the sperm plasma membrane by flow cytometry [10]. Significant levels of C_{11}-Bodipy$^{581/591}$ peroxidation were noted in the deteriorated sperm subpopulation at the lowest level of H_2O_2 addition (100 µM, Fig. 2B) and increased to approximately 50% of the deteriorated sperm subpopulation under conditions with the oxidant cocktail. The C_{11}-Bodipy$^{581/591}$ was better protected against the pro-oxidants in the living sperm subpopulation. Only the oxidant cocktail induced a significant increase in plasma membrane lipid peroxidation (Fig. 2B).

Increased intracellular and mitochondrial oxidation after ROS exposure.

The degree of oxidation in the sperm cytosol and the sperm mitochondria was assessed by flow cytometry after labelling the cells with to the cytosolic oxidation probe CM-H_2DCFDA [24] and the mitotracker oxidation probe CM-H_2XRos[25], respectively. In the IVF control group, the majority of cells did not show high oxidation levels of both probes (80 % of the cells, Fig. 2C). However, a 3 h incubation, independent of the presence of H_2O_2, increased the peroxidation of the mitotracker probe. The amount of cytosolic specific oxidation remained similar to the IVF control treated sperm (Fig. 2C). On top of this induction of mitochondrial oxidation the incubation with the oxidant cocktail induced a significant increase in the oxidation of the cytosolic probe (Fig. 2C). Remarkably, cytosolic oxidation was not detected in cells that did not show oxidation of the mitotracker probe. This may implicate that cytosolic oxidation processes depend on radicals formed in the mitochondria.

Mild oxidative stress to sperm impairs zygote development but does not alter fertilization competence of sperm

After detecting the impact of the pro-oxidative stress on sperm cells we studied the fertilization competence of the sperm resulting from those incubations and followed the developmental competence of the zygotes formed. The control incubation without addition of oxidants gave similar cleavage rates followed by development of the zygote to 8 cell and blastocyst stadia as the IVF control (Table 1). The mildest oxidant treatment (100 µM H_20_2) did not affect fertilization rate and the developmental competence of the zygote formed (Table 1). Higher levels of oxidants (500 µM H_20_2) significantly affected the developmental capacity of the zygote as was detected at day 5 (% of embryos formed with ≥8 cells) and day 9 (% of blastocysts formed). The blastocyst rates dropped with 65 % compared to the IVF control (Table 1). Despite of the dramatic drop there was only a non-significant

and limited drop in the fertilization rate and amount of oocytes that reached a 8 cell stage at day 5 (a drop of a factor 16 % compared to the IVF control; Table 1). A much more pronounced effect was seen when sperm samples were used that were exposed to the oxidant cocktail. A total developmental block was detected at the 2 cell stage leading to the complete absence of 8 cell stage formation at day 5 and likewise also a full absence of blastocyst formation at day 9 (100% inhibition). Note here that the amount of cleaved embryos was also reduced approx. 50 % compared to the other incubations) thus the cocktail conditions also affected fertilization rate or zygote formation at an earlier stage than the first cleavage. From these data we derive that mild oxidative stress imposed on sperm negatively influences the reproductive processes at the level post-fertilization. However, the fertilization competence of the sperm cell and the activation of the fertilized oocyte remains intact.

Table 1
Percentages of embryos formed days 5 and 9 after fertilization of oocytes with sperm exposed to different conditions. Numbers are mean percentages of 8 independent experiments. * differences from the no oxidants control that are significant are indicated by * ($p<0.05$) and ** ($p<0.01$).

		Day 5		Day 9
Incubation	Nr of oocytes	% Cleaved	% ≥ 8 cells	% Blastocysts
IVF control	567	53 ± 3.9	14.5 ± 1.6	19 ± 2.4
No oxidants	562	51 ± 6.1	13.2 ± 3.3	16 ± 4.6
H_2O_2 100 µM	616	47 ± 6.5	15.6 ± 3.6	14 ± 3.6
H_2O_2 500 µM	594	44 ± 4.2*	5.1 ± 1.4**	6.8 ± 4.6**
Cocktail	608	26 ± 4.7**	0**	0**

The integrity of the developing embryos was further analysed by staining 5 days old embryos with Sytox-green (DNA), Phalloidine (microfilaments) and an anti-tubulin antibody (microtubules), followed by confocal laser scanning microscopy (CLSM). Embryos that were derived from control IVF sperm were at the 8-cell stage exhibited normal blastomeres with normal DNA and microfilament distribution (Figure 3A). Embryos that were derived form sperm cells that had been exposed to 500 µM H_20_2 were either normal or showed small abnormalities such as cellular extrusions. However, in the majority of these embryos, the blastomeres appeared

healthy and were cleaving normally (Figure 3B,C). Embryos that were derived from the sperm exposed to the oxidant cocktail did not develop beyond the two-cell stage (Figure 3D).

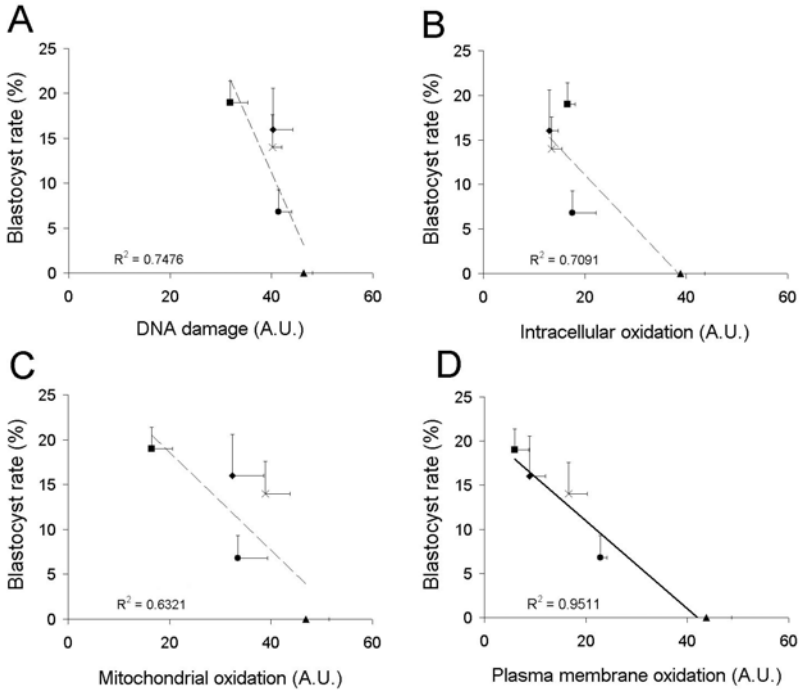

Figure 3
Correlation between the percentages of formed blastocysts and A) sperm DNA oxidation measured by 8-oxoguanine formation; B) sperm intracellular oxidation (CM-H_2DCFDA); C) sperm mitochondrial oxidation (CM-H_2Xros) and D) sperm plasma membrane oxidation (C11 Bodipy 581/591). ■ = IVF control; ♦ = No oxidants; x = 100 µM H_2O_2; ● = 500 µM H_2O_2; Oxidants cocktail. All correlations are negative (dotted lines), but only the correlation between blastocyst percentage and plasma membrane oxidation is significant (solid line, $p<0.05$) Values are means of three independent experiments. Vertical error bars are SEM for percentage of blastocyst mean, horizontal error bars are SEM of oxidation level means.

The degree of lipid peroxidation in the sperm plasma membrane significantly correlates with the developmental capacity of the zygote post-fertilization

Finally we correlated the type of oxidative damage observed in sperm cells with the percentages of blastocysts formed at day 9 in order to study which of the flow cytometric assays will be the most accurate for predicting fertilization competence of sperm. Clearly the degree of DNA damage was a poor indicator for

the degree blastocyst development (Fig. 4). The difference between 8-oxoguanine levels in IVF control sperm on the one hand, and sperm treated with the oxidants cocktail on the other was quite low (increase of approx. 50 %). In contrast blastocyst formation was completely blocked. Thus 8-oxoguanine formation on sperm DNA is a poor parameter for predicting the blastocyst formation. Both plasma membrane oxidation and mitochondrial oxidation in sperm negatively correlated with the blastocyst formation rate (Fig. 4). However, only the degree of C_{11}-Bodipy$^{581/591}$ probe oxidation at the sperm plasma membrane significantly correlated with the impaired blastocyst development. Finally, the blastocyst development did not correlate well with the degree of cytosolic oxidation most likely because cytosolic oxidation only occurred during sperm incubation in the oxidant cocktail oxidant but was independent from the amount of hydrogen peroxide in the other treatments (Fig. 4). Thus our results suggest that detection of the levels of lipid peroxidation at the sperm plasma membrane best predicts the developmental competence of oocytes that will be fertilized with a given sperm sample.

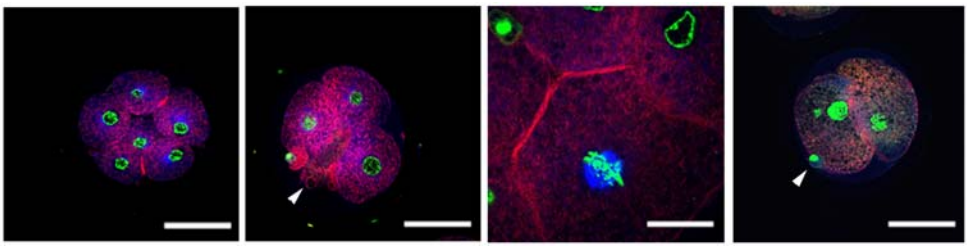

Figure 4
Confocal laser scanning microscopy (CLSM) photographs of day 5 cleaved embryos after fertilization with sperm under the different oxidant treatment and stained with Sytox green (chromatin); Alexa Fluor™ 568-phalloidine (red, actin) and indirect immunostaining with Alexa Fluor™ 633 goat anti-mouse antibody (blue, tubuline). A) Embryo originated from sperm under IVF control conditions with normal and evenly distributed blastomeres; B) Four-cell stage embryo originated from sperm exposed to 500 µM H_2O_2. Apart from the presence of cellular extrusions (arrowhead) the embryo is morphologically normal; C) Embryo originated equally from sperm under treatment with 500 µM H_2O_2 (8-cells embryo) with a detail of a mitotic spindle, indicating normal development; D) Embryo originated from sperm exposed to the oxidants cocktail arrested at the 2-cell stage and showing abnormal chromatin distribution and absence of tubulin asters. Arrow head denotes reminiscence of a polar body. Scale bar 50 µm in A,B,D, 20 µm in C.

Discussion

Many aspects of infertility and subfertility, both in human as in farm animal species are unknown but can be caused by oxidative damage, either in the oocyte or in sperm cells. The exact mechanisms of ROS-induced damage to gametes are

unclear but it has been described that for instance the presence of immature sperm cells [32] or leukocytes in the ejaculate [33,7] can lead to oxidative damage. Additionally, cryopreservation of sperm cells can reduce the tolerance for oxidative stress as the activities of antioxidant enzymes are decreased [34]. Particularly in farm animals, artificial insemination is most efficient when frozen-thawed sperm can be used. However, the efficiency of frozen-thawed sperm in artificial insemination is suboptimal, only about 35% of sperm cells survive freezing-thawing procedures and the quality of frozen-thawed sperm is less than that of fresh sperm (reference). Here, we exposed sperm cells to different reactive oxygen species that simulated possible scenarios these cells encounter and investigated subsequent cellular damage, fertilizing capacity and developmental potential after fertilization.

Lipid peroxidation was induced by the pro-oxidants ferrous (II) sulphate, ascorbate and H_2O_2 that can trigger LPO [35] via hydroxyl radical formation and by increasing the levels of thiobarbituric acid reactive substances (TBARS) such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), that originate from breakdown of lipid peroxides [35]. In sperm cells, spontaneous LPO occurs but at a lower level than that induced by ferrous sulphate and ascorbate [36] and MDA production is positively correlated with the sperm concentration and with the presence of immotile spermatozoa, but negatively correlated with superoxide dismutase (SOD) activity [37]. The instigated reactions for ROS formation were as follows:

- Fenton reaction: $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$ (1)
- Fe^{3+} + Ascorbate $\rightarrow Fe^{2+}$ + ascorbyl radical ($asc^{\bullet}$) (2)
- Haber-Weiss: $O_2^{-\bullet} + H_2O_2 \rightarrow O_2 + H_2O + OH^{\bullet}$ (3)
- ROOH (lipid hydroperoxyde) + $Fe^{2+} \rightarrow Fe^{3+} + RO^{\bullet}$ (alkyl radical)+ OH^{-} (4), or
  + $Fe^{3+} \rightarrow Fe^{2+} + ROO^{\bullet}$ (peroxy radical)+ H^{+} (5)

Despite its short half life the hydroxyl radical $OH^{\bullet}$ is a highly reactive molecule which can react with membrane phospholipid acyl chains (RH), initiating a series of reactions to adjacent lipids (see eqs. 4 and 5) but also to protein and DNA molecules [38]. The superoxide radical $O_2^{-\bullet}$ is a by-product of normal functioning mitochondria and can equally originate $OH^{\bullet}$ when in combination with H_2O_2, mediated by SOD (see eq. 3).

In our experiments mitochondrial and cytosolic oxidation were not strongly associated, possibly since ROS production rates are higher in the core of the mitochondria and only cause cytosolic oxidation after leaking from dysfunctional mitochondria. The fact that plasma membrane peroxidation (as demonstrated with C_{11}-Bodipy$^{581/591}$) correlated with mitochondrial peroxidation probably relates to the fact that the plasma membrane peroxidation is occurring in the mid-piece surface area [10] at the same area where mitochondria are localized. This may implicate as

suggested earlier that the mid-piece is the part of the sperm cells were radicals are less hampered by antioxidants to attack their substrates [10].

Curiously, the pro-oxidant treatments did not affect the integrity of the acrosome before fertilisation since the percentage of cells with an intact acrosome was similar to that of sperm cells with a non-oxidised plasma membrane after oxidative stress. This is an interesting observation, since a precocious acrosome reaction by pro-oxidants would impair fertilization [39]. On the other hand concentrations of 100 µM H_2O_2 are known to stimulate tyrosine phosphorylation and could therefore be beneficial for sperm capacitation without affecting the motility whilst 500 µM H_2O_2 has an inhibitory effect on tyrosine phosphorylation and a negative effect on motility [40,31,17]. At any rate our pro-oxidants treatments did not significantly alter fertilization potential nor the membrane integrity of the incubated sperm.

Despite the induction of molecular oxidation in sperm cells by the pro-oxidant treatments our data show that those subjected to hydrogen peroxide sperm did not affect their potential to fertilize bovine oocytes. However, in combination of Fe^{2+}/ascorbate the fertilization rate was inhibited and the developmental competence of the zygote was blocked at the 2 cell stage. In absence of hydrogen peroxide Kodama *et al.* [41] who described that incubation of sperm with increasing concentrations of Fe^{2+}/ascorbate, improved fertilization rates with 36% and 50% for 20/100 µM and 0.4/2.0 mM of Fe^{2+}/ascorbate, respectively. On the other hand incubation in presence of hydrogen peroxide was reported to decrease sperm – oocyte fusion [31]. Despite of these two finding we did not detect effects of pro-oxidants on fertilization and first cleavage rates but they adversely affected zygote developmental competence.

Importantly, our observations showed that the developmental competence of the oocytes that were fertilized with sperm that was treated with low concentrations of H_2O_2 [100 µM] showed no decrease in developmental competence whereas higher [500 µM] were very inhibitory. It has been described that zygotes are more susceptible to H_2O_2 than 8-16-cells embryos [12]. Possibly the zygote may still develop after fertilization by pro-oxidant attacked spermatozoa when a sufficient activation of a repair mechanism in the oocyte can restore the peroxidized molecules [11,12]. In the bovine, the embryonic genome is activated at the 8-16cell stage and is accompanied by de novo glutathione (GSH) synthesis [42], an increased requirement for ATP, and increased protein synthesis [43,44]. We showed that at higher levels of hydrogen peroxide indeed inhibited or blocked embryo development before the 8 cell stage. With this respect it is important to consider the conditions for maturation of oocytes: The maturation medium may raise the intracellular content of molecules (such as ß-mercaptoethanol, cystein, and GSH) that have been demonstrated to increase the resistance to oxidative stress and maintaining the redox-regulated electron flux [45]. In our study cysteamine was

present in the maturation media. Cysteamine can increase the intracellular GSH concentration in oocytes [46] and can enhance the resistance to oxidative damage [42].
In all the incubations studied the oocyte played an important role in the ability to buffer the damage brought by the sperm, given that except for the cocktail treatment all other incubations of sperm cells showed embryo development up to day 9 after fertilization. The 2-cell block observed in zygotes fertilized with sperm exposed to the cocktail may have been caused by the strong peroxidation inflicted on the sperm by the pro-oxidants mixture. The sperm cell although viable may contained intracellularly trapped radicals that may have worked on the fertilized oocyte and compromised metabolic pathways. We believe that the first cleavage block has become manifest for cocktail treated sperm as they contain molecular peroxidation above the threshold of reparable levels for the antioxidants in the oocyte. In analogy a naturally occurring developmental block at the 2-cell stage has been associated with a reduced concentration of anti-oxidant enzymes [47].
Our results also indicated that the degree peroxidation of C_{11}-Bodipy$^{581/591}$ in bovine sperm has the best predictive value to the competence of the oocyte that becomes fertilized with the corresponding sperm samples. Assays for mitochondrial, cytosolic and DNA oxidation were less predictive or not suitable to this end.
In conclusion, oxidative stress of sperm cells can result in damaged cell structures. Oxidative damage, particularly intracellular oxidation and plasma membrane oxidation, diminishes to a small extent the sperm's capacity to fertilize an oocyte and form an embryo after fertilization. Apparently mechanisms in the oocyte can repair the oxidative damage of the sperm. When sperm cells have received more oxidative damage, the cells are still ably to fertilize, although to a reduced extent, but further embryo development of these fertilized oocytes is severely limited. A further understanding of the mechanisms of sperm oxidative damage might help to prevent or rescue the negative consequences of oxidative sperm damage that occurs in vivo and in vitro.

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CHAPTER 6

Summarizing discussion

P.F.N. Silva

Reactive oxygen species (ROS) are produced during normal cellular activity under aerobic conditions. The intracellular homeostasis is, among other factors, a balance between ROS production and antioxidant systems. Conditions where ROS production surpasses the antioxidant capacity are related to various pathologies, for instance certain types of cancer, neurodegenerative diseases, atherosclerosis, and reproductive disorders. Results from studies of ROS effects on reproduction have been important for understanding human infertility and may help to find solutions to overcome it. An increasing number of couples worldwide face subfertility problems and have to resort to assisted reproductive techniques (ART), particularly artificial insemination (AI) or in vitro fertilization (IVF). In addition, AI using cryopreserved sperm has been applied for more than 50 years in animal breeding [1,2]. The main benefits of the use of sperm cryopreservation are the dissemination of genetic values across continents and the improvement of the efficiency in animal production systems while simultaneously reducing the risk of disease transmission. Moreover, gamete cryopreservation has become a resource for banking germ cells of species and breeds facing danger of extinction. For human reproduction, sperm cryopreservation is most valuable as a tool for couples with infertility problems, to store sperm from donors but also to preserve sperm from men that have to undergo cancer treatment.

Although cryopreservation presents several advantages it still faces serious handicaps as freezing protocols cause damage to the sperm plasma membrane. Freeze-thawing does not only affect lipid phase behavior, but also causes lateral redistribution and conformational changes of membrane proteins possibly affecting their functions [3-5]. These post-thaw membrane deviations may compromise adequate progression of sperm cells in the female tract and their fertilizing ability. Addition of egg yolk to the freezing extender before cryopreservation reduces membrane damage possibly due to the high content of antioxidant compounds (vitamins E, A) and lipids [6,7] . A relatively high amount of cholesterol in the sperm plasma membrane relates to improved cryoresistance [8]. Other lipid components of sperm membranes also determine the cryoresistant properties of sperm, for instance the degree of unsaturated fatty acids in phospholipids [9,10]. The aim of this thesis is to relate molecular peroxidation and sperm damage upon cryopreservation.

Assaying lipid peroxidation, from TBARS to MS.

It is generally believed that cryopreserved sperm is more susceptible to oxidative stress than fresh sperm. Hence it is important for AI and particularly for IVF that valid methods are available and developed to assess sperm resistance to cryopreservation or to detect post-thawing damage. Until recently the thiobarbituric acid reactive substances (TBARS) assay was the common method to assess lipid peroxidation (LPO), by accounting the levels of malondialdehyde (MDA). Unfortunately, this method can only give an estimation of LPO. The main pitfalls with this assay reside on the difficulty to distinguish between oxidized lipid classes and an overestimation of LPO due to MDA production by substrates other than lipids. In chapter 2 it is shown how new assays using mass spectrometry (MS) alone or hyphenated with high performance liquid chromatography (HPLC) can detect LPO. With these techniques we resolved oxidation patterns of cholesterol and molecular species of phospholipids such as phosphatidylcholine (PC). Oxidised phospholipids and cholesterol were identified in the plasma membranes of bovine frozen-thawed sperm, in particular hydroxyl- and hydroperoxyphosphatidylcholine and ketocholesterol and epoxycholesterol (α and β), respectively. These oxidized lipid species are produced through the actions of lipid peroxides (ROOH), peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals on membrane lipids. In addition, the fluorescent fatty acid analogue C_{11}Bodipy $^{581/591}$ has been implemented to detect fatty acid peroxidation. We have used this probe to localize and quantify fatty acid peroxidation in sperm cells by confocal laser scanning microscopy and flow cytometry respectively. Fatty acid peroxidation was found predominantly at the surface of the midpiece and cytoplasmic droplets of porcine sperm cells. Importantly probe peroxidation was virtually absent in the sperm head. This implies that the sperm head is more protected against radical attack to lipids. In analogy it is possible that the paternal DNA present in the sperm head is better protected against radical attack by the same defense mechanisms.

Taken these assays together one can say that assessing LPO profiles prior to and after preservation is useful to determine the resistance of sperm to cryopreservation and what types of antioxidants are appropriate to protect sperm from oxidation thus increasing post-thaw survival. It should be noted that there are studies showing that mild LPO formation predisposes the sperm to capacitation and acrosome reaction [11]. Apparently, the sperm needs to go through this chaotic lipid rearrangement to acquire fertilizing ability. The described assays may therefore also be useful to assess how mild oxidation can improve IVF outcome.

What types of damage can we detect? Is it all that bad?

Detecting damage in mammalian sperm cells is extremely valuable when sperm samples are to be used for cryopreservation or IVF. Particularly in human medicine it is important to detect DNA damage before fertilization. Chapter 3 gives a comprehensive description of the techniques used to assess sperm damage at different cellular levels (integrity assessment of organelles, membrane lipids and DNA). The integrity of sperm DNA is of importance for the normal development of the future embryo. DNA deterioration may inhibit or even block embryonal development after the first cleavage(s) when embryonic DNA transcription is initiated. In contrast, sperm membrane integrity (at the level of the sperm's surface, acrosome or mitochondria) is of importance for processes leading to fertilization. Their functional integrity is required for zona pellucida binding, zona penetration and the fertilization fusion of both gametes. After this sperm membrane structures are degraded by the fertilized oocyte. Besides sperm integrity assays this chapter also describes how the organisation and peroxidation of lipids in the sperm plasma membrane can be analysed and followed during sperm capacitation or before and after cryopreservation.

Cholesterol oxidation - Is the COPs brigade friend or foe?

Lipids are main components of biological membranes and thus are crucial for biological functions ranging from membrane trafficking to signal transduction. Consequently, the lipid composition affects the organization and properties of membranes, so it is not surprising that disorders in lipid metabolism and transport have a role in human disease such as atherosclerosis. Cholesterol oxidation products (COPs) are no exception and are associated with degenerative diseases such as cancer and Alzheimer's disease. In chapter 4 the profiles of COPs in fresh ejaculated sperm, frozen-thawed sperm and sperm exposed to oxidative stress were assessed. Surprisingly, COPs are already present in ejaculated sperm and their levels remained similar after the aforementioned treatments. This suggests that cholesterol oxidation is different from phospholipid oxidation. The latter was almost absent in fresh ejaculated sperm but became drastically increased when sperm underwent freeze-thawing or was exposed to oxidative stress.
Desmosterol was shown to represent 16 % of all total sperm sterol content. Desmosterol only differs from cholesterol by one extra double bond between carbons 24 and 25. Due to this extra double bond desmosterol is expected to be a better peroxidation substrate. However, no desmosterol oxidation products (DOPs) were detected. It is hypothesized that desmosterol may be located in intracellular membranes that may not be exposed to extracellular oxidative stress.

Exposure to pro-oxidants dramatically increased the total COPs content, but surprisingly did not elevate the concentration of 3,5,6 cholestane-triol, the most toxic COP. In contrast, reconstituted lipid vesicles from sperm membranes exposed to the same pro-oxidants showed a high increase of 3,5,6 cholestane-triol. These findings suggest that sperm can metabolize toxic COP, however, cholesterol and COP metabolic studies are required to proof this suggestion.

LPO may cause changes in the lipid arrangements in the membrane that enhance the membrane fluidity and the bilayer-permeability to substances that normally need special channels to pass through the sperm plasma membrane's lipid bilayer (e.g. Ca^{2+} ions). Such rearrangements may also inactivate membrane-bound enzymes. In addition, LPO may cause protein oxidation (for instance by cross-linking membrane proteins thereby limiting their lateral and rotational mobility) [12]. Similarly, capacitation of sperm is also a multi-step process in which membrane rearrangements occur and result in increased membrane permeability and fluidity. These membrane rearrangements may be similar to oxidation-mediated rearrangements and appear to correlate with alterations in sperm motility and receptiveness for acrosome reaction inducers. The effects endured by LPO or capacitation on the sperm's plasma membrane have some degree of similarity and may therefore be connected.

Interestingly, cholesterol-enriched microdomains of the plasma membrane that are also designated as lipid rafts have been implicated in membrane binding including zona pellucida (ZP) binding [13,41]. Cholesterol depletion destabilizes lipid rafts and suppresses protein tyrosine phosphorylation [14,41]. During capacitation cholesterol loss coincides with redistribution of raft marker proteins in the sperm head [14,41] and this redistribution might be a key regulatory step in capacitation [15]. Cholesterol oxidation probably disturbs the normal function of membranes [16] and signal transduction pathways likewise may become altered when lipid rafts are disrupted by cholesterol depletion [17]. Hence, in sperm cells post-ejaculatory cholesterol oxidation may be needed for signaling cascades that enable the sperm cell to capacitate. In analogy, other cell types have been shown to be able to convert cholesterol into oxysterols (important signaling molecules) [18]. Another aspect to consider is that once cholesterol is oxidised, desmosterol may play a more important role in raft stability, and thus may participate in (re)organization of sperm lipid rafts [15]. This hypothesis is corroborated by studies in membranes lacking cholesterol, where biophysical properties like lipid packing were not affected by replacement of cholesterol with desmosterol and where organism homeostasis did not suffer any impact [19,20].

Sperm under stress, "savoir faire" of the oocyte, fertilization beats the odds. A 4-well-dish story.

Despite what is known about deleterious and beneficial effects of ROS/RNS on molecular substrates and the roles they might play in sperm to acquire fertilizing ability, there is a lack of information concerning effects for the embryo in case of fertilization. As discussed previously, freezing-thawing inflicts a certain membrane injury by lipid oxidation affecting lipid phase behavior and membrane stability, enabling possible DNA damage. How does the embryo develop when sperm has been damaged prior to fertilization? And can this damage be compensated by the oocyte?

In chapter 5 we examined how oxidation would influence sperm fertilizing ability and subsequent embryo development. The oxidative stress imposed imitated conditions that sperm cells can encounter within either the male and female reproductive tract both in physiological and pathological conditions (inflammation). Surprisingly, we demonstrated that despite oxidation fertilization still took place.

At mild oxidation conditions (100 µM H_2O_2 and 500 µM H_2O_2) a certain percentage of embryos still developed to blastocysts. When sperm was exposed to an oxidative cocktail (500 µM H_2O_2 and 20/100 µM Fe^{2+}/Ascorbate) fertilization took place but first cleavage rates were low. Possibly the sperm cells, although viable, may still contained inside-trapped radicals, that were brought into the oocyte upon fertilization and compromised metabolic pathways after pronucleus fusion. In the oxidative cocktail a transition metal (Fe^{2+}) is available to trigger further radical formation (e.g. $OH^{\bullet}$) via Fenton and Haber-Weiss chemistry. Moreover H_2O_2 easily diffuses through cell membranes, and thus can trigger further oxidation, either directly or via $OH^{\bullet}$ itself, to molecular substrates like lipids (lipid peroxides), proteins (protein peroxides, oxidation of amino acid side chains) and DNA (base oxidation, nicks, strand breaks). This additional oxidative damage possibly causes the observed impaired development. Indeed, a 2-cell block has been associated with abnormal cell division, mitochondrial dysfunction and an increase of lipid peroxides by an increased H_2O_2 concentration [21,22,23]. It is also known that susceptibility to H_2O_2 is higher in zygotes than in 8 to16-cell embryos [24], and in case of fertilization with DNA damaged spermatozoa, the embryos may still recover by using a repair mechanism mainly inherited from the oocyte [25,24]. Supplementation of maturation media with the components ß-mercaptoethanol, cystein, or glutathione (GSH) limit peroxidative processes by boosting the antioxidant capacity of cumulus-oocyte complexes (COCs) and maintaining the redox potential (radical-trapping antioxidant ability) of the oocyte [26]. For that reason we used cysteamine in the maturation media known to improve the intracellular GSH content of oocytes [27]. Indeed the oocyte played an important role to buffer the

damage brought by the sperm, given that except for the oxidative cocktail all sperm treatments showed embryo development to blastocysts although at a reduced level. Interestingly, adverse reproductive effects of pro-oxidant treatments to sperm only became manifest after the the first cleavage of fertilized oocytes. This could indicate that sperm DNA damage caused these effects as is shown for radiated sperm [42]. Experimental data do not support this as DNA oxidation to sperm was minimal under the pro-oxidative conditions and showed no or only weak correlations with the two cell embryonic development block.

Curiously, the degree of LPO did significantly correlate with impaired embryo development. Despite of this LPO did not affect the integrity of the acrosome before fertilisation, in fact the percentages of cells with intact acrosomes were similar to the percentages of cells with non-oxidized plasma membranes. This is an important observation, since acrosome damage or a precocious reaction (LPO induced or not) would impair fertilization [28]. We do not have an explanation for the correlation of sperm LPO to the post-fertilisation developmental competence of the embryo. Nevertheless, the results suggest that the oocyte possesses repair mechanisms for oxidative damage of sperm molecules.

It is known that at lower concentrations (100 µM) H_2O_2 stimulates tyrosine phosphorylation and can be thus beneficial to sperm capacitation, without affecting sperm motility whereas higher concentrations (500 µM) of H_2O_2 can have a reducing/inhibitory effect on tyrosine phosphorylation and negative effect on sperm motility [29,30,31]. Regardless of the opposite effects on capacitation of these two H_2O_2 concentrations in our experiments there were no differences between sperm exposed to 100 µM or to 500 µM H_2O_2 when cleavage rates and embryo development were concerned, despite the 3 h incubation prior to IVF. In contrast, cleavage rate (within this class the percentage of embryos $\geq$ 8 cells) and percentage of blastocysts were decreased for sperm subjected to 500 µM H_2O_2 comparatively to No-oxidants and IVF control groups.

Occurrence of capacitation prior to IVF was unlikely since sperm was incubated in absence of bicarbonate and 5% CO_2 in air (sperm capacitation ingredients). However the oxidative effects of H_2O_2 when present and brought to the oocyte upon fertilization, may have been repaired by the anti-oxidant system of the female gamete. This is obvious with absence of differences between 100 µM and 500 µM H_2O_2 and the significant differences between 500 µM H_2O_2 and the groups that were not incubated with any oxidants.

Other aspects of oxidized substrates in reproduction remain unclear. One is the nitrosylation (via $NO^{\bullet}$) of tyrosine residues in sperm proteins and its impact on sperm physiology. In chapter 1 it is mentioned that $NO^{\bullet}$ can improve the capacity of sperm to fuse with the oocytes but $NO^{\bullet}$ can also affect tubal contractibility [32] and have a paracrine function in the uterus [33]. Inhibition of nitric oxide synthase (NOS)

significantly reduced fertilization without affecting zona pellucida binding [34,35]. Although several studies demonstrated the presence of neuronal NOS on the acrosome and tail of sperm cells [36] and endothelial NOS activity in the endometrium, fallopian tubes and follicular fluid [33], there are no reports about localization of nitrosylated tyrosine residues or possible effects of this modified amino acid on reproductive outcome. Although not presented in this thesis, pilot experiments were performed to localize nitrosylated tyrosine residues in bovine sperm using immunocytochemistry. Nitrosylated tyrosine residues were localized in the acrosome cap of the sperm cell, despite the presence of the NOS inhibitor L-NAME [37,38,39,40,34]. Future experiments should include FACS analyses to assess percentages of cells exhibiting nitrosylated tyrosine residues under capacitating and non-capacitating conditions. With this respect, immunoblot analysis revealed three proteins with molecular weights of approximately 64, 70 and 116 KDa. It is known that $NO^{\bullet}$ regulates sperm capacitation and tyrosine phosphorylation via a cAMP-PKA signalling pathway [37,38] and double phosphorylation of the threonine-glutamine-tyrosine motif in p81 and p105 KDa sperm proteins occurs during capacitation by $NO^{\bullet}$ action [40]. Even though the protein molecular weights were different between the blots with anti-phosphotyrosine and anti-nitrotyrosine, as were their amino acid moieties, it is hypothesized that tyrosine nitrosylation and phosphorylation may occur hand in hand during sperm capacitation and be a necessary step for acrosome reaction and fusion with the oolema. A research topic for the Future…

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Samenvatting

Gewoonlijk worden de effecten van vrije radicalen in cellen geassocieerd met ziekte en celdood. Dit ontstaat door een verkeerde balans tussen vrije radicaalproductie enerzijds en de werking van anti-oxidanten anderzijds, en het onvermogen om deze balans te herstellen.
Gedurende de differentiatie en rijping van gameten komen zowel eicellen als spermacellen in contact met zuurstofradicalen en stikstofradicalen. De mate van radicaal gemedieerde beschadiging van moleculen zal voor een deel bepalen welke geslachtscellen als beste geselecteerd worden en daardoor de rijping kunnen voltooien. Bovendien is aangetoond dat bepaalde radicaalconcentraties de activiteit van diverse signaaltransductiepaden kunnen beïnvloeden. Deze signaaltransductiepaden zijn betrokken bij de capacitatie van de zaadcel, de rijping van de eicel en het gereedmaken van de eicel voor de bevruchting. Mede als gevolg hiervan is de eicel na de bevruchting in staat het arrest van het metafase II stadium van de meiose op te heffen.
In dit proefschrift worden allereerst methoden beschreven om schade in spermacellen op te sporen, vervolgens worden studie's beschreven waarin de detectie van peroxidatie van moleculaire substraten wordt beschreven en de effecten van oxidatieve stress op de bevruchtingscapaciteit van spermacellen en op de vroeg-embryonale ontwikkeling.
In hoofdstuk 1 wordt een uitgebreid overzicht gegeven van de verschillende aspecten van moleculaire oxidatie en de effecten hiervan op zoogdierreproductie. Oxidatieve modificaties die optreden bij lipiden, eiwitten en DNA van zaadcellen worden in dit hoofdstuk beschreven. Daarbij wordt speciale aandacht besteed aan de mogelijke invloed van deze processen op de differentiatie en de rijping van geslachtscellen.
In hoofdstuk 2 worden nieuwe technieken beschreven die gebruikt kunnen worden om oxidatie van lipiden in de plasmamembranen van zaadcellen te detecteren. Het gaat hierbij voornamelijk om oxidatie van cholesterol en van het fosfolipide, fosfatidylcholine. De mate van oxidatie is bestudeerd zowel voor als na een invries-ontdooi procedure. Invriezen en ontdooien ten behoeve van het cryopreserveren van zaadcellen lijkt de oxidatieve schade aan fosfolipiden te versterken en kan daardoor de bevruchtingscapaciteit van ontdooide zaadmonsters verminderen.
In hoofdstuk 3 worden andere technieken beschreven om de integriteit van zaadcellen te bestuderen. De principes van bepalingen voor de integriteit van de plasma membraan, de acrosoom en de mitochondriën alsmede van het DNA van de zaadcel worden beschreven. De integriteit van het zaadcel DNA is van belang

voor een normale ontwikkeling van het toekomstige embryo. Beschadigingen van het DNA in de zaadcel kan resulteren in een remming of blokkering van de embryonale ontwikkeling na de eerste klievingsdeling(en). Echter, de integriteit van de mitochondria, de acrosoom en de plasmamembraan van zaadcellen is slechts voorafgaand en tijdens de bevruchting van belang. De integriteit ervan is van belang voor de binding aan de zona pellucida, de zona penetratie en de binding en fusie aan de eicelplasmamembraan. Daarna worden deze structuren door de bevruchte eicel afgebroken. Naast deze integriteitsbepalingen wordt in dit hoofdstuk beschreven hoe de organisatie en de oxidatie van lipiden in de plasmamembraan van zaadcellen kan worden geanalyseerd en gevolgd gedurende de zaadcelcapacitatie en voor en na cryopreservering.

In dit proefschrift wordt de oxidatie van cholesterol in het plasmamembraan van de spermacel in detail beschreven. De specifieke aandacht voor cholesterol komt voort uit het gegeven dat dit molecuul enerzijds van belang is voor de stabilisatie van membranen en anderzijds een belangrijke precursor is voor verschillende steroïd-hormonen die betrokken zijn bij de rijping van gameten. Daarnaast is de concentratie en laterale organisatie van cholesterol van belang bij de capacitatie van zaadcellen en daarmee tevens in een optimale zaadcel-zona pellucida interactie die cruciaal is voor de bevruchting van de eicel. In hoofdstuk 4 wordt geconcludeerd dat oxidatie van cholesterol al heeft plaatsgevonden in geëjaculeerd sperma, maar dat niveau's van oxysterolen of oxidatieproducten van cholesterol (COPs) gelijk bleven na invriezen-ontdooien van sperma. Dit is in tegenstelling tot geoxideerde niveau's van fosfatidylcholine. Als zaadcellen werden blootgesteld aan pro-oxidanten namen de niveau's van COPs significant toe, maar de concentratie van de meest toxische COP (3,5,6-cholestaan-triol) bleef gelijk. Dit suggereert dat de spermacellen oxysterolen kunnen metaboliseren maar om dit hard te kunnen maken is nader onderzoek noodzakelijk.

De organisatie van membraanlipiden in zaadcellen kan veranderen na cryopreservering van zaadmonsters of door incubatie van zaadmonsters met oxidatieve stressoren. Deze veranderingen zouden de bevruchtingskans van een eicel kunnen verminderen. Zowel cryopreservering als pro-oxidantia kunnen tevens het zaadcel DNA beschadigen hetgeen de embryonale ontwikkeling kan remmen/blokkeren. Daarom is onderzocht of en hoe pro-oxidanten de bevruchting en de vroege embryonale ontwikkeling kunnen beïnvloeden. Hiervoor werden zaadcellen blootgesteld aan pro-oxidanten voordat ze gebruikt werden voor in vitro fertilisatie. De bevruchtingspercentages werden gescoord, de bevruchte eicellen vervolgens in vitro gekweekt en de ontwikkeling van deze embryo's vastgesteld tot 9 dagen na bevruchting. In hoofdstuk 5 werden spermacellen experimenteel blootgesteld aan oxidatieve stress waarbij scenario's nagebootst werden van oxidatieve stress waaraan spermacellen worden blootgesteld in het mannelijk of

vrouwelijk geslachtsapparaat, zowel onder fysiologische als pathologische (ontsteking) omstandigheden. Er werd waargenomen dat blootstelling van spermacellen aan pro-oxidanten het proces van bevruchting niet beïnvloedde. Er werd echter een groter percentage blastocysten gevormd wanneer spermacellen waren blootgesteld aan lagere concentraties van oxidanten, terwijl de embryonale ontwikkeling tot blastocysten volledig geblokkeerd was op het twee-cellige stadium wanneer spermacellen vóór bevruchting waren blootgesteld aan drie verschillende oxidanten. Deze blokkade in embryonale ontwikkeling was geassocieerd met abnormale celdeling, abnormaal functioneren van mitochondria en verhoging in de oxidatie van lipiden. Op eerste gezicht zouden deze effecten verklaard kunnen worden door de oxidatie van zaadcel DNA die er zorg voor draagt dat de embryonale ontwikkeling na de eerste klieving gaat stoppen (na begin van de expressie van het embryonale genoom dus post-fertilisatie). Echter experimenteel kon er geen correlatie aangetoond worden tussen de mate van DNA oxidatie (gedetecteerd met 8-oxoguanine) en de remming van de embryonale ontwikkeling. De mate van lipiden-peroxidatie in zaadcellen correleerde daarentegen juist wel met verslechterde embryonale ontwikkeling. Hoewel er geen verklaring voor deze correlatie is suggereert dit resultaat dat de eicel een belangrijk herstelmechanisme heeft om de oxidatieve schade die een spermacel heeft ondergaan teniet te doen na de bevruchting.

Deze resultaten kunnen bruikbaar zijn voor de humane voortplantingsgeneeskunde waarbij spermacellen van mannen met verminderde vruchtbaarheid gebruikt kunnen worden in IVF klinieken. Nadat deze spermacellen blootgesteld zijn aan een specifieke cocktail van anti-oxidanten kan door de vermindering van oxidatieve schade mogelijk het succes van bevruchting, de hoeveelheid embryo's, de kwaliteit van de gevormde embryo's en uiteindelijk de kans op een succesvolle zwangerschap vergroot worden.

Sumário

Os efeitos de radicias livres em células está geralmente associado a patologia e morte celular. A patologia é derivada de um desequilíbrio entre a produção de radicais livres e a capacidade antioxidante, associado à inaptidão da célula de restabelecer o equilíbrio.
Gâmetas são células muito particulares, na fêmea o número de oócitos já está estabelecido aquando do nascimento, enquanto que nos machos a produção de esperma dá-se de forma continua durante a vida reprodutiva.
Durante a diferenciação e maturação, ambos esperma e oócito são alvo de espécies reactivas de oxigénio e nitogénio (ROS e RNS, respectivamente) que dentro de certos limites contribuem para a selecção das melhores células germinais que terão a chance de prosseguir para o último estágio de maturação. Para além disso, estudos têm demonstrado que dentro de determinadas concentrações ROS e RNS podem ter um papel nas cascadas de sinalização que conduzem o esperma à aquisição de capacidade fertilizadora e os oócitos na progressão da maturação até ao estágio em que se encontram receptivos para serem fertilizados pelo espermatozóide e consequentemente sairem da clausura da metaphase da meiose II.
Os capítulos apresentados nesta tese descrevem primeiramente metodologias usadas na detecção de danos em células espermáticas e secondariamente estudos sobre detecção de peroxidação de determinados substratos moleculares e efeitos do stress oxidativo na capacidade fertilizadora do esperma e subsequente desenvolvimento embrionário.
No capítulo 1 são revistos extensivamente os aspectos da oxidação molecular e os seus efeitos nos resultados reprodutivos de mamíferos. Aqui são descritos mecanismos de oxidação de lípidos, proteinas, DNA e como estes podem afectar as células germinais durante diferentes estágios de diferenciação e maturação.
No capítulo 2 são então descritas novas metodologias para detectar oxidação na membrana plasmática da célula espermática, nomeadamente fosfolipidos (fosfatidilcolina) e cholesterol antes e depois de congelamento-descongelamento. Este último está inerente à criopreservação, o que parece agravar os padrões de oxidação daquele determinado fosfolipido, reduzindo o potencial fertilizador do esperma.
Para além dos lipidos, é descrito no capítulo 3 outros métodos para detectar danos em células espermáticas. Por exemplo, em DNA e mitocondria, o primeiro devido à sua importância no futuro *conceptus*, o segundo porque é um organelo

responsável pela produção de energia que permite à célula espermática movimentar-se dentro do aparelho reprodutivo feminino e fertilizar o oócito.
É também descrito como avaliar a integridade do acrosoma no semen fresco, armazenado em forma liquida e criopreservado. Para além disso são também descritos métodos de avaliação da organização dos lipidos na membrana plasmática e a sua relação com os estados de capacitação e integridade do acrosoma.
Nesta tese é também descrita detalhadamente a oxidação de cholesterol em membranas de células espermáticas. Este foco de interesse é principalmente devido ao papel estabilizador desta molécula nas membranas e ao papel importante como precursor de várias hormonas implicadas na maturação de células germinais. No capítulo 4 é concluido que a oxidação de cholesterol já está presente em esperma ejaculado, e os níveis de oxiesterois ou COPs (produtos de oxidação de colesterol) permanecem semelhantes após congelamento-descongelamento, contrariamente ao que se observou com a fosfatidilcolina. Contudo, quando o esperma é exposto a pro-oxidantes as concentrações de COPs aumentaram significativamente, mas a concentração do COP mais tóxico (3,5,6-cholestanetriol) permaneceu inalterada. Isto sugere a presença de um potencial mecanismo metabólico na célula espermática que retira este COP da membrana.

Dados estes resultados prévios sobre a forma como os lipidos da membrana podem ser afectados pelo congelamento-descongelamento e incubação com agentes oxidativos stressantes, procuramos determinar a extensão em como os pro-oxidantes podem afectar a fertilização e subsequente desenvolvimento embrionário. Células espermáticas foram incubadas com pro-oxidantes antes de se proceder a IVF e o desenvolvimento embrionário avaliado até ao dia 9 *post*-fertilização. É demonstrado no capítulo 5 que o stress oxidativo imposto ao esperma imita possíveis cenários que as células espermáticas pode encontrar quer no aparelho reprodutivo masculino quer no feminino tanto em condições fisiológicas como patológicas (inflamação).
Foi observado que os pro-oxidantes não impediram a fertilização de tomar lugar, mas que a colheita de blastocistos foi maior para concentrações mais baixas de oxidantes e esteve completamente ausente quando três tipos de oxidantes foram usados simultâneamente (concentração mais elevada), apresionando o zigoto no estágio de 2-células. Este bloqueio foi associado com divisão celular anormal, disfunção da mitochodria e um aumento em peroxidação lipídica. Para a maior concentração de H_2O_2 observou-se uma queda na percentagem de blastocistos no dia 9, apesar de não ter sido significantemente diferente da concentração mais baixa de H_2O_2. Apesar destes efeitos sugerirem que o DNA do esperma esteve

sujeito à oxidação danificadora e por isso impediu o desenvolvimento embrionário após a primeira clivagem, não foi encontrado forte correlação entre estes efeitos através da avaliação da oxidação de DNA (detecção de 8-oxoguanine). Em contraste, os niveis de peroxidação de lipidos esteve fortemente correlacionada com o impedimento do desenvolvimento embrionário. Apesar de não termos uma explicação para esta correlação, este resultado sugere que o oócito possue um importante mecanismo de reparação do dano oxidativo transportado pelo espermatozóide aquando da fertilização, sobretudo devido à sua capacidade antioxidante.

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Curriculum Vitae

Patrícia da Fonseca Nunes da Silva was born on 22nd September 1972 in Lisbon, Portugal. In 1991 she initiated her University degree in Agricultural Engineering, specialization Animal Husbandry, at the Instituto Superior de Agronomia, Technical University of Lisbon. During her study she was an active member of the International Association of Agricultural Students as an activity platform coordinator, representing Portugal in international meetings of this association. She was also a member of the Faculty football and basketball teams.

In 1996-97 she did an Erasmus exchange program with the Wageningen Agricultural University where she developed a research project concerning hormone profiles and follicular growth in cycling sows. In 1997 she graduated in the top 30% of her Course and later that year she was awarded with a research grant to initiate young scientists in research, by the Rector of the Technical University of Lisbon. She worked at the Lisbon Veterinary Faculty, where she developed a molecular biology project entitled "Identification and cloning of new xylanase encoding genes from the anaerobic bacterium *Clostridium thermocellum*".

In the end of 1998 she got a grant from the University of Aberdeen to do a further specialization with an MSc in Animal Production. There she did a research project concerning changes in the relationship between fetal size and organ development during pregnancy in the pig. She also represented her classmates at the University's PostGrad Agricultural and Forestry Society.

In 1999 she got a position as a research assistant at De Montfort University in England, and was involved in a project dealing with effects of free radicals in domestic livestock semen during liquid storage. Later she was awarded a PhD grant from the Foundation for Science and Technology, Portuguese Ministry for Science, Technology and Higher Education. She initiated her PhD in July 2001 at the Faculty of Veterinary Medicine in Utrecht under supervision of Prof. Dr. Ben Colenbrander and Prof. Dr. Bert van Golde who later retired and was substituted by Prof. Dr. Bernd Helms.

Canto I dos Lusíadas (Luís Vaz de Camões, 1524-1580)

As armas e os Barões assinalados
Que da Ocidental praia Lusitana
Por mares nunca de antes navegados
Passaram ainda além da Taprobana,
Em perigos e guerras esforçados
Mais do que prometia a força humana,
E entre gente remota edificaram
Novo Reino, que tanto sublimaram;

E também as memórias gloriosas
Daqueles Reis que foram dilatando
A Fé, o Império, e as terras viciosas
De África e de Ásia andaram devastando,
E aqueles que por obras valerosas
Se vão da lei da Morte libertando,
Cantando espalharei por toda parte,
Se a tanto me ajudar o engenho e arte.

Cessem do sábio Grego e do Troiano
As navegações grandes que fizeram;
Cale-se de Alexandro e de Trajano
A fama das vitórias que tiveram;
Que eu canto o peito ilustre Lusitano,
A quem Neptuno e Marte obedeceram.
Cesse tudo o que a Musa antiga canta,
Que outro valor mais alto se alevanta.

E vós, Tágides minhas, pois criado
Tendes em mi um novo engenho ardente,
Se sempre em verso humilde celebrado
Foi de mi vosso rio alegremente,
Dai-me agora um som alto e sublimado,
Um estilo grandíloco e corrente,
Por que de vossas águas Febo ordene
Que não tenham enveja às de Hipocrene.

Dai-me üa fúria grande e sonorosa,
E não de agreste avena ou frauta ruda,
Mas de tuba canora e belicosa,
Que o peito acende e a cor ao gesto muda;
Dai-me igual canto aos feitos da famosa
Gente vossa, que a Marte tanto ajuda;
Que se espalhe e se cante no universo,
Se tão sublime preço cabe em verso.

E, vós, ó bem nascida segurança
Da Lusitana antiga liberdade,
E não menos certíssima esperança
De aumento da pequena Cristandade;
Vós, ó novo temor da Maura lança,
Maravilha fatal da nossa idade,
Dada ao mundo por Deus, que todo o mande,
Pera do mundo a Deus dar parte grande;

(...)