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Review Articles

Expression of Specific Mitochondrial Proteins in Germ Cells during Spermatogenesis

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The current review is focused on the identity and function of some specific mitochondrial proteins/enzymes, their localization, gene expression, properties and activity, as well as their significance in the normal development of different germ cell populations during spermatogenesis and thus, sperm quality and fertility. Some of these molecules could be used as valuable markers for the diagnosis/prognosis of male infertility. Characterization of the potential of the mitochondrial proteins could open infinite possibilities for future research in the field of andrological clinical practice, but it may also be successfully applied for assisted reproduction procedures.

Key words: mitochondrial membrane proteins, mitochondrial genome/mtDNA, spermatogenesis, spermatozoa.

Introduction

Among the most widespread hypotheses is that the distant ancestors of mitochondria are bacteria from the α-proteobacterial lineage [23]. However, the events leading to endosymbiosis between bacteria (prokaryotes) and eukaryotes, and the subsequent evolution of mitochondria as integrated cellular organelles is not still clearly recognized [18]. The mitochondrion possesses its own independent genome (mtDNA) that shows substantial similarity to bacterial genomes. Its internal compartment, protein-rich liquid matrix, is bounded by the highly structured inner membrane, which in turn is bounded by the intermembrane space and the outer membrane. The inner mitochondrial membrane (IMM) possesses many cristae or invaginations, which increases the surface area available for energy production. The IMM is the site of ATP-production via oxidative

phosphorylation (OXPHOS), but it also controls the transport of ions and metabolites via transporters, as well as the oxidative status of the cell [50]. The mitochondrial membrane potential (MMP) presents the proton gradient formed by the flow of electrons along the electron transport chain (ETC), which is then used by the enzyme ATP-synthase for ATP synthesis. Decreased membrane potential (or depolarization) indicates mitochondrial damage and inability to meet energy demands of the cells, whereas hyperpolarization can lead to increased reactive oxygen species (ROS) production and cell damage [83].

The outer mitochondrial membrane (OMM) possesses non-specific protein pores, called porins, which are necessary for the passage of ions and other metabolites less than 10 kDa into the mitochondria (for example, those from VDACs family) [50]. The import of mitochondrial proteins synthesized in the cytoplasm and their homing to the appropriate compartments, membranes and correct membrane spaces/topologies involve multiple pathways and macromolecular mechanisms. Most proteins then pass through the translocase of the OMM to the intermembrane space, where divergent pathways sort them to the outer membrane, inner membrane and matrix, or trap them in the intermembrane space. Mitochondrial proteins (transcribed by the mtDNA) vary depending on both tissues and species. In humans, 615 distinct types of proteins have been identified from cardiac mitochondria [74], whereas in rats have been reported 940 proteins [86]. The mitochondrial proteome has been proposed as dynamically regulated [87]. In yeasts approximately 1000 proteins are available in these organelles, from which eight major constituents are coded and synthesized in the matrix. Roughly 25% of the mitochondrial proteins participate in the mitochondrial genome maintenance and/or expression at the inner surface of the IMM, providing 7 membrane proteins whose synthesis nucleates the assembly of three respiratory complexes.

Several mitochondria proteins are known to be synthesized in the germ cells during their morphological and functional development. These transit proteins, such as heat shock proteins (Hsp) hsp 60 and hsp 70, Lon protease, sulphydryl oxidase (SOx) and cytochrome c_t, serve as germ cell markers in different stages of spermatogenesis.

1. Heat shock proteins (HSPs). The physiological data indicate that their production is essential for cell survival and recovery from heat shock or other stress factors [54]. In the testis, these proteins (cytoplasm/ct-hsp90 and 70, mitochondria/mt-hsp60 and 70, nucleus/nuc-hsp70, etc.) are important for the heat protection of the germ cells at temperature above 35°C by binding unfolded or partially folded proteins to prevent their aggregation or irreversible thermal denaturation. The long-term rise in temperature above 35°C could lead to inevitable male infertility [63].

Mt-hsp60. Most of the hsp are also expressed by normal (non-stressed) cells, where they usually function as molecular chaperones. Mt-hsp60 is essential for the correct folding of the native structures of imported mitochondrial proteins followed by a stepwise process of ATP-dependent release. According to studies on human testicular biopsies, mt-hsp60 is located in the spermatogonia, early primary spermatocytes, Sertoli and Leydig cells in normal unaffected tubules [46]. The expression of this protein also correlates positively with the mitochondria type and activity, and it is increased in orthodox, but decreased in condensed and intermediate organelles [81]. The elevated mt-hsp60 levels in stages with proliferating spermatogonia suggest the existence of highly active mitochondrial protein import and assembly machinery, which is necessary for general new mitochondria for the daughter cells. Apparently, production of mt-hsp60 is required primarily during the initial steps of spermatogenesis, when most of the cell divisions occur, while its expression during the differentiation of spermatids and spermatozoa is not necessary. In general, the number of mt-hsp60 positive spermatogonia decreases with the loss of spermatogenic function, as with maturation arrest of spermatogenesis at the level of primary spermatocytes [81].

Hsp70-family. Numerous studies have been conducted to clarify the expression of hsp70 and its isoforms during different stages of the spermatogenesis, as well as during the proliferation, differentiation and maturation of germ cells [16, 35, 88]. Two specific forms are found in rodent testes: hsp70-2 (spermatocyte-specific) and HSC70t (testis-specific) proteins, expressed during spermatogenesis [16]. In the mouse testes, hsp70-2 is first detectable in pachytene spermatocytes and post-meiotic round spermatids (from day 17th postnatally), but in rat testes it is found in late pachytene spernatocytes, diakinesis and in steps 1-7 of spermiogenesis (day 22 postnatally) [35]. These data prove the active function of this chaperone during the differentiation of spermatocytes into spermatids [16]. According to other experiments, hsp70-2 participates in synaptonemal complex (SC) desynapsis, as a component of SC lateral elements [16], and it also interacts with the cyclin B-dependent CDC2 kinase, an enzyme with a key role in triggering the G2/M-phase transition during mitotic and meiotic cell cycle (in pachytene spermatocytes) [88]. Disruption of this formation could prevent G2-M-phases transition during meiosis and to cause increased apoptosis levels [16]. The chaperon protein mt-hsp70 from hsp70-family also plays an essential role during the import of mitochondrial matrix proteins. It is involved in a variety of different processes, as protein folding, disassembly of oligomeric protein complexes, delivery of proteins to proteases and translocation of polypeptides across the intracellular membranes.

In immunofluorescence experiments with application of monoclonal antibody to mt-hsp70, localization in the mitochondria of all germ cells up to pachytene spermatocytes has been shown [46]. Mt-hsp70, an element from the inner import complex, has been found to bind to the precursor sequence after the translocation of the N-terminal segment to avoid premature folding [29]. Most of the proteins (after cleavage of the pre-sequence by metalloproteinase) imported into the mitochondrial matrix are refolded directly by mt-hsp70 in co-operation with DnaJ (hsp40, Mdj-1) and GrpE (mt-hsp23) [29]. Besides the translocation of proteins across the mitochondrial inner membrane, mt-hsp70 also mediates the folding of these precursor molecules in the matrix. This fact correlates with the existence of two different populations of mt-hsp70: one bound to the inner membrane and the other one - soluble in the matrix [29]. Additionally, this protein supports the assembly of proteins, synthesized in the mitochondria to supra-molecular complexes. Another important function is that misfolded or denatured proteins exclusively bind to it and are then degraded by mitochondrial ATP-dependent protease (LON protease, PIM-1 protease) [80]. This is an indication that mt-hsp70 and Lon protease expression probably overlap in early primary spermatocytes [80].

2. Lon protease. Lon protease is a proteolytic ATP-dependent mitochondrial matrix enzyme involved in catabolic activity of the cells [70]. The appearance of this protein has been established in the orthodox mitochondria of leptotene spermatocytes, but it has also been determined as a specific marker for the intermediate type of the organelles (which are transit to condensed form), and have been found in zygotene and early pachytene spermatocytes [46]. The enzyme Lon protease has been connected with the protection of the mtDNA integrity, but also with degraded malfolded proteins in the mitochondrial matrix [46].

3. Cytochrome *c* (Cyc) and Cytochrome C oxidase (COX). Mitochondrial genome (mtDNA) encoded 13 polypeptides (subunits) that are included in the complexes of the ETC (except complex II), whereas the remainder of the subunits (totally 67) are encoded by the nucleus [72]. Mitochondrial polypeptides formed seven subunits of NADH CoQ reductase (complex I), cytochrome b (complex III); subunits I, II, and III of cytochrome C oxidase (complex IV), as well as subunits VI and VIII of the H+ ATPase [3]. Specific enzyme isoforms, such as cytochrome c_t and subunit VIb-2 of the cytochrome *c* oxidase

(COX), have also been found in the testicular mitochondria [27, 30], which underlines namely the increased mitochondrial activity in the germ cells. Cytochrome c (Cyc) has been determined as the only component of the mitochondrial respiratory chain known to express a testes-specific isoform in mammals. It is located in the intermembrane space of mitochondria.

Testicular cytochrome c_t isoform (T-Cyc) starts its expression in zygotene spermatocytes slowly taking the place of the somatic cytochrome c_s [27]. Cells in transition states during the sperm maturation have been found to express both Cyc isoforms [27]. Despite the equal function, indicated for both isoforms, cytochrome c_t isoform has shown higher pro-apoptotic activity and ability to catalyze the reduction of H_2O_2 three-fold faster than its counterpart in somatic cells. These findings strongly imply that T-Cyc can protect sperm from the damages caused by H_2O_2 [40]. The dramatically stronger apoptotic activity of T-Cyc might be important for the suicide activity of male germ cells, which has been considered as a physiological mechanism, necessary to regulate the number of sperm produced and to eliminate those with damaged DNA. Besides in primary spermatocytes, cytochrome c_t isoform has also been found in cell types, comprising the later stages of spermatogenesis, unlike the interstitial cells, Sertoli cells and spermatogonia, which have been established to contain the somatic form of cytochrome c_s [27, 40]. The appearance and action of T-Cyc during the stages of spermatogenesis correlates with the condensed and intermediate mitochondrial configuration, but also with the activated metabolism of germ cells.

Cytochrome C oxidase (COX) or Complex IV, has been characterized as the terminal enzyme in the electron transport chain located in the inner membrane of mitochondria. Its main function is connected with generation of ATP, required for the cellular living processes [26]. Complex IV consists of thirteen subunits, the genes for three of which (I, II and III) are localized within the mitochondrial genome, whilst the rest are located in nuclear chromosomes [71]. In the biochemical processes, COX receives electrons from each of the four cytochrome c molecules and transfers them to an oxygen molecule, converting molecular oxygen into two molecules of water, and in addition transporting four protons through the membrane (thus helping to establish proton electrochemical potential), used by the enzyme *ATP synthase* for ATP synthesize [21]. Importantly, COX has been shown to be the rate-limiting enzyme in the mitochondrial ETC [79], thus controlling respiration rate and the membrane potential [30].

Subunit Vib-2 of the COX is a testicular isoform of COX subunit Vib-2 (nuclear genome-encoded mitochondrial polypeptide). Similarly to cytochrome c, it is solely located in the intermembrane space [30]. Sampson and Alleyne (2001) have suggested a direct interaction between Cyc and COX subunits II, IV and VIb [60]. Later, Hüttemann et al. (2003) have reported that COX subunit VIb-2 (and/or VIb-1), analogically to *cytochrome c_t*, displays a testes-specific isoform in human, bull, rat and mouse, but unlike the rodent testes, where a lack of subunit VIb-1 has been established, both isoforms (VIb -2 and VIb-1) have been found to be transcribed in human testes. *In situ* hybridizations of testes sections has revealed VIb-2 transcripts in all testicular cell types (in human, rat and mouse), but within the seminiferous tubules, VIb-1 has shown stronger signals in the periphery than in the lumen. The authors have proposed any interaction between the isoforms of Cyc and VIb sub-unit in the context of sperm-specific energy requirements [30].

Messages for a specific stage-dependent change in the expression of another COX component – COXII (subunit II) during the spermatogenic cycle in testes from rat, have also been obtained [64]. According to this study, pachytene spermatocytes in seminiferous tubules at stage VII have shown the highest level of expression of mitochondrial COXII mRNA, which probably reflected the higher energy requirements of this cells entered the final stages of their meiosis [64].

4. Sulphydryl oxidase (*SOx*). Sulphydryl oxidase (*SOx*) is an enzyme that catalysis the oxidation of sulphydryl components (as glutathione, cysteine and thioglycerol), utilizing molecular oxygen as an electron acceptor, and newly formed disulphide bonds are thought to result in conformational changes of membrane proteins [67, 77]. This enzyme has first been established in mitochondria of condensed type in pachytene spermatocytes at stage I in the seminiferous epithelium of rat and hamster testes [36]. Compared to both rodent species (where SOx appears in a stage-dependent manner, presented mainly in pachytene spermatocytes and early spermatids), SOx distribution in the human testis is less dependent of the stage and not confined to certain germ cell types [8]. These authors investigated SOx in the seminiferous epithelium of human biopsy material in order to evaluate the possible value of this mitochondrial marker in the diagnosis of male infertility. In biopsies of oligozoospermic men (with hypospermatogenesis), the observed significant increase of SOx-labeled spermatogonia was associated with a significant decrease of sperm concentration in the ejaculate [8]. The assessed significant difference in the presence of SOx in human seminiferous epithelium has been established predominantly in A_{dark} spermatogonia (regarded as stem cell population) [52], whereas in A_{pale} spermatogonia only in stage V of spermatogenesis are significantly positive [6]. In the mature human testis moderate SOx amounts have been found in Leydig cells, whereas no SOx has been reported for the Sertoli cells and peritubular cells. In the mitochondria in leptotene (stages IV and V) and zygotene (stage VI) primary spermatocytes only moderate SOx amounts have been found, which are highest in pachytene spermatocytes of stages I-IV, but they decrease in stage V and are low during diakinesis and in secondary spermatocytes. The midpieces of the human spermatozoa are free of SOx-positive mitochondria, whereas in residual bodies small amounts of SOx are visible [6].

5. Other proteins, associated with mitochondrial function and sperm quality. Many mammalian genes expressing testis- or sperm-specific isoforms, have been identified [69]. Some of the examples are mitochondrial transcription factor A (Tfam) [37], lactate dehydrogenase (LDH-X), phosphoglycerate kinase 2 (pgk-2) [19] and etc. Genes, coding these proteins, are transcribed post-meiotically, but their expression is regulated at the translational level [10].

***Mitochondrial transcription factor A* (*mtTFA or Tfam*)**, particularly in humans (h-mtTFA), has been characterized as the focal point for regulation of human mtDNA copy number. It is a high mobility group- (HMG-) box protein that is a key activator of mitochondrial transcription [53]. It is likely that h-mtTFA also plays a role in the mtDNA replication since a transcript of a main mitochondrial promoter performs the role as an RNA primer for the initiation of this process [14]. Expression of germ cell-specific Tfam transcript isoforms occurs during spermatogenesis in mice and humans [37]. These Tfam transcript isoforms possess a structure that could prevent protein translation and their expression correlates with the down-regulation of the mitochondrial Tfam protein values. Unlike in mice, where the testis-specific mtTFA transcripts encode protein isoforms that are imported to the nucleus rather than into the mitochondria of spermatocytes and elongating spermatids, in humans the same transcripts predict a nuclear protein isoforms, and Western blot analysis identified only the mitochondrial form of h-mtTFA. Both mtTFA protein and mtDNA have been found to exhibit parallel gradients with high levels in undifferentiated male germ cells and low levels or absence in differentiated male germ cells, but on the other hand**,** testis-specific transcripts exhibit the opposite pattern. Thus probably in both humans and mice, these testis-specific mtTFA transcripts down-regulate mtTFA protein levels in mammalian mitochondria. In round and elongating spermatids this down-regulation coincides with

reduction of mtDNA copy number, which shows that Tfam is probably involved also in the regulation of the mtDNA amount during spermatogenesis [37].

Lactate dehydrogenase-X (LDH-X or C4). Enzyme lactate dehydrogenase catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+. It also converts pyruvate (the final product of glycolysis) to lactate in the absence or in short supply of oxygen, and it performs the reverse reaction during the Cori cycle in the liver. At high concentrations of lactate, the enzyme exhibits feedback inhibition, and the rate of conversion of pyruvate to lactate is decreased. It also catalyzes the dehydrogenation of 2-hydroxybutyrate and α-hydroxyvalerate, but these substrates are much poorer than lactate. In investigation on the catalytic properties and subcellular distribution of lacto-dehydrogenase isozyme-specific for testicles and spermatozoa (LDH X or C4) [9], isozyme X has been found to be located in the cytosol, plasma membrane and in the matrix of a special type of mitochondria, presented in spermatozoa of many mammalian species (including bovine and human) [9], which represents more than 80% of LDH activity in these germ cells [51]. Because of its multiple locations, LDH-C4 is a strong candidate for generation of NADH (by oxidation of lactate into pyruvate) in different sperm compartments. Because of its participation in the energetic metabolism of mature gametes, LDH-C4 has been associated with fertility [9], but also with capacitation in studies with bull and mouse spermatozoa [17]. Moreover, the low activity of LDH-C4 has been associated with partial or total reductions in sperm motility and concentration [11]. Spermatozoa from the bull (and other species) utilize pyruvate and lactate (generated by LDH-C4) as oxidative substrates for mitochondrial respiration and capacitation [51]. The cytosolic isoform of LDH-C4 converts lactate into pyruvate and NADH. The so received pyruvate can then enter into the mitochondria, where it converts into acetyl-CoA by pyruvate dehydrogenase and enters into the Krebs cycle to generate reducing equivalents that would be used in the respiratory chain for ATP-generation. The so produced ATP would be further used for energy purposes and to provide phosphate groups, necessary to support a series of phosphorylation events required during sperm capacitation [51]. The pyruvate not used for energy purposes could be converted into lactate by mitochondrial LDH-C4 and would diffuse to the cytosol to refeed the production of $O2^{\cdot}-$ by oxidase enzymes [51].

According to many studies, the five main types of LDH in mammals are formed by a random combination of two different subunits into tetramers. Each subunit (A and B) is a product of a distinct gene locus [25]. There is substantial evidence that the isoenzyme LDH-X of the male germ cell is also a tetramer, and that it is composed of a third polypeptide type (C), which is also encoded by a separate gene [25]. It is believed that this gene is active only during the primary stage of spermatocytes in the spermatogenic process and is inactive in all other cells of the organism [25], but the presence LDH-X in stem cells of spermatogonia has also been proposed [44]. The finding that LDH-X is indeed an unique protein of the sperm cell may be of considerable practical significance by providing a new approach to contraception in the male. Experimental studies are aimed to determine whether the interference with LDH-X synthesis and/or activity would affect the spermatogenesis or fertilization processes.

Protein kinases. The mitochondrial electron transport chain (ETC) in mammalian spermatozoa has been identified to undergo capacitation dependent tyrosine phosphorylation [34, 68]. The activity of enzyme protein kinase A (PKA) has been proposed as essential for sperm tyrosine phosphorylation, hyperactivation and acrosome reaction [38]. Other kinases implicated in capacitation are protein kinase C (PKC), protein kinase B (PKB/Akt), mitogen activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K) and extracellular signal regulated kinase (ERK1) [41]. Functional analysis of some of the tyrosine-containing phosphoproteins may predict

similar functions as observed for the proteins in somatic cells. However, the situation is complex for those proteins which are localized in the mitochondria of somatic cells, performing well known functions, but in spermatozoa they are non-canonical in their localization such as for example the enzymes dihydrolipoamide dehydrogenase (DLD), pyruvate dehydrogenase A2 (PDHA2) and glycerol-3-phosphate dehydrogenase 2 (GPD2) [34]. This is a problem in determination of the functional relevance of such proteins. The enzymes of the ETC such as phospholipid hydroperoxide glutathione peroxidase (PHGPx) and voltage-dependent anion channel (VDAC/mitochondrial porins) are localized into the mitochondrion while other enzymes as DLD, GPD2, and PDHA2 (pyruvate dehydrogenase/lipoamide alpha 2) are extra-mitochondrial [68]. Another important aspect is the ascertainment of identical localization of these mitochondrial proteins between human and animal spermatozoa before the establishment of corroboration between findings in both mammalian species [57].

***Phospholipid hydroperoxide glutathione peroxidase* (*PHGPx/GPx4*)** is a unique antioxidant enzyme, able to reduce directly phospholipid hydroperoxide in mammalian cells. This is in contrast with the most antioxidant enzymes, which cannot reduce intracellular phospholipid hydroperoxides directly [32]. Enzyme GPx4 belongs to the family of glutathione peroxidases (GPxs), it also contains selenocysteine at the active site and regulatory domain, which is successively oxidized and then reduced during the catalytic cycles. This selenoenzyme is distinct from cellular glutathione peroxidase (cGPx or GPx1, which has been characterized as "classical") on the basis of molecular weight, amino acid composition and substrate specificity [42]. Among the main structural differences is that PHGPx is a 20–22 kDa monomer protein, whereas the other GPxs are tetramers [78]. Furthermore, PHGPx is the only GPx that use phospholipid hydroperoxides as substrates, it is able to react with hydrogen peroxide and a wide range of lipid hydroperoxides, including with those derived from cholesterol and cholesteryl esters [43]. Kinetic data suggest that cGPx and PHGPx play different roles in hydroperoxide (H_2O_2) metabolism of cells, since in the aqueous phase the reaction of cGPX with peroxides is favored over that with PHGPx [42]. PHGPx, on the other hand, reduces specifically hydroperoxides in membranes [49] and mitochondria that are totally resistant to cGPx [66]. PHGPx has also been proved as able to reduce thymine hydroperoxide [7] and unlike the most GPxs, it can use a wide range of reducing substrates besides glutathione [5]. Similar to cGPx, replacing selenocysteine at the active site of PHGPx with cysteine significantly reduces its catalytic activity. It was also found that L-form of PHGPx (as opposed to its S-form) is mitochondrial PHGPx because it includes a signal peptide for transport to the mitochondria at the N-terminus of the protein, and as well as that the amount of PHGPx is significantly increased in the mitochondria, whileS-form PHGPx was highly expressed in nuclei, endoplasmic reticulum, and cytosol, but not in mitochondria [4].

Human spermatozoa contain high concentrations of polyunsaturated fatty acids (PUFAs), in particular, docosahexaenoic acid with six double bonds of the molecule [33], which makes the sperm vulnerable to peroxidative damage by the oxygen radicals. The ROS generation and sperm lipids peroxidation often have negative effects on the sperm motility [2], they could also cause midpiece abnormalities [58] and sperm-oocyte fusion, each one of which could disturb the fertilization in vertebrates [1]. In the mitochondria of spermatozoa, the main role of PHGPx is connected with the suppression of the effects of ROS that are produced by the mitochondrial respiratory chain [4]. PHGPx is distributed mainly in the midpiece of tails in the human ejaculated spermatozoa (and rat epididymal sperm), but it also presents in the sperm heads [24,31]. In the most somatic cells, hydrogen peroxide (H_2O_2) is reduced primarily by cytosolic glutathione peroxidase (cGPx) [2]. However, the reduction of H_2O_2 by PHGPx is similar

to that by cGPx in normal human spermatozoa. In rat epididymal spermatozoa, PHGPx represents at least 50% of the capsule material that embeds the helix of the sperm mitochondria. Studies have shown that infertile men with insufficient PHGPx expression in spermatozoa (PHGPx-defective gametes, according to WHO criteria) belong to the oligoasthenozoospermia group in which both number and motility of germ cells are significantly below normal and might, and therefore, could lead to serious impairment in fertilization and/or male fertility [31]. When the membrane potential in mitochondria of PHGPx-deficient spermatozoa is decreased compared to that of normal spermatozoa, the ultrastructure of the organelles (assessed by transmission electron microscopy) in the midpiece of PHGPx-defective gametes is extremely variable and abnormal compared to that of normal spermatozoa [31]. Hence, mitochondrial dysfunction could be a direct cause of the spermatozoa functions impairment. Furthermore, PHGPx has been suggested to play a role in the maturation of sperm during their passing through the epididymis by hydroperoxide metabolism and thiol oxidation in processes such as chromatin condensation, in addition to its role as an antioxidant. The oxidation of protamine thiols plays an important role in the sperm maturation. Pfeifer et al. identified a 34 kDa selenoprotein as a sperm-nucleus GPx (snGPx), a new type of PHGPx with a nuclear localization signal that is highly expressed in the nuclei of late spermatids [55]. Both PHGPx and snGPx might play an important role in the stabilization of condensed chromatin and the protection of sperm DNA against oxidation [55]. All of the sperm nuclei in selenium-deficient rats have shown abnormal DNA-condensation. PHGPx is expressed at high levels in rat, mouse and human testes [31, 59], but little is known about its regulation on the molecular level during the process of spermatogenesis. Expression of mitochondrial PHGPx in testes is induced after puberty [59] and an extraordinarily high rate of transcription of the gene for this enzyme, has been detected in the layer, containing late spermatocytes and round spermatids in human and other species [31]. A number of studies with knockout mice have suggested a close link of apoptosis with this regulation [56]. Mutations in genes for proteins from Bcl-2 family such as Bax, Bcl-2, Bcl-xL and Bcl-w lead to the accumulation of pre-meiotic germ cells and disruption of their differentiation [56]. Accelerated levels of apoptosis in germ cells during the formation of rat pachytene spermatocytes and spermatids have been found in experimentally-diminished PHGPx activity (via ethane dimethanesulphonate/EDS) and expression of mRNA for it in rat testis [84]. These results suggest a role of mitochondrial PHGPx as an anti-apoptotic factor in spermatogenesis and also suggest that failed induction of the mitochondrial PHGPx expression (as an anti-apoptotic factor) in late spermatocytes might have a major effect on spermatogenesis.

6. Paracrine mitochondrial maturation factors (PMMFs). The mechanisms that regulate or trigger the various steps of mitochondrial differentiation and metabolism during spermatogenesis are still not studied very well. Here belong different processes and factors as hormonal regulation, essential cell-to-cell interactions and testis-specific enzymes, which are of key importance about the regular testicular functions [20]. Both Sertoli and germ cells secrete various growth factors, which act on a local or paracrine manner [65]**.** Some of the proteins, produced by the Sertoli cells, affect the mitochondrial activity, and are generally determined as paracrine mitochondrial maturation factor (PMMF) [46]. Such a molecule is activin A, which acts on a paracrine fashion on primary spermatocytes.

Activin A. Activin A is the first Sertoli cell product that has been identified to influence differentiation of male meiotic germ cells. It is described as an inducer of the condensed form of mitochondria, contributing to the regulation of the germ cells' differentiation by the Sertoli cells. The mitochondria in spermatogonia change

their morphology as those cells proceed into the prophase of meiosis (primary as spermatocytes). The principal change is the dilatation of the intracristal space to give a vacuolated appearance of a mitochondrion. The role of activin A to control this change has been suggested [46]. This factor has been found to be included in many other functions, including cell proliferation, differentiation, apoptosis [13], metabolism, homeostasis, and immune response. Activin A stimulates both biosynthesis and secretion of pivotal for the spermatogenesis process hormones as follicle-stimulating hormone (FSH) and gonadotropin-releasing hormone, but it also activates the spermatogonia and preleptotene spermatocytes proliferation and regulates the pre-meiotic and meiotic DNA-synthesis as well. On the other hand, a possibility of this protein to inhibit FSH-stimulated conversion of the testosterone to estradiol has been proved. Besides the paracrine effect on spermatogenesis this molecule also has indicated the autocrine mechanism of action on the Sertoli cells. Receptors for activin A have been established besides in these cells, also on the spermatogonia, spermatocytes and spermatids. A cyclic activin secretion has been assessed in the seminiferous tubules – at stage VI, with maximal values at stage VIII of the seminiferous epithelium (in rat). Dibutyryl-cAMP stimulates the secretion of activin A in late stages VII and VIII. As a local mediator of activin A has been characterized interleukin-1alpha (IL-1α), also found to be produced by the Sertoli cells. Another important activator of activin is also the follistatin, which is secreted by the hypophysis, binds to activin and in this way acts as a local buffer.

Follistatin (FS). Follistatin also known as activin-binding protein is an autocrine glycoprotein that in humans is encoded by the *FST* gene [76], consisting of six exon. At least six forms of this protein are known: two basic forms - FS344 and FS317, as well as a few derivatives - FS315, FS300, FS288, etc. [73]. The main function of FS is connected with a complex influence on the activin functions. The finding of this factor in cells, different of spermatids at steps 1-11 (in rodents) suggests that activin A neutralization by follistatin probably accounts for the observed changes in mitochondrial morphology to the intermediated type in post-meiotic germ cells by activin neutralization (in fact, other factors have also been proposed to be involved). In rat testis, follistatin mRNA has been located in many germ cells. Small amounts of follistatin mRNA were found in preleptotene and leptotene primary spermatocytes but declined expression has been noted in the late leptotene and early zygotene stage (stages XI and XII), with slight activation in early pachytene and diplotene stage (stages VIII-XIV). FS levels remain relatively high in spermatids from step 1 to step 10, declined thereafter and from step 16 to step 19 have not been detected. This protein has also been found in Sertoli cells and endothelial cells, but no mRNA has been detected in Leydig cells [46].

Mitochondrial Ferritin (FtMt) is a ferroxidase enzyme (metal-binding protein) which is located within the mitochondria and in humans it is encoded by gene *FTMT*, mapped in chromosome 5q23.1 [39]. FtMt differs from the cytosolic H- (FtH) and L-ferritin chains (FtL) by the presence of a long amino acid N-terminal sequence for mitochondrial import, which is cleaved during the processing. After the protein is taken up by the mitochondria, it can be processed into a mature protein and to assemble functional ferritin shells. Although excess iron is stored primarily in the cytoplasm (by FtH and FtL chains), most of the metabolically active iron in the cells is processed in the mitochondria. Ferritin mitochondrial expression is restricted to specific cells and tissues such as testis and brain. This protein is highly expressed in human testis, particularly in the spermatocytes and Leydig cells [61], and it appears to correlate with the management of ATP burst in a short-time period. As the main function of FtMt in cell types, characterized by high metabolic activity and oxygen consumption (such as spermatocytes, Leydig cells, spermatozoa), is proposed its role in the protection of mitochondria against iron-dependent oxidative damage, not storing iron for the

synthesis of mitochondrial iron enzymes. A high metabolic rate seems to be a common characteristic of the cells positive on FtMt. For example, FtMt-rich Leydig cells in the testis require a high energy levels for the biosynthesis of hormones, and in the seminiferous tubules, the germinal cells with the highest FtMt content have been determined the spermatozoa in the latest phases of maturation in the tail region that lay freely in the lumen. Mitochondria of spermatozoa are highly active in order to meet the requirements for energy-dependent movement, but in few of them, imply very high local FtMt concentration. These cells are exposed to oxygen tension much higher than other internal cells, and thus, FtMt might offer further protection against oxidative damage. The epithelial cells in the epididymis are also rich of FtMt, similarly to all tissues, composed mainly of ciliated cells (which compose bronchus, Fallopian tubes, etc.) that use high energy for motility/transport or absorption/phagocytosis. This specific high-energy requirement is associated with the production of reactive oxygen species (ROS), derived by the Fenton reaction that involves the Fe(II)-catalyzed production of the highly toxic hydroxyl radicals from hydrogen peroxide. The expression of FtMt might be a cellular response to avoid this harmful reaction [61].

7. Proteins that control the mitochondrial fusion and fission. Frequent cycles of fusion and fission adapt the morphology of mitochondria (or of the cellular mitochondrial compartment) to the metabolic needs of the cells, optimizing their bioenergy capacity. The increased fusion activity leads to mitochondrial elongation, whereas the increased fission activity results in mitochondrial fragmentation [12]. The changes in balance between the rates of these two processes directly affect the wide range of mitochondrial length that can be observed in different cell types. The mechanisms of mitochondrial fusion and fission are regulated by proteolysis and post-translational modifications. Many gene products that control them have been identified and they belong to three core groups. These groups of proteins include mitofusins **(**mitochondrial membrane fusion/ MMF family**)**, OPA1 and Drp1. All of these molecules are GTP-hydrolyzing enzymes (GTPases) that belong to the dynamin family. Mitochondrial dynamics (mitochondrial morphology, distribution, fusion and fission) in different cells could be understood by the way in which these proteins regulate and bind to each other [85]. Mitochondrial fusion is particularly important in the respiratory-active cells. In this way, it allows the spreading of metabolites, enzymes and mitochondrial gene products throughout the entire mitochondrial compartment and serves to maintain the function of these organelles, but also to control the accumulation of mtDNA mutations during aging [82].

Mitofusins (Mfn1, Mfn2 and OPA1). Mfn1 and Mfn2 are specific membrane-anchored dynamin family members, which mediate fusion between mitochondrial outer membranes (in mammals) [62, 82]. Both proteins can act either together or separately during the mitochondrial fusion. It is not yet determined whether the two proteins perform the same function in the process or each of them performs separate functions. It is however shown that within the Mfn1–Mfn2 hetero-oligomeric complex, each molecule is functionally distinct [48, 15]. In addition, Mfn2 has been established to associate specifically with Bax and Bak (from Bcl-2 family), resulting in altered Mfn2 activity, indicating that probably the mitofusins possess unique functional characteristics. Cells lacking one or the other protein have reduced mitochondrial fusion possibility and show a mitochondria subpopulation with lacking mtDNA nucleoids. Such mtDNA defects lead to severe cellular injuries as accumulation of respiration-deficient mitochondria in the cells, with decreased cellular respiration respectively and leads to poor cell growth. Indeed, the expression levels of Mfn1 and Mfn2, as well as the mitochondrial morphology, vary according to the cell or tissue type [28]. These features suggest that control on the expression levels of each protein likely represents the most basic form

of regulation to alter mitochondrial dynamics in mammalian tissues. Mitochondrial fusion mediators differ between the outer and inner membranes of the organelle. Opa1 (belonging to dynamin-related protein family) is localized in the intermembrane space and it mediates the fusion between the inner mitochondrial membranes (in mammals) [45]. Fragmented mitochondria are frequently found in resting cells, and mitochondrial fission plays an important role in the removal of damaged organelles by autophagy. Some of the identified proteins that are involved in the mitochondrial fission are associated with mitochondrial diseases [85]. Mitochondrial fission has significant implications in stress response and apoptosis [12, 85].

***Proteins of mitochondrial fission* (*Drp1, Mff*)**. Drp1 controls the final part of the mitochondrial fission, pinching off the membrane stalk between two forming daughter mitochondria. This molecule functions by binding to other mitochondrial fission proteins. Several Drp1-binding proteins have been identified [22]: Mff (called mitochondrial fission factor) binds Drp1 and promotes mitochondrial fission [12]; FIS1 (mitochondrial fission 1 protein) is located on the outer mitochondrial membrane might attract Drp1 to sites of fission [12], but it might require association with another protein, such as MIEF1 (mitochondrial elongation factor 1) to promote mitochondrial division. In contrast, when bound to Drp1, MIEF1 might prevent mitochondrial fission and thus shift the balance towards the fusion of the organelles [12]. Mitochondrial fission occurs at the points of ER-mitochondrial associations that have been related with the formation of Drp1 complexes. On the other hand, the binding of the endoplasmic reticulum (ER) to mitochondria in the ER-mitochondrial association includes a protein complex with the presence of Mfn2. According to recent study, actin polymerization through ER-localized inverted formin 2 (INF2) is required for efficient mitochondrial fission in mammalian cells. This function of the protein has been assessed at the stage before Drp1 perform its function. In this way, the INF2-induced actin filaments may drive initial mitochondrial constriction, which allows Drp1-driven secondary constriction [48]. The activity of the key proteins of mitochondrial dynamics is regulated at multiple levels, including transcription and post-translational modification, and to respond directly to the mitochondria bioenergetic state.

Mitochondrial fission regulator 1 (Mtfr1) is associated with the mitochondrial inner membrane that induces mitochondrial fission [47]. In mice, it is encoded by a nuclear gene located on mouse chromosome 3 A3, containing a short chondrocyte protein with a polyproline region polyproline-rich region (CHPPR) [75]. In mice, the highest expression levels have been observed in pubertal and adult testis, particularly by the haploid spermatids and Leydig cells. In Mtfr1-deficient mice, the lack of this molecule is associated with markedly reduced levels of several proteins involved in the defense against oxidative stress (e.g., an enzyme glutathione peroxidase 3, specifically expressed in the endocrine compartment of the testis), probably due to the protective role of Mtfr1 on the male gonads against oxidative stress, which is important in the regulation of ROS-scavenging genes expression [47].

The activity of the key proteins of all mitochondrial dynamics is regulated at multiple levels, including transcription, post-translational modification and direct response to the bioenergetic state of mitochondria.

Conclusion

We make a review of the existing literature, covering the role of some specific mitochondrial proteins/enzymes from spermatogenesis to mature spermatozoa. Changes in mitochondrial proteins levels (or gene expression) are important to

elucidate mitochondrial function with respect to complex processes of the germ cell differentiation through the separate spermatogenesis stages. Clarification of functional identification and relevance of the proteins, especially in sperm capacitation, is a necessity for better understanding of the relationship between sperm protein tyrosine phosphorylation, hyperactivation, and acrosome reaction, and hence, in elucidating the molecular mechanisms of male infertility. Some of these enzymes could be used as specific biomarkers for the normal development of germ cell populations and for monitoring of sperm fertilization ability. Characterization of the potential of these proteins could open infinite possibilities for future studies in the field of andrological clinical practice, but it could also be applied successfully in the assisted reproduction practice.

References

1. **Aitken, R. J., J. S. Clarkson, S. Fishel**. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. – *Biol. Reprod.,* **41**, 1989, 189–197.
2. **Alvarez, J. G., B. T. Storey.** Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. – *Gamete Res*., **23**, 1989, 77-90.
3. **Anderson, S., A. T. Bankier, B. G. Barrell, M. H. De Bruijn, A. R. Coulson, J. Drouin, I. C. Eperon, D. P. Nierlich, B. A. Roe, F. Sanger, P. H. Schreier, A. J. Smith, R. Staden, I. G. Young**. Sequence and organization of the human mitochondrial genome. – *Nature,* **290**, 1981, 457-465.
4. **Arai, M., H. Imai, T. Koumura, M. Yoshida, K. Emoto, M. Umeda, N. Chiba, Y. Nakagawa**. Mitochondrial phospholipid hydroperoxide glutathione peroxidase plays a major role in preventing oxidative injury to cells. – *J. Biol. Chem*., **274**, 1999, 4924- 4933.
5. **Aumann, K. D., N. Bedorf, R. Brigelius-Flohe, D. Schomburg, L. Flohe**. Glutathione peroxidase revisited simulation of the catalytic cycle by computer-assisted molecular modelling. – *Biomed. Environ. Sci*. **10**, 1997, 136-155.
6. **Aumüller, G., M. Bergmann, J. Seitz**. Immunohistochemical distribution of sulfhydryl oxidase in the human testis. – *Cell Tissue Res*., **266**, 199, 123-128.
7. **Bao, Y., P. Jemth, B. Mannervik, G. Williamson**. Reduction of thymine hydroperoxide by phospholipid hydroperoxide glutathione peroxidase and glutathione transferases. – *FEBS Lett*., **410**, 1997, 210-212.
8. **Bergmann, M., G. Aumüller, J. Seitz, E. Nieschlag**. Sulfhydryl oxidase immunoreactivity in seminiferous tubules of infertile men. – *Cell Tissue Res*., **267**, 1992, 209-214.
9. **Blanco A.** On the functional significance of LDH X. – *Johns Hopkins Med J.,* **146**(6), 1980, 231-235.
10. **Braun, R. E., R. R. Behringer, J. J. Peschon, R. L. Brinster, R. D. Palmiter**. Genetically haploid spermatids are phenotypically diploid. – *Nature*, **337**, 1989, 373-376.
11. **Burgos, G. N. M., C. Burgos, C. E. Coronel, A. B. de Camusso, T. Pigini T**, **A. Blanco**. Correlation of lactate dehydrogenase isoenzyme C4 activity with the count and motility of human spermatozoa. – *J. Reprod. Fertil.,* **55,** 1979, 107-111.
12. **Chan, D. C**. Mitochondrial fusion and fission in mammals. – *Ann. Rev. Cell Develop. Biol.*, **22**, 2006, 79-99.
13. **Chen, Y. G., Q. Wang, S. L. Lin, C. D. Chang, J. Chuang, J. Chung, S. Y. Ying**. Activin signaling and its role in regulation of cell proliferation, apoptosis, and carcinogenesis. – *Experim. Biol. Med.*, **231**(5), 2006, 534-544.
14. **Clayton, D. A**. Transcription and replication of mitochondrial DNA. - *Human Reprod*., **15**(2), 2000, 11–17.
15. **Detmer, S. A., D. C. Chan**. Complementation between mouse Mfn1 and Mfn2 protects mitochondrial fusion defects caused by CMT2A disease mutations. – *J. Cell Biol.*, **176** (4), 2007, 405-414.
16. **Dix, D. J., J. W. Allen, B. W. Collins**, **P. Poorman-Allen, C. Mori, D. R. Blizard, P. R. Brown, E. H. Goulding, B. D. Strong, E. M. Eddy.** HSP70-2 is required for desynapsis of synaptonemal complexes during meiotic prophase in juvenile and adult mouse spermatocytes. **–** *Development*, **124**, 1997, 4595-4603.

17. **Duan, C., E. Goldberg**. Inhibition of lactate dehydrogenase C4 (LDH-C4) blocks capacitation of mouse sperm *in vitro*. – *Cytogenet. Genome Res*., **103**, 2003, 352-359.
18. **Embley, T. M., W. Martin**. Eukaryotic evolution, changes and challenges. – *Nature*, **440**, 2006, 623-630.
19. **Erickson, L. D., S. A. Rizza, E. R. Bergert, M. C. Charlesworth, D. J. McCormick, R. J. Ryan**. Synthetic alpha-subunit peptides stimulate testosterone production in vitro by rat Leydig cells. – *Endocrinology*, **126**, 1990, 2555-2560.
20. **Erkkila, K., S. Kyttanen, M. Wikstrom, K. Taari, A. P. Hikim, R. S. Swerdloff, L. Dunkel**. Regulation of human male germ cell death by modulators of ATP production. – *Am. J. Physiol. Endocrinol. Metab*., **290**, 2006, 1145-1154.
21. **Ferguson-Miller, S., G. T. Babcock**. Heme/copper terminal oxidases. – *Chem. Rev*., **96**(7), 1996, 2889-2908.
22. **Frezza, C., S. Cipolat, O. M. de Brito, M. Micaroni, G. V. Benznoussenko, T. Rudka, D. Bartoli, R. S. Polishuck, N. N. Danial, B. De Strooper, L. Scorrano**. OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. – *Cell,* **126**(1), 2006, 177-189.
23. **Gray, M. W., B. F. Lang, G. Burger**. Mitochondria of protists. – *Ann. Review Genetics,* **38,** 2004, 477-524.
24. **Godeas, C., F. Tramer, F. Micali, A. Roveri, M. Maiorino, C. Nisii, G. Sandri, E. Panfili**. Phospholipid hydroperoxide glutathione peroxidase (PHGPx) in rat testis nuclei is bound to chromatin. Biochem. – *Mol. Med*., **59,** 1996, 118-124.
25. **Goldberg, E**. Immunochemical specificity of lactate dehydrogenase-X. - *National Acad. Sci*., 68 (2), 1971, 349-352.
26. **Herrmann, J. M., M. W. Woellhaf, N. Bonnefoy**. Control of protein synthesis in yeast mitochondria: the concept of translational activators. – *Biochem. Biophys. Acta*, **1833** (2), 2013, 286-294.
27. **Hess, R. A., L. A. Miller, J. D. Kirby**, **E. Margoliash, E. Goldberg.** Immunoelectron microscopic localization of testicular and somatic cytochromes c in the seminiferous epithelium of the rat. – *Biol. Reprod*., **48**, 1993, 1299-1308.
28. **Hoppins, S., J. Horner, C. Song, J. M. McCaffery, J. Nunnari**. Mitochondrial outer and inner membrane fusion requires a modified carrier protein. – *J. Cell Biol*., **184**(4), 2009, 569-581.
29. **Horst, M., W. Oppliger, S. Rospert, H. J. Schönfeld, G. Schatz, A. Azem**. Sequential action of two hsp70 complexes during protein import into mitochondria. – *EMBO J*., **16**(8), 1997, 1842-1849.
30. **Hüttemann, M., S. Jaradat, L. I. Grossman.** Cytochrome c Oxidase of mammals contains a testes-specific isoform of subunit Vib – the counterpart to testes-specific cytochrome c? – *Mol. Reprod. Dev.,* **66**, 2003, 8-16.
31. **Imai, H., K. Suzuki, K. Ishizaka, S. Ichinose, H. Oshima, I. Okayasu, K. Emoto, M. Umeda, Y. Nakagawa**. Failure of the expression of phospholipid hydroperoxide glutathione peroxidase in the spermatozoa of human infertile males. – *Biol. Reprod.,* **64**, 2001, 674-683.
32. **Imai**, **H., Y. Nakagawa**. Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells. – *Free Radic. Biol. & Med.*, **34(**2), 2003, 145-169.
33. **Kim, J. G., S. Parthasarathy**. Oxidation and the spermatozoa. – *Semin. Reprod. Endocrinol*., **16**, 1998, 235-239.
34. **Kota, V., V. M. Dhople, S. Shivaji**. Tyrosine phosphoproteome of hamster spermatozoa: role of glycerol-3-phosphate dehydrogenase 2 in sperm capacitation. – *Proteomics,* **9**, 2009, 1809-1826.
35. **Krawcyk, L. A., P. Mali, M. Parvinen**. Expression of a testis-specific hsp 70 gene-related RNA in difined stages of rate seminiferous epithelium. – *J. Cell. Biolol*., **107**, 1988, 1317-1323.
36. **Kumari, M., G. Aumüller, M. Bergmann, A. Meinhardt, J. Seitz**. Stage-dependent appearance of sulfhydryl oxidase during spermatogenesis in the testis of rat and hamster. – *Histochem.*, **94**(4), 1990, 365-371.
37. **Larsson, N. G., A. Oldfors, J. D. Garman, G. S. Barsh, D. A. Clayton**. Down-regulation of mitochondrial transcription factor A during spermatogenesis in humans. – *Human Mol. Genet*., **6**, 1997, 185-191.
38. **Lefièvre, L., K. N. Jha, E. de Lamirande, P. E. Visconti, C. Gagnon**. Activation of protein kinase A during human sperm capacitation and acrosome reaction. – *J. Androl*., **23**, 2002, 709-716.
39. **Levi, S., B. Corsi, M. Bosisio, R. Invernizzi, A. Volz, D. Sanford, P. Arosio, J. Drysdale**. A human mitochondrial ferritin encoded by an intronless gene. – *J. Biol. Chem*. **276** (27), 2001, 24437-24440.

40. **Liu, Z., H. Lin, S. Ye, Q-y. Liu, Z. Meng, C-m. Zhang, Y. Xia, E. Margoliash, Z. Rao, X. J. Liu**. Remarkably high activities of testicular cytochrome c in destroying reactive oxygen species and in triggering apoptosis. – *Proc. Natl. Acad. Sci. USA*, **103**, 2006, 8965-8970.
41. **Luconi, M., F. Marra, L. Gandini, E. Filimberti, A. Lenzi, G. Forti, E. Baldi**. Phosphatidylinositol 3-kinase inhibition enhances human sperm motility. – *Human Reprod*. **16**, 2001, 1931-1937.
42. **Maiorino**, **M., F. F. Chu, F. Ursini, K. J. A. Davies, J. H. Doroshow, R. S. Esworth**. Phospholipid hydroperoxide glutathione peroxidase is the 18-kDa selenoprotein expressed in human tumor cell lines. – *J. Biol. Chem*., **266**(12), 1991a, 7728-7732.
43. **Maiorino, M., J. P. Thomas, A. W. Girotti, F. Ursini**. Reactivity of phospholipid hydroperoxide glutathione peroxidase with membrane and lipoprotein lipid hydroperoxides. – *Free Radic. Res. Commun*., **12–13**, 1991, 131-135.
44. **McKee, R.W.**, **E. Longstaff, A. L. Latner**. Lactate dehydrogenase in human testes. – *Clinica Chimica Acta*, **39**(1), 1972, 221-227
45. **Meeusen, S., R. DeVay, J. Block, A. Cassidy-Stone, S. Wayson, J. M. McCaffery, J. Nunnari.** Mitochondrial inner-membrane fusion and crista maintenance requires the dynamin-related GTPase Mgm1. – *Cell,* **127**, 2006, 383-395.
46. **Meinhardt, A., B. Wilhelm, J. Seitz**. Expression of mitochondrial marker proteins during spermatogenesis. – *Human Reprod. Update*, **5**, 1999, 108-119.
47. **Monticone, M., L. Tonachini, S. Tavella, P. Degan, R. Biticchi, F. Palombi, R. Puglisi, C. Boitani, R. Cancedda, P. Castagnola**. Impaired expression of genes coding for reactive oxygen species scavenging enzymes in testes of Mtfr1/Chppr-deficient mice. – *Reproduction*, **134**, 2007, 483-492.
48. **Naotada I., E. Yuka, M. Katsuyoshi**. Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions via GTPase activity. – *J. Cell Sci*. **117** (Pt 26), 2004, 6535-6546.
49. **Nomura, K., H. Imai, T. Koumura, Y. Nakagawa**. Involvement of mitochondrial phospholipid hydroperoxide glutathione peroxidase as an antiapoptotic factor. – *Biol. Signals Recept.*, **10**, 2001, 81-92.
50. **Nicholls, D.G., S. J. Ferguson**. *Bioenergetics* (Ed. D. Nicholls), 4th edition, San Diego, Academic Press, , 2013, 288.
51. **O'Flaherty, C.** Redox regulation of mammalian sperm capacitation. – *Asian J. Androl*., **17,** 2015, 583-590.
52. **Paniagua, R., J. Codesal, M. Nistal**, **M. C. Rodrigues, L. Santamaria**. Quantification of cell types throughout the cycle of the human seminiferous epithelium and their DNA content. A new approach to the spermatogonial stem cell in man. – *Anat. Embryol*., **176**, 1987, 225-230.
53. **Parisi, M. A., D. A. Clayton**. Similarity of human mitochondrial transcription factor 1 to high mobility group proteins. – *Science*, **252**, 1991, 965-969.
54. **Parsell, D.A., S. Lindquis**. The function of heat shock proteins in stress tolerance: reactivation of damaged proteins. – *Annu. Rev. Genet*., **27**, 1993, 437-496.
55. **Pfeifer, H., M. Conrad, D. Roethlein, A. Kyriakopoulos, M. Brielmeier, G.W. Bornkamm, D. Behne**. Identification of a specific sperm nuclei selenoenzyme necessary for protamine thiol cross-linking during sperm maturation. – *FASEB J.,* **15**, 2001, 1236-1238.
56. **Print, C. G., K. L. Loveland, L. Gibson, T. Meehan, A. Stylianou, N. Wreford, D. de Kretser, D. Metcalf, F. Kontgen, J. M. Adams, S. Cory**. Apoptosis regulator bcl-w is essential for spermatogenesis but appears otherwise redundant. – *Proc. Natl. Acad. Sci. USA*, **95,** 1998, 12424-12431.
57. **Rajender, S., P. Rahul, A. A. Mahdi**. Mitochondria, spermatogenesis and male infertility. – *Mitochondrion*, **10**, 2010, 419-428.
58. **Rao, B., J. C. Soufir, M. Martin, G. David**. Lipid peroxidation in human spermatozoa as related to midpiece abnormalities and motility. – *Gamete Res*., **24**, 1989, 127-134.
59. **Roveri, A., A. Casasco, M. Maiorino, P. Dalan, A. Calligaro, F. Ursini**. Phospholipid hydroperoxide glutathione peroxidase of rat testis. Gonadotropin dependence and immunocytochemical identification. – *J. Biol. Chem*., **267**, 1992, 6142-6146.
60. **Sampson, V., T. Alleyne**. Cytochrome c/cytochrome c oxidase interaction. Direct structural evidence for conformational changes during enzyme turnover. – *Eur. J. Biochem*., **268**(24), 2001, 6534-6544.
61. **Santambrogio, P., G. Biasiotto, F. Sanvito, S. Olivieri, P. Arosio, S. Levi.** Mitochondrial ferritin expression in adult mouse tissues. – *J. Histochem. & Cytochem*., **55**(11), 2007, 1129-1137.

62. **Santel, A., M. T. Fuller**. Control of mitochondrial morphology by a human mitofusin. – *J. Cell Sci*., **114,** 2001, 867-874.
63. **Sarge, K. D., K. E. Cullen**. Regulation of hsp expression during rodent spermatogenesis. – *Cell. Mol. Life Sci.,* **53**, 1997, 191-197.
64. **Saunders, P. T., M. R. Millar, A. P. West, R. M. Sharpe**. Mitochondrial cytochrome C oxidase II messenger ribonucleic acid is expressed in pachytene spermatocytes at high levels and in a stage-dependent manner during spermatogenesis in the rat. – *Biol. Reprod*., **48**, 1993, 57-67.
65. **Schlatt, S., A. Meinhardt, E. Nieschlag.** Paracrine regulation of cellular interactions in the testis: factors in search of a function. – *Eur. J. Endocrinol*., **137**, 1997, 107-117.
66. **Schneider, M., H. Förster, A. Boersma, A. Seiler, H. Wehnes, F. Sinowatz, C. Neumüller, M. J. Deutsch, A. Walch, M.H. de Angelis, W. Wurst, F. Ursini, A. Roveri, M. Maleszewski, M. Maiorino, M. Conrad**. Mitochondrial glutathione peroxidase 4 disruption causes male infertility. – *FASEB J*., **23**(9), 2009, 3233-3242.
67. **Seitz, J., J. Moëbius, M. Bergmann, A. Meinhardt**. Mitochondrial differentiation during meiosis of male germ cells. – *Int. J. Androl*., **18**, 1995, 7-11.
68. **Shivaji, S., V. Kota, A. B. Siva**. The role of mitochondrial proteins in sperm capacitation. – *J. Reprod. Immunol*., **83**, 2009, 14-18.
69. **Siva, A. B., D. B. Kameshwari, V. Singh, K. Pavani, C.S. Sundaram, N. Rangaraj, M. Deendayal, S. Shivaji**. Proteomics-based study on asthenozoospermia: differential expression of proteasome alpha complex. – *Mol. Hum. Reprod*., **16**, 2010, 452-462.
70. **Sonezaki, S., K. Okita, T. Oba**, **Y. Ishii, A. Kondo, Y. Kato**. Protein substrates and heat shock reduce the DNA-binding ability of *Escherichia coli* Lon protease. – *Appl. Microbiol. Biotechnol*., **44**, 1995, 484-488.
71. **Soto, I. C., F. Fontanesi, J. Liu, A. Barrientos**. Biogenesis and assembly of eukaryotic cytochrome c oxidase catalytic core. – *Biochim. Biophys. Acta*, **1817** (6), 2012, 883-897.
72. **St. John, J. C., D. Sakkas, C. L. Barratt**. A role for mitochondrial DNA and sperm survival. – *J. Androl*., **21**, 2000, 189-199.
73. **Sugino, K., N. Kurosawa, T. Nakamura**, **K. Takio, S. Shimasaki, N. Ling, K. Titani, H. Sugino**. Molecular heterogeneity of follistatin, an activing-binding protein. – *J. Biol. Chem*., **268**, 1993, 15579-15587.
74. **Taylor, S .W., E. Fahy, B. Zhang, G. M. Glenn, D. E. Warnock, S. Wiley, A. N. Murphy, S. P. Gaucher, R. A. Capaldi, B. W. Gibson, S. S. Ghosh**. Characterization of the human heart mitochondrial proteome. – *Nature Biotechnol*., **21**(3), 2003, 281-286.
75. **Tonachini, L., M. Monticone, C. Puri, C. Tacchetti, P. Pinton, R. Rizzuto, R. Cancedda, S. Tavella, P. Castagnola**. Chondrocyte protein with a polyproline region (CHPPR) is a novel mitochondrial protein and promotes mitochondrial fission. – *J. Cell. Physiol.,* **201**, 2004, 470-482.
76. **Tortoriello, D. V., Y. Sidis, D. A. Holtzman, W. E. Holmes, A. L. Schneyer**. Human follistatin-related protein: a structural homologue of follistatin with nuclear localization. – *Endocrinol.*, **142** (8), 2001, 3426-3434.
77. **Tury, A., G. Mairet-Coello, A. Esnard-Fève, B. Benayoun, P. Y. Risold, B. Griffond**. Cell-specific localization of the sulphydryl oxidase QSOX in rat peripheral tissues. - *Cell Tissue Res.*, **323**(1), 2006, 91-103.
78. **Ursini, F., M. Maiorino, C. Gregolin**. The selenoenzyme phospholipid hydroperoxide glutahtione peroxidase. – *Biochim. Biophys. Acta*, **839**, 1985, 62-70.
79. **Villani, G., G. Attardi.** In vivo control of respiration by cytochrome c oxidase in human cells. – *Free Radic. Biol. Med*., **29**, 2000, 202-210.
80. **Wagner, I., H. Arlt, L. van Dyck**, **T. Langer, W. Neupert.** Molecular chaperone cooperate with P1M1 protease in the degradation of misfolded proteins in mitochondria. – *EMBO J*., **1**, 1994, 5135-5145.
81. **Werner, A., A**. **Meinhardt, J. Seitz, M. Bergmann.** Distribution of heat shock protein 60 immunoreactivity in testes of infertile men. - *Cell Tissue Res*., **288**, 1997, 539-544.
82. **Westermann, B**. Bioenergetic role of mitochondrial fusion and fission. – *Biochim. Biophys. Acta*, **1817,** 2012, 1833–1838.
83. **Wolken, G. G., E. A. Arriaga.** Simultaneous measurement of individual mitochondrial membrane potential and electrophoretic mobility by capillary electrophoresis. - *Anal Chem*., **86**(9), 2014, 4217-1426.

84. **Woolveridge, I., M. de Boer-Brouwer, M. F. Taylor, K. J. Teerds, F. C. Wu, I. D. Morris**. Apoptosis in the rat spermatogenic epithelium following androgen withdrawal: changes in apoptosis-related genes. – *Biol. Reprod.,* **60**, 1999, 461-470.
85. **Youle, R. J., A. M. van der Bliek**. Mitochondrial Fission, Fusion, and Stress. – *Sci*., **337**(6098), 2012, 1062-1066.
86. **Zhang, J., X. Li, M. Mueller, Y. Wang, C. Zong, N. Deng, T. M. Vondriska, D.A. Liem, J. I. Yang, P. Korge, H. Honda, J. N. Weiss, R. Apweiler, P. Ping**. Systematic characterization of the murine mitochondrial proteome using functionally validated cardiac mitochondria. – Proteomics, **8**(8), 2008, 1564-1575
87. **Zhang, J., D. A. Liem, M. Mueller, Y. Wang, C. Zong, N. Deng, T. M. Vondriska, P. Korge, O. Drews, W. R. Maclellan, H. Honda, J. N. Weiss, R. Apweiler, P. Ping**. Altered proteome biology of cardiac mitochondria under stress conditions. – J.Proteome Research., **7**(6), 2008, 2204-2214.
88. **Zhu, D., D. J. Dix, E. M. Eddy.** HSP70-2 is required for CDC2 kinase activity in meiosis I of mouse spermatocytes**.** – *Development*, **124**, 1997, 3007-3014.