Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Acta morphologica et anthropologica, 25 (3-4)
Sofia ● 2018

Sperm Mitochondrial Biology During Spermatogenesis and Fertilization (Review)

E. Zvetkova[2], I. Ilieva[1], I. Sainova[1], B. Nikolov[1]*

[1] Dept. Experimental Morphology, Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria
[2] Bulgarian Society of Biorheology, Institute of Mechanics, Bulgarian Academy of Sciences, Bulgaria

* Corresponding author e-mail: iilieva@abv.bg

The aim of our review article is related to the sperm mitochondrial biology during living cycle – spermatogenesis, when the sperm mitochondria are independent biomarkers of male germ cell differentiation, sperm capacitation and motility, as well as of spermatozoa health and fertility. An increased understanding of sperm mitochondrial structure, functions, bioenergetics, remodeling/plasticity and especially studies of the own mitochondrial genome (mtDNA), may be useful in the routine andrological clinical practice and assisted reproduction.

Key words: remodeling/plasticity, sperm mitochondrial genome, proteomics, fertilization, male pronucleus

Introduction

The mitochondrial morphology, functions, biodynamics, remodeling and plasticity have been elucidated on cellular, biochemical and molecular levels in plants, animals and humans, and could be characterized as species-specific features [4, 6, 18, 39, 40, 43, 57, 72]. During the mitotic- and meiotic cell divisions, as well as in the process of fertilization, the mitochondria form complexes with other cellular organelles (cytoskeleton, endoplasmic reticulum, ribosomal components, etc.) [5, 41, 43, 56, 59, 74, 81].

The mitochondria contain their own genome with a modified genetic code [74]. The human mitochondrial DNA/mtDNA (double-stranded circular molecule in the mitochondrial matrix) encodes two rRNAs, 22 tRNAs and many specific polypeptides [4, 74], related with normal cell development. In the last decades, new modern technologies give possibilities to be examined and evaluated the influence of mtDNA-mutations/ polymorphism on human diseases (genetic cases of "mitochondrial diseases") [16, 79], as well as with ageing [19].

Mitochondrial structure, functions and plasticity are closely related to the cellular oxidative metabolism and bio-energy production [46], but organelles also participate in the processes of apoptosis, reactive oxygen species ROS-generation, Ca^{2+} (calcium-)

homeostasis [2, 3, 21, 39, 40, 68, 72]. Having in view this specific mitochondrial functions the authors pointed out the significance of organelles as independent biomarkers of different health disorders ("mitochondrial diseases" including) [6, 18, 26, 46, 47, 50, 59, 60, 73, 78].

Sperm mitochondria differ from the same organelles of somatic cells participating in important spermatozoon functions, such as sperm viability, motility, activation, acrosomal reaction and fertilization. Further studies could be performed to determine the role of mitochondrial functions in male fertility/infertility. Recent data [45] supported idea that mitochondria during spermatogenesis affect sperm survival and preservation for the purposes of assisted reproduction. The question arises whether or not there are implications of spermatozoa damage due to the increasing ROS - simultaneously with high mitochondrial activity [12, 17, 45, 77]. As additional criteria for stratification of human sperm quality and fertility were proposed mitochondrial DNA amplification, but also further studies are necessary to understand mtDNA- (mitochondrial genome-) potential as a new independent biomarker for sperm health [2, 33, 39, 49]. The biological reactions occurring inside mitochondria as changes in their proteome, metabolome, respiratory functions, redox signaling and bioenergetics metabolism, cover their principal role as biomarkers – in health and diseases [4, 5, 12, 41, 45, 52, 53, 74].

The aim of this review article is to present and compare data from various studies focusing on mitochondria as special sperm organelles and independent biomarkers of male germ sells/spermatozoa health during spermatogenesis and fertilization, as well as to be used as new diagnostic tools of male fertility/infertility in clinical andrology.

Male germ cells mitochondrial morphology, functions, biodynamic and plasticity during spermatogenesis

During spermatogenesis, the mitochondrial plasticity/dynamics is related to organelle capability to change size, shape and number [58, 73]. Three different morphological types of mitochondria have been described during spermatogenesis – "orthodox", "condensed" and "intermediate", according to the states of expansion and/or contraction of the mitochondrial matrix (according to earlier data of Hackenbrock, 1968) [22, 43].

The mitochondria of "orthodox" type (characterized with a few cristae, contracted and dispersed in electron-translucent matrix) have been found in the various types of spermatogonia, in preleptotene and leptotene spermatocytes, as well as in the Sertoli cells [74]. In this male germ cells the number of mitochondria is small, the organelles are dispersed in the cytoplasm, and their shape is most often oval [13, 43, 53, 74].

In the early stages of meiosis – zygotene and early pachytene, the mitochondria with "intermediate" configuration prevail in the spermatocytes [43, 53, 55].

The "condensed" mitochondrial configuration could serve as biomarker of organelles in active functional state. The round-shaped "condensed" mitochondria – with small size, compressed matrix and dilated christae [43, 53, 55, 74], are localized in the male germ cells (including middle- and late pachytene spermatocytes) during meiotic cell division, as well as in the secondary spermatocytes and the early round spermatids [13, 43, 74].

The biological characteristics of mitochondria in male germ cells in the early prophase-I of meiosis (increased number, round/elongated shape, aggregation of mitochondria in clusters, located near to the nuclear membrane) [13, 43, 74], support the idea for active participation of the organelles in the early spermatogenesis.

In male germ cells (zygotene and pachytene spermatocytes) mitochondria form clusters and are associated with an amorphous electron-dense structure in the cytoplasm – without limiting membrane, termed "nuage" or germinal granules [9, 16, 58, 79]. Germinal granules are aggregates characterized as ribinucleoproteins (RNPs) – associated with RNA- and

protein metabolism, and interplaying with mitochondria. Some germ cell-specific proteins, including GASZ-, PIWI-, MIWI-families are associated with "nuage" [8, 9, 16, 19, 39, 58, 79]. Loss of function of these proteins usually leads to disrupted formation of "nuage", defective piRNA biosynthesis and male infertility [8, 9, 16, 19]. The role of "nuage" (first described ultrastructurally by Eddy et al. [16] and called also inter-mitochondrial cement – IMC or pi-body) in the small RNAs (sRNAs) biosynthesis and in the maintenance of genome integrity, was largely examined during male germ cell differentiation [16, 58, 79]. IMC was found in the embryonic gonocytes, in the post-natal spermatogonia and in spermatocytes with clustered mitochondria [79].

In the secondary spermatocytes, no mitochondrial clusters have been established near the cell nucleus, and the translocation of the organelles to the plasma membrane has been observed [74]. During spermatids' maturation (elongated spermatids) and their moving to the lumens of the seminiferous tubules of testes, the appearance of "intermediate" forms of mitochondria could be assessed [43, 74]. Some of the organelles are localized in the developing sperm flagellum, whereas others form the "residual bodies", subsequently phagocyted by Sertoli cells [14, 23, 43].

At the end of spermiogenesis, the sperm mitochondrial are again transformed into "orthodox" type, as well as could be "elongating" or "fusing" into larger organelles. They are grouped and helically arranged in the region of the sperm neck, as well as "enveloped" into the mitochondrial sheath, localized around the outer dense fibers (ODFs) and axoneme in the middle-piece (mid-piece) of flagellum (sperm tail) (Fig.1) [24, 26, 45, 53]. In the mitochondrial sheath the organelles are surrounded by specific selenoproteins and attached to the fibrose sheath. Additionally, the mitochondrial sheath provides structural support to the flagellum [53]. In the scientific literature a discussion existed if the sperm mid-piece tail is discarded outside the ovum in fertilization or the paternal mitochondria are degraded inside the zygote, following male gamete penetration [53]. The examination of this phenomenon needs of future investigations. (**Fig. 1**)

The male germ cells' survival in the testis depends also of the carbohydrate metabolism – anaerobic (glycolysis) and aerobic (OXPHOS) pathways [53]. In the inner mito-

Fig. 1. Electron image showing the immature human spermatozoa with mitochondrial sheath in mid-piece of the sperm tail: longitudinal section. TEM, × 20000 [24].

chondrial membrane (IMM) are arranged the respiratory enzyme complexes including NADH-dehydrogenase, succinate dehydrogenase, cytochrome oxidase, cytochrome C, etc. [45]. One of the respiratory complexes is responsible for ATP synthesis. The IMM and christae enclose a dense protein-rich mitochondrial matrix. Lactate dehydrogenase (LDH) activity within the mitochondrial matrix is unique to sperm cells and is related to the mitochondrial energy production too [70]. For energy production sperm mitochondrial matrix needs lactate/pyruvate, aspartate/malate and glycerol-3 phosphate: addition of lactate and pyruvate to the sperm media increases mitochondrial functions [12].

Dynamics and variations in mitochondrial morphology during spermatogenesis could be influenced by OXPHOS and respiration mitochondrial activity: in conditions of active respiration and functionality, most of the mitochondria rest in "condensed" state [41]. The changes in the mitochondrial dynamics are closely dependent to the metabolic and energetic status of cells and have shown a transition from slow to intensive male gamete metabolism [21, 43, 49]. The morphological mitochondrial transformations (orthodox configuration) in both spermatogonia and spermatocytes' in prophase stages (preleptotene, leptotene) include predominantly the glycolysis as a main source of ATP [21, 43, 49]. Condensed and intermediate mitochondria in the male germ cells during spermatogenesis are preliminary involved in the production of ATP by OXPHOS mechanism [7, 44, 47, 54]. Enhanced OXPHOS leads also to increased number and size (hypertrophy) of organelles [1, 5, 13].

A hypothesis about changes in the mitochondria architecture as a result of osmotic changes in the local cell tissue environment, has been also proposed [40]. The localization of many mitochondria in the mid-piece of the flagellum at the end of spermiogenesis is related to diffusion of more oxygen and mitochondrial energy (ATP) to the flagellum - of importance for spermatozoa motility [23, 27, 74].

Additionally to energy production, the mitochondria are also engaged in the processes of calcium homeostasis and apoptosis or "apoptotic-like" events in male germ cells during spermatogenesis [45]. All biological reactions occurring inside mitochondria lead to changes in their proteome-, metabolome-redox signaling, respiratory functions and bioenergetics [5, 40, 74].

Mitochondrial genome (mtDNA, RNAs and proteins) during spermatogenesis and fertilization

In the scientific literature was indicated the significance of mitochondrial DNA (mtDNA) as a non-invasive biomarker of sperm quality and fertility, but it is not clear whether mtDNA has a functional role in sperm when in cases of completed spermatogenesis [42, 64, 67].

Mitochondrial DNA copy number of sperm has been examined in human and mammalian and data demonstrated an increase in mtDNA copy number simultaneously with reduced sperm motility and quality [42, 64, 68]. Fragmented sperm genomes were also described within mitochondria [1]. The cellular and molecular mechanisms of epiphenomenon has not been elucidated but authors hypothesized that the mitochondrial transcription factor A (TFAM) is a key regulator of the mtDNA copy number in mammalian and human: the down-regulation of TFAM correlated to the lower mtDNA copy number – examined at the level of the round and elongated spermatides during spermatogenesis [2, 33, 34, 54]. Recently, as additional criteria for sperm quality were proposed mtDNA amplification and mtDNA/beta-globin ratio, which could be biomarkers for male infertility [41, 47].

Some types of mtDNA damages as specific point mutations and deletions in the mitochondrial genome (containing in mammalian 37 genes, which encode 13 peptides,

22 tRNAs and 2 rRNAs [50]) could affect synthesis of RNAs, proteins and respiratory enzymes also participating in the regulation of spermatogenesis in mammalians and humans [11, 20, 44, 46, 48]. Microarray analysis suggested that sperm from asthenozoospermic patients has altered levels of specific mtRNAs, as well as of nuclear transcripts, encoding several sperm mitochondrial proteins [28, 62, 80].

Nakada et al. [48] evaluated induction of mitochondrial dysfunction in the human spermatogenic cells due to accumulation of the pathogenic mutant DNAs in the testes – leading to the "meiotic arrest" in male germ cells during spermatogenesis. Accumulated mtDNA mutations in the spermatocytes and early spermatids during spermatogenesis in mammalians and humans suppress the respiratory functions of the sperm mitochondria, increase the number of apoptotic spermatogenic cells, induce respiration deficiency and spermatocyte degeneration and thus, male infertility [26, 46, 48, 55, 58, 78]. The presence of mutant mtDNA has been also evaluated in animal models [15, 46]. During spermatogenesis the mutant mtDNA accumulate preliminary in the early spermatids with induced mitochondrial dysfunction [26, 46, 48]. Deletions and other types of mutations in the mtDNA occur at a frequency of less than 1% in men and increase with aging [44]. However, few studies on the special role of mtDNA mutations/polymorphism in male fertility/infertility (disorders as oligozoospermia and asthenozoospermia) have been performed [31, 42, 48, 73, 78]. mtDNA-amplification was proposed as an additional criterion for human sperm quality and male fertility by WHO (1999) [42, 48].

Recent studies [1, 29, 46, 49, 50, 64, 66, 69] showed that pathological changes in the mtDNA during spermatogenesis affect male spermatogenic cell homeostasis and induce their degeneration and apoptosis. Degeneration of germ cells affects the quantitative and qualitative aspects of spermatogenesis [29, 30]. A significant male germ cells loss (36–45%) by degeneration occurs during the meiotic cell division [30]. The mitochondria thereby assuring that good quality meiotic products enter the process of spermatogenesis to yield good quality mature sperm [52]. MtDNA mutations in the etiopathogenesis of male infertility have been recently reviewed [1, 41, 59, 62, 73]. Data existed that remained in the egg cytoplasm paternal mitochondria after fertilization are eliminated, for example by ubiquitinization [68, 71].

The morphological and functional development of male germ cells is a reflection of the changes in the testicular microenvironment during spermatogenesis when expression of specific mitochondrial marker proteins was evaluated: with attention to hsp60, hsp70, protamin-1, Lon-protease, sulfhydryl-oxidase, cytochrome C, cytochrome Ct, endopeptidases, chaperonin 60, transition proteins 1, 2, etc. [32, 43]. The expression of several sperm mitochondrial proteins may be altered in asthenozoospermic patients [62]. The synthesis of the sperm mitochondrial fusion and fission factors – mitofusins 1, 2 (Mfn1 and Mfn2) and dynamin-related protein 1 (Drp1) was examined at the levels of the round and elongated spermatids (steps 8 - 12) of spermatogenesis [24].

Mitochondrial functions are preliminary related to the oxidative metabolism of cells and free oxidative radicals (ROS-) generation. To meet their energy demands, sperm utilize both pathways – oxidative and/or glycolytic energy metabolism. For example, bull sperm utilizes both OXPHOS and glycolysis with high efficiency [38]. The question arises if the stimulated and increased sperm mitochondrial function prior to sperm use in assisted reproduction may improve male fertility [12]. In this sense, mild oxidative stress (produced by ROS) is considered necessary for some sperm functions as motility, acrosome reaction and fertility [1]. On the other hand, high ROS activity/ oxidative stress are related to the respiration defects in mitochondria – including to the mitochondrial genome (mtDNA-) mutations and low male fertility: ROS production is necessary for normal sperm function [45], but in excess it could damage spermatozoa – with negative influence on fertility.

Evidence that human sperm mitochondria have the potential to generate ROS was obtained using a peroxide-based hemiluminescent system to detect the release of H_2O_2 into the extracellular space. Fluorescent probes (flow-cytometry) and ApoAllert mitochondrial membrane sensor kits – JC-1, TMRM and Mito Tracker Green (MT-G), were employed to assess mitochondrial membrane potential [12, 25, 45, 65]. The authors showed that MT-G-positive sperm has better fertilization potential: green-stained mid-pieces of spermatozoa could be biomarkers for the presence of high functional mitochondria, successfully participating in the processes of fertilization and early embryonic development. By this way MT-G test gives new possibilities to determine whether sperm would constitute a better - (MT-G-positive-) functional sperm subpopulation, or MT-G-negative non-functional male gametes - with low quality/ fertility parameters.

According to many studies, the changes in the mitochondrial morphology are dependent on the protein synthesis in the Sertoli cells (actively synthesizing paracrine mitochondrial maturation factors/PMMFs) [14, 43, 60]. These data have pointed out the protein activin A as a major paracrine modulator in the early male spermatogenesis. In addition, the Sertoli cells are the main lactate/pyruvate source required for OXPHOS and ATP-synthesis of mitochondria during meiosis and post-meiosis phases of spermatogenesis [13, 21, 43].

Sperm mitochondria during fertilization (male pronucleus development)

In this review we try to summarize insufficient literature data on the role of sperm mitochondria in the biology of fertilization [37]. Having in view our own *in vitro* studies in this field, we pointed out especially on data for sperm mitochondria participation in the development of the male pronucleus (breakdown of the sperm nuclear envelope; dispersion of the condensed sperm nuclear chromatin preliminary at the perinuclear regions; development of the male pronuclear envelope (**Fig. 2**).

Following fusion of male and female gametes and subsequent breakdown of the sperm nuclear envelope, the male nuclear chromatin is associated directly – without membranous boundary, with components of the egg cytoplasm. The conical sperm nucleus changes into spherical MP with decondensed (dispersed) nuclear chromatin. In the literature exists a hypothesis [37] related to the probable "agents" responsible for sperm nuclear chromatin dispersion – a process starting at the periphery of the nucleus (**Fig. 2D**). The authors concluded that events related to the MP formation are similar to those occurring in prophase and telophase of mitotically-active cells. There are lack of accurate data on the fate of incorporated sperm mitochondria, flagellum and perinuclear structures – well visualized on **Fig. 2**. For example data existed that after sperm incorporation in the egg the mitochondria are displaced from the sperm mid-piece and destruction of mitochondrial sheath occurs [71]. An aggregate of clustered unstained sperm mitochondria (as mitochondrial "crown") could be visualized around the sperm nucleus in the early stages of sperm penetration (**Fig. 2B**). These mitochondria are of very small size – probably result of fission [24]. Organelles are probably in active functional state – fusing in the MP and thus, participating in the chromatin activation/decondensation. Similar results were obtained in highly proliferated somatic cells [82, 84]. According to data of other authors about the fate of organelles - the mitochondrial clusters of this type remain in the vicinity of the MP and can later be seen at one of the poles of the metaphase spindle, as well as in one blastomer of the four-cell embryo, and are no longer detected in early embryogenesis [37]. Finally, a zygote nucleus formation is a result of fusion of the outer and inner membranes of the two pronuclear (MP-, FP-) envelopes.

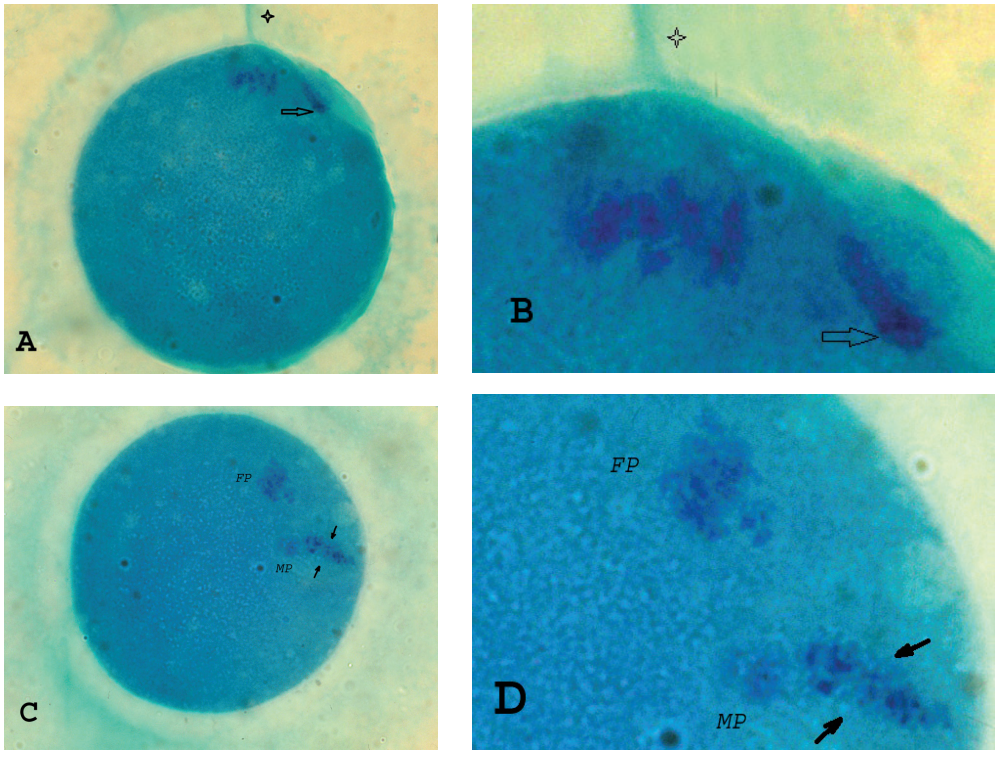

Fig 2. *In vitro* fertilization in mice. Remodeling of sperm nucleus into a male pronucleus during fertilization. A, B – The spermatozoon enters the cytoplasm of oocyte through the cone of fertilization (B – empty arrow). Desoxyribonucleoproteins (DNP) are blue-violet stained (with methylene blue) in both pronuclei (female – FP and male – MP). Decondenzation of the sperm nuclear chromatin could be visualized. Basic proteins in sperm head, neck and tail (asterisks) are green-stained (with fast green). C, D – DNP in the FP, MP and mitochondrial genome (mitochondria localized in the mid-piece of sperm tail – arrows), are blue-violet stained and one could see condensed chromosomal chromatin in FP and perinuclear chromatin decondensation in MP. Staining with methylene blue – fast green, after cold acid hydrolysis with 5N HCL for RNP-extraction (Zvetkova and Zvetkov [83]); × 200, 450.
Images have been originally made by assoc. prof. Elissaveta Zvetkova and prof. Pascale Debay – Institut de Biologie Physico-Chimique – INSERM, Paris – France, 1991.

One question remains - related to confusions still persisting in the literature: evaluation that the paternal sperm-born mitochondria of most mammalian species are targeted for lysosomal degradation by ubiquitin system (ubiquitination) during fertilization. Other authors supported the thesis that such widely disputed evidence warrants further investigations. The problem is also related to the major paradox in the developmental biology - for maternal inheritance of mtDNA and subsequent paternal genome elimination in the zygote, by unknown degradation mechanisms [71]. New methods for cytological/cytochemical *in situ* studies on sperm mitochondria could be recommended for better understanding of the sperm mitochondrial biology - in health and diseases.

Conclusion

Nowadays it is possible to evaluate sperm mitochondrial biology (structure, functions, biochemistry, biodynamics, remodeling, plasticity, etc.) during spermatogenesis/spermiogenesis. Biological characteristics of sperm mitochondria could serve as biomarkers and diagnostic tools for spermatozoa health and fertility in the clinical practice.

In conclusion, we could point out that morphological, biochemical, physiological and bioenergy parameters of sperm mitochondria correlate with sperm quality, health, functionality and fertilization properties. From epigenetic's point of view, it is important that a cross-talk between mitochondrial metabolism, functionality and genome activities could also exist.

References

1. **Amaral, A., B. Lourenço, M. Marques, J. Ramalho-Santos**. Mitochondria functionality and sperm quality. – *Reproduction*, **146**, 2013, 163-174.
2. **Amaral, A., J. Ramalho-Santos, J. C. St John**. The expression of polymerase gamma and mitochondrial transcription factor A and the regulation of mitochondrial DNA content in mature human sperm. – *Human Reprod.*, **22**, 2007, 1585-1596.
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. **Arismendi-Morillo, G**. Electron microscopy morphology of the mitochondrial network in human cancer. – *Int. J. Biochem. Cell Biol.*, **41**, 2009, 2062-2068.
5. **Bereiter-Hahn, J., M. Vöth**. Dynamics of mitochondria in living cells: shape changes, dislocations, fusion, and fission of mitochondria. – *Microsc. Res. Tech*., **27**, 1994, 198-219.
6. **Boussouar, F., M. Benahmed**. Lactate and energy metabolism in male germ cells. – *Trends Endocrinol. Metab*., **15**, 2004, 345-350.
7. **Campello, S., L. Scorrano**. Mitochondrial shape changes: orchestrating cell pathophysiology. – *EMBO Reports*, **11**, 2010, 678-684
8. **Carmell, M. A., A. Girard, H. J. van de Kant, D. Bourc'his, T. H. Bestor, D. G. de Rooij, G. J. Hannon**. MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. – *Dev. Cell*, **12**, 2007, 503-514.
9. **Chuma, S., M. Hosokawa, T. Tanaka, N. Nakatsuji**. Ultrastructural characterization of spermatogenesis and its evolutionary conservation in the germline: Germinal granules in mammals. – *Mol. Cell. Endocrinol*., **306**, 2009, 17-23.
10. **Clayton, D. A**. Transcription and replication of mitochondrial DNA. – *Hum. Reprod*., **2**, 2000, 11-17.
11. **Creasy, D., A. Bube, E. de Rijk, H. Kandori, M. Kuwahara, R. Masson, T. Nolte, R. Reams, K. Regan, S. Rehm, P. Rogerson, K. Whitney**. Proliferative and nonproliferative lesions of the rat and mouse male reproductive system. – *Toxicol. Pathol*., **40**, 2012, 40S-121S.
12. **Darr, C. R., G. A. Cotopassi, S. Datta, D. Varner, S. A. Meyers**. Mitochondrial oxygen consumption is a unique indicator of stallion sperm spermatozoal health and varies with cryopreservation media. – *Theriogenology*, **86**, 2016, 1382-1392.
13. **De Martino, C., A. Floridi, M. L. Marcante, W. Malorni, P. Scorza Barcellona, M. Bellocci, B. Silvestrini**. Morphological, histochemical and biochemical studies on germ cell mitochondria of normal rats. – *Cell Tissue Res*., **196**, 1979, 1-22.
14. **de Winter, J. P., H. M. J. Vanderstichele, M. A. Timmerman**. Activin is produced by rat Sertoli cells *in vitro* and can act as an autocrine regulator of Sertoli cell function. – *Endocrinology*, **123**, 1993, 975-982.
15. **Dunn, D. A., M. V. Cannon, M. H. Irwin, C. A. Pinkert**. Animal models of human mitochondrial DNA mutations. – *Biochim. Biophys. Acta*, **1820**, 2012, 601–607.
16. **Eddy, E. M**. Fine structural observations on the form and distribution of nuage in germ cells of the rat. – *Anat. Rec*., **178**, 1974, 731–197757.
17. **Gibb, Z., S. R. Lombourne, R. G. Aitken**. The paradoxal relationship between stallion fertility and oxidative stress. – *Biol. Reprod*., **91**, 2014, 77.

18. **Greaves, L. C., A. K. Reeve, R. W. Taylor, D. M. Turnbull**. Mitochondrial DNA and disease. – *J. Pathol.*, **226**, 2012, 274-286.
19. **Grivna, S. T., B. Pyhtila, H. Lin**. MIWI associates with translational machinery and PIWI-interacting RNAs (piRNAs) in regulating spermatogenesis. – *Proc. Natl. Acad. Sci. U.S.A.*, **103**, 2006, 13415-13420.
20. **Grivna, S. T., E. Beyret, Z. Wang, H. Lin**. A novel class of small RNAs in mouse spermatogenic cells. – *Genes Dev.*, **20**, 2006, 1709-1714.
21. **Grootegoed, J. A., R. Jansen, H. J. van der Molen**. Spermatogenic cells in the germinal epithelium utilize alpha-ketoisocaproate and lactate, produced by Sertoli cells from leucine and glucose. – *Ann. N. Y. Acad. Sci.*, **438**, 1984, 557-560.
22. **Hackenbrock, C. R**. Ultrastructural bases for metabolically linked mechanical activity in mitochondria. II. Electron transport-linked ultrastructural transformations in mitochondria. – *J. Cell Biol.*, **37,** 1968, 345-369.
23. **Ho, H. C., S. Wey.** Three dimensional rendering of the mitochondrial sheath morphogenesis during mouse spermiogenesis. – *Microsc. Res. Tech.*, **70**, 2007, 719-723.
24. **Honda, S., S. Hirose**. Stage-specific enhanced expression of mitochondrial fusion and fission factors during spermatogenesis in rat testis. – *Biochem. Biophys. Res. Commun.*, **311(2)**, 2003, 424-432.
25. **Hu, C. H., X. J. Zhuang, Y. M. Wei, M. Zhang, S. S. Lu, Y. Q. Lu, X. G. Yang, K. H. Lu**. Comparison of mitochondrial function in boar and bull spermatozoa throughout cryopreservation based on JC-1 staining. – *Cryo. Lett.*, **38**, 2017, 75-79.
26. **Ilieva, I**. Morphological changes in the structure of sperm in male infertility. – PhD Thesis, Bulgarian Academy of Sciences, Sofa, 2012, 215.
27. **Ilieva, I., I. Sainova, E. Zvetkova**. Sperm mitochondria-associated male infertility: sperm quality defects and mitochondria (mtDNA) anomalies. – *Acta morphol. anthropol.*, **24**, 2017, 114-122.
28. **Jodar, M., S. Kalko, J. Castillo, J. L. Ballesca, R. Oliva**. Differential RNAs in the sperm cells of asthenozoospermic patients. – *Hum. Reprod.*, **27**, 2012, 1431-1438.
29. **Johnson, L**. Increased daily sperm production in the breeding season of stallions is explained by an elevated population of spermatogonia. – *Biol. Reprod.*, **32**, 1985, 1181-1190.
30. **Johnson, L., C. S. Petty, W. B. Neaves**. Further quantification of human spermatogenesis: germ cell loss during postprophase of meiosis and its relationship to daily sperm production. – *Biol. Reprod.*, **1**, 1983, 207-215.
31. **Kao, S. H., H. T. Chao, Y. H. Wei**. Multiple deletions of mitochondrial DNA are associated with the decline of motility and fertility of human spermatozoa. – *Mol. Human Reprod.*, **4**, 1998, 657-666.
32. **Kleene, K. C., R. J. Distel, N. B. Hecht**. Translational regulation and deadenylation of a protamine mRNA during spermiogenesis in the mouse. – *Dev. Biol.*, **105**, 1984, 71-79.
33. **Kolmer, M., M. Pelto-Huikko, M. Parvinen, C. Höög, H. Alho**. The transcriptional and translational control of diazepam binding inhibitor expression in rat male germ-line cells. – *DNA Cell Biol.*, **16**, 1997, 59-72.
34. **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 Molec. Genet.*, **6**, 1997, 185-191.
35. **Larsson, N. G., J.D. Garman, A. Oldorfs, G.S. Barsh, D.A. Clayton.** A single mouse gene encodes the mitochondrial transcription factor A and a testisspecific nuclear HMG box protein. – *Nat. Genet.*, **13**, 1996, 296-302.
36. **Lindemann, C. B., K. A. Lesich**. Functional anatomy of the mammalian sperm flagellum. – *Cytoskeleton*, **73,** 2016, 652-669.
37. **Longo, F. J., E. Anderson**. The fine structure of pronuclear development and fusion in the sea urchin, *Arbacia punctulata*. – *J. Cell Biol.*, **39**(2), 1968, 339-368.
38. **Losano, J. D. A., J. F. Padin, I. Mendez-Lopez, D. S. R. Angrimani, A. G. Garcia, V. H. Barnabe, M. Nichi**. The stimulated glycolytic pathway is able to maintain ATP levels and kinetic patterns of bovine epididymal sperm subjected to mitochondrial uncoupling. – *Oxid. Med. Cell. Longev*. **ID 1682393**, 2017, 1-8.
39. **Ma, L., G. M. Buchold, M. P. Greenbaum, A. Roy, K. H. Burns, H. Zhu, D. Y. Han, R. A. Harris, C. Coarfa, P. H. Gunaratne, W. Yan, M. M. Matzuk**. GASZ is essential for male meiosis and suppression of retrotransposon expression in the male germline. – *PLoS Genet.*, **5**, 2009, e1000635.

40. **Mannella, C. A**. Structural diversity of mitochondria, functional implications. – *Ann. N.Y. Acad. Sci*., **1147**, 2008, 171-179.
41. **Mannella, C. A**. Structure and dynamics of the mitochondrial inner membrane cristae. – *Biochim. Biophys. Acta – Molecular Cell Research*, **1763**, 2006, 542-548.
42. **May-Panloup**, **P., M. F. Chretien, F. Savagner, C. Vasseur, M. Jean, Y. Malthiery, P. Reynier**. Increased sperm mitochondrial DNA content in male infertility. – *Hum. Reprod*., **18**, 2003, 550-556.
43. **Meinhardt, A., B. Wilhelm, J. Seitz**. Expression of mitochondrial marker proteins during spermatogenesis. – *Human Reprod. Upd*., **5**, 1999, 108-119.
44. **Michikawa, Y., F. Mazzucchelli, N. Bresolin, G. Scarlato, G. Attardi**. Aging dependent large accumulation of point mutations in the human mtDNA control region for replication. – *Science*, **286**, 1999, 774-779.
45. **Moraes, C. R., S. Meyers**. The sperm mitochondrion: Organelle of many functions. – Animal Reprod. Sci., 2018, https://doi.org/10.1016/j.anireprosci.2018.03.0242018.
46. **Mota, P. C., S. Amaral, L. Martins, M. L. Pereira, P. J. Oliveira, J. Ramalho-Santos**. Mitochondrial bioenergetics of testicular cells from the domestic cat (felis catus) – a model for endangered species. – *Reprod. Toxicol*., **11**, 2009, 111-116.
47. **Moussa, F., R. Oko, L. Hermo**. The immunolocalization of small nuclear ribonucleoprotein particles in testicular cells during the cycle of the seminiferous epithelium of the adult rat. – *Cell Tissue Res*., **278**, 1994, 363-378.
48. **Nakada, K., A. Sato, K. Yoshida, T. Morita, H. Tanaka, S. Inoue, H. Yonekawa, J. Hayashi**. Mitochondria - related male infertility. – *Proc. Natl. Acad. Sci. U.S.A.*, **103**, 2006, 15148-15153.
49. **Nakamura, M., S. Okinaga, K. Arai**. Metabolism of pachytene primary spermatocytes from rat testes: pyruvate maintenance of adenosine triphosphate level. – *Biol. Reprod*., **30**, 1984, 1187-1197.
50. **Park, C. B., N.-G. Larsson**. Mitochondrial DNA mutations in disease and aging. – *J. Cell Biol*., **193**, 2011, 809-818.
51. **Pelliccione, F., A. Micillo, G. Cordeschi, A. D'Angeli, S. Necozione, L. Gandini, A. Lenzi, F. Francavilla S. Francavilla**. Altered ultrastructure of mitochondrial membranes is strongly associated with unexplained asthenozoospermia. – *Fertil. Steril.*, **95**, 2011, 641-646.
52. **Rajender, S., P. Rahul, A. A. Mahdi**. Mitochondria, spermatogenesis and male infertility. – *Mitochondrion*, **10**, 2010, 419-428
53. **Ramalho-Santos, J., S. Varum, S. Amaral, P. C. Mota, A. P. Sousa, A. Amaral**. Mitochondrial functionality in reproduction: from gonads and gametes to embryos and embryonic stem cells. – *Human Reprod. Upd*., **15**, 2009, 553-572.
54. **Rantanen, A., N. G. Larsson**. Regulation of mitochondrial DNA copy number during spermatogenesis. – *Hum. Reprod*., **15** (2), 2000, 86-91.
55. **Rato, L., M. G. Alves, S. Socorro, A. I. Duarte, J. E. Cavaco, P. F. Oliveira**. Metabolic regulation is important for spermatogenesis. – *Nat. Rev. Urol*., **9**, 2012, 330-338.
56. **Rube, D.A., A. M. van der Bliek**. Mitochondrial morphology is dynamic and varied. – *Mol Cell. Biochem*., **256/257**, 2004, 331-339.
57. **Ruiz-Pesini, E., C. Diez, A. Lapena, A. Perez-Martos, J. Montoya, E. Alvarez, J. Arenas**, **M. Lopez-Perez**. Correlation of sperm motility with mitochondrial enzymatic activities. – *Clin. Chem.*, **44**, 1998, 1616-1620.
58. **Russell, L., B. Frank**. Ultrastructural characterization of nuage in spermatocytes of the rat testis. – *Anat. Rec.*, **190**(1), 1978, 79-97.
59. **Scott, I., D. C. Logan**. Mitochondrial dynamics: the control of mitochondrial shape, size, number, motility, and cellular inheritance. – In: Plant Mitochondria, (Ed. David C Logan). – *Blackwell Publishing Ltd*., Oxford, UK, 2007, 1-35.
60. **Seitz, J., J. Möbius, M. Bergmann, A**. **Meinhardt**. Mitochondrial differentiation during meiosis of male germ cells. – *Int. J. Androl*., **2**, 1995, 7-11.
61. **Shamsi, M. B., R. Kumar, A. Bhatt, R. Bamezai, R. Kumar, N. Gupta, T. Das, R. Dada**. Mitochondrial DNA mutations in etiopathogenesis of male infertility. – *Ind. J. Urol.*, **24** (2), 2008, 150-154.
62. **Siva, A. B., D. B. Kameshwari, V. Singh, K. Pavani, C. S. Sundaram, N. Rangaraj, M. Deenadayal, S. Shivaji**. Proteomics-based study on asthenozoospermia: differential expression of proteasome alpha complex. – *Mol. Human Reprod.*, **16**, 2010, 452-462.
63. **Söderstrom**, **K. O., Parvinen, M**. Incorporation of (^{3}H) uridine by the chromatoid body during rat spermatogenesis. – *J. Cell Biol*., **70**, 1976, 239-246.

64. **Song, G. J., V. Lewis.** Mitochondrial DNA integrity and copy number in sperm from infertile men. – *Fert. Steril*., **90**, 2008, 2238-2244.
65. Sousa, A. P., A. Amaral, M. Baptista, R. Tavares, P. C. Campo, P. C. Peregrín, A. Freitas, A. Paiva, T. Almeida-Santos, J. Ramalho-Santos. Not all sperm are equal: functional mitochondria characterize a subpopulation of human sperm with better fertilization potential. – *PloS ONE*, 6(3), 2011, e18112.
66. **Srivastava, N., P. Megha**. Mitochondrion: Features, functions and comparative analysis of specific probes in detecting sperm cell damages. – *Asian Pacif. J. Reprod*., **5**(6), 2016, 445-452.
67. **St John, J. C., E. Bowles, A. Amaral**. Sperm mitochondria and fertilisation. – *Soc. Reprod. Fertil. Suppl*., **65**, 2007, 399-416.
68. **St John, J. C., R. P. Jokhi, C. L. Barratt.** The impact of mitochondrial genetics on male infertility. – *Int. J. Androl.*, **28**, 2005, 65-73.
69. **St John, J. C., J. Facucho-Oliveira, Y. Jiang, R. Kelly, R. Salah**. Mitochondrial DNA transmission, replication and inheritance: a journey from the gamete through the embryo and into offspring and embryonic stem cells. – *Human Reproduction Update*, **16**, 2010, 488-509.
70. **Storey, B. T.** Mammalian sperm metabolism: oxygen and sugar, friend and foe. – *Int. J. Dev. Biol*., **52**, 2008, 427-437.
71. **Sutovsky, P., R. D. Moreno, J. Ramalho-Santos, T. Dominko, C. Simerly, G. Schatten**. Ubiquitin tag for sperm mitochondria. – *Nature*, **402**, 1999, 371-372.
72. **Taanman, J. W**. The mitochondrial genome: structure, transcription, translation and replication. – *Biochim. Biophys. Acta,* **1410**(2), 1999, 103-123.
73. **Thangaraj, K., M. B. Joshi, A. G. Reddy, A. A. Rasalkar, L. Singh**. Sperm mitochondrial mutations as a cause of low sperm motility. – *J. Androl*., **24**, 2003, 388-392.
74. **Tzagoloff, A**. Mitochondria. Cellular organelles. (Ed. Philip Siekevitz), Plenum Press, New York and London, 1982, 15-23.
75. **Venkatesh, S., M. Deecaraman, R. Kumar, M. B. Shamsi, R. Dada**. Role of reactive oxygen species in the pathogenesis of mitochondrial DNA (mtDNA) mutations in male infertility. – *Indian J. Med. Res.,* **129**, 2009, 127-137.
76. **Wai, T., A. Ao, X. Zhang, D. Cyr, D. Dufort, E. A. Shoubridge**. The role of mitochondrial DNA copy number in mammalian fertility. – *Biology of Reproduction*, **83**(1), 2010, 52-62.
77. **Yeste, M., E. Estrada, L. G. Rocha, H. Marin, J. E. Rodriguez-Gil, J. Miro**. Cryotolerance of stallion spermatozoa is related to ROS production and mitochondrial membrane potential rather than to the integrity of sperm nucleus. – *Andrology*, **3**, 2015, 395-407.
78. **Zeviani, M., C. Antozzi**. Mitochondrial disorders. – Mol. Human Reprod. **3**, 1997, 133-148.
79. **Zhang, J., Q. Wang, M. Wang, M. Jiang, Y. Wang, Y. Sun, J. Wang, T. Xie, C. Tang, N. Tang, H. Song, D. Cui, R. Chao, S. Ding, B. Ni, X. Chen, Y. Wang**. GASZ and mitofusin-mediated mitochondrial functions are crucial for spermatogenesis. – *EMBO Reports*, **17**(2), 2016, 220-234.
80. **Zhao, C., R. Huo, F. Q. Wang, M. Lin, Z. M. Zhou, J. H. Sha**. Identification of several proteins involved in the regulation of sperm motility by proteomic analysis. – *Fertil. Steril.*, **87**, 2007, 436-438.
81. **Zvetkova, E.** Cytochemical detection and distribution of ribonucleoprotein complexes (rnpcs) during mitosis. – *Genetics and breeding*, **25**(3), 1992, 282-287.
82. **Zvetkova E**. Unstained mitochondria in nuclei of myeloblast-patients with CML; Unstained mitochondria in granulocytes' nuclei of cancer patients. – In: Thein S. L. (Ed.), BloodMed, www.bloodmed.com-SlideAtlas, 2006, Blackwell Publishing, Oxford, UK.
83. **Zvetkova, E., I. Zvetkov**. A cytological method for the simultaneous staining of nucleoproteins and some cationic proteins. – *Acta Histochem*., **57**, 1976, 1-13.
84. **Zvetkova E., R. Toshkova**. Unstained mitochondria in the cytoplasm and nucleus of atypical myeloid cell – *Graffi* myeloid tumor, induced in hamster. – In: Thein S. L. (Ed.), BloodMed, www.bloodmed.com-SlideAtlas, 2006, Blackwell Publishing, Oxford, UK.