Contribution of electron microscopy to study *in vitro* inositol effects on human spermatozoa

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**Abstract.** – Infertility is a worldwide problem and male partner contributes to almost 30% of cases of infertility. The term oligoasthenoterato-spermia is related to defective spermatogenesis and is characterized by a reduction of motility and number of spermatozoa and a change in their morphology. Electron microscopes are frequently used in order to evaluate sperm pathology and overall to establish a correlation between structural and functional deficiencies of altered sperm. High levels of reactive oxygen species endanger sperm function and viability. The correlation between male infertility, reactive oxygen species levels and the innovative therapeutic strategy employing inositol has been highlighted through analysis of literature data.

Key Words: Male infertility, Oxidative stress, Sperm morphology, Electron microscopy, Inositol.

**Introduction**

The process through which spermatozoa are produced from a no differentiated germ cell population in the testis is called spermatogenesis. In the multicellular epithelium of the seminiferous tubule, there are two cell types, the germ cells and the somatic Sertoli cells. The Sertoli cells create two compartments within the seminiferous epithelium. The barrier between these compartments is produced by the formation of specialized tight junctions between adjacent Sertoli cells¹.

It is a common assumption that infertility is primarily related to the woman. Indeed, only one-third of infertility cases are related to women alone. Statistically, one-third of infertility problems are related to men and the remaining one-third is a combination of fertility factors involving both partners or unknown causes. Up to 90% of man infertility is correlated to low sperm count or quality or both².

Oligoasthenoteratospermia (OAT) is characterized by the reduction of number, motility, and alterations of the morphology of spermatozoa; several are the therapies suggested for counteracting such a disease, however, they are often ineffective³. Much research remains to be performed on the topic of male infertility, as many cases still receive an “unknown cause” diagnosis. It usually occurs because of the abnormal size or inadequate number of spermatozoa, or because there are problems with ejaculation. One of the factors that may influence male infertility is the production of reactive oxygen species (ROS). Spermatozoa are more susceptible than other cell species to the harmful activity of ROS. In particular, they can affect motility, morphology and DNA stability of spermatozoa.

**Contribution of the Electron Microscopies**

The electron microscope allowed overcome the physical limit of the resolving power of light microscopes (about 200 nm). There are mainly two types of electron microscopes: the transmission (TEM) and the scanning electron microscope (SEM). TEM was devised around the same year as SEM but was marketed only 25 years later (1965). In the field of biomedical research both TEM and SEM play the same important role, especially when they are used in association, yielding complementary morphological and ultrastructural information. TEM enters the cells and explores the ultrastructure of the intracellular components, SEM supplies information on the general morphology and surface architecture of biological samples. SEM has generally a poorer resolution than TEM, but it provides a three-dimensional representation of the sample⁴,⁵.

During the 17th century, Antonie van Leeuwenhoek was the first to report the existence of spermatozoa, that he called *animalcules*, in the seminal fluid of animals and men⁶. Light microscopy (LM), as well as electron microscopy, has been largely employed to study sperm morphology that...
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represents an important factor in determining the fertilizing properties of spermatozoa. LM serves as the routine method for morphological examination of spermatozoa, whereas through TEM single sections of sperm cells can be easily analyzed. SEM is usually employed to study the spermatozoa surface morphology obtaining three-dimension images (Figure 1). Also, microscopic studies are often correlated with molecular biology, proteomic and genomics to understand the male infertility causes and to establish a correlation between structural and functional deficiencies of altered sperm. Sperm motility and morphology have long been recognized as indicators of the fertility potential of human spermatozoa. The recent introduction of micro-fertilization techniques provides access to the structural and functional features of spermatozoa that are being used for fertilization. Overall, TEM is an important adjunct to the traditional methods of semen analysis. Indeed, LM is not able to provide the detailed information required to characterize the morphological and functional state of the mitochondria, dense fibers, fibrous sheath and axoneme. Detailed morphological information regarding sperm flagellum or tail spermatozoa, nuclear and acrosomal structures has been obtained through ultrastructural studies. Furthermore, SEM analysis contributed to demonstrate a significant reduction in the length and/or important morphological alterations of the midpieces associated with multiple ultrastructural abnormalities in the sperm (Figure 2).

**Oxidative Stress and Sperm’s Damages**

Oxidative stress is defined as an imbalance between ROS generation and antioxidant capacity of the mammalian cells. In the past few years, the scientific community studying male infertility addressed the research towards oxidative stress field, since it had been observed that one of the factors which may influence this pathology is linked to the production of ROS by morphologically altered spermatozoa. It is well known that human spermatozoa contain especially high levels of polyunsaturated fatty acids that are very sensible to oxidative stress. Moreover, these cells discharge their cytoplasm during maturation, so they do not contain high levels of antioxidant enzymes that protect from ROS
attack (glutathione peroxidase, catalase and superoxide dismutase)\textsuperscript{37,38}.

Oxidative stress can induce DNA damage such as nucleotide modifications, base loss, deletions, frame-shifts mutations, DNA cross-links and chromosomal rearrangements. These aberrations in the chromatin structure can expose the genome to further oxidative insult\textsuperscript{39}. DNA damage correlates with the lower reproductive potential of sperm, and the assessment of this parameter has a better predictive score than conventional semen analysis. A recent meta-analysis suggests that antioxidant therapy improves male fertility\textsuperscript{37,20-27}.

A substantial number (up to 40\%) of males diagnosed with semen disorders shows high levels of ROS\textsuperscript{34}. Furthermore, human spermatozoa can undergo the effects of ROS released by white blood cells, such as neutrophils, that infiltrate the male tract as a consequence of infection\textsuperscript{30}. Consequently, the presence of free-radical generating leukocytes in human sperm suspensions prepared for Assisted Reproductive Therapy represents a highly significant determinant of fertilization success \textit{in vitro}\textsuperscript{39}.

Moreover, Najafi et al\textsuperscript{30} have demonstrated that changes in the redox status can induce apoptosis. Apoptosis is a physiological phenomenon characterized by cellular morphological and biochemical modifications that cause cells to die in a controlled manner. There are two main stages of apoptotic cell death. The first one involves the interaction of a death receptor, such as the TNF receptor-1 or the Fas receptor, with its ligand and the subsequent one depends on the signaling molecule cytochrome c, which triggers caspasess, such as caspasess 3 and 9\textsuperscript{30}. Apoptosis is activated when the intracellular status becomes more oxidized and caspases, belonging to cysteine protease family, are sensitive to redox state\textsuperscript{30,32}.

The rationale for treating infertile men with oral antioxidants is based on the premise that seminal oxidative stress (common in infertile men) is due in part to a deficiency in seminal antioxidants. However, the levels of seminal ROS should not be entirely suppressed (by oral antioxidants) as this may impair normal sperm functions (e.g., sperm capacitation and hyperactivation) that normally require low levels of ROS\textsuperscript{33}. Consequently, there is great interest in evaluating antioxidant agents that could protect spermatozoa from ROS. Some studies have reported a dose-response relationship between the daily intake of antioxidant nutrients (carotenoids, lycopene and lutein) and semen quality\textsuperscript{34,35}. Recently, resveratrol, a natural phytoalexin, has been studied for its antioxidant properties. It is widely consumed in the Mediterranean diet in the form of peanuts, grapes and wine\textsuperscript{36-38}.

Another possible antioxidant agent is represented by inositol. It is a carbocyclic polyol with 9 different stereoisomers synthesized from glucose-6-phosphate, the first product of glycolysis, and is eliminated by the kidney. Inositol is a precursor of secondary messengers, such as diacylglycerol and inositol triphosphate, which are involved in the cellular signal transduction system and the regulation of calcium intracellular concentration\textsuperscript{39,40}. It is widely distributed in nature and is involved in many systemic mechanisms of signal transduction in the plasma membrane. Inositol is synthesized by 2 enzymes present in high concentrations in the testes, which synthesize inositol from glucose-6-phosphate previously transported into cells by a sodium/inositol cotransport protein, whose expression is sensitive to osmolar changes\textsuperscript{40}.

Inositol, due to the antioxidant activity, could preferentially influence the mitochondria functionality. Mitochondria are sub-cellular organelles present in the cell cytoplasm. They have an outer and an inner membrane separated by an inter-membrane space. The inner membrane forms numerous folds inside mitochondrial matrix (cristae)\textsuperscript{41-51}. An apoptotic stimulus induces the depolarization of mitochondrial membrane potential (MMP), an early and reversible event, following the opening of mitochondrial permeability transition pore. Therefore, the permeability of the mitochondrial membrane is altered leading to the release of cytochrome c and other apoptogenic factors into the cytosol and loss of oxidative phosphorylation.

Sperm mitochondria, as well as the somatic ones, are the location of the oxidative phosphorylation process, which is necessary for the production of metabolic energy in the form of adenosine triphosphate (ATP). Sperms use ATP to sustain their motility, which is one of the major factors to determine male fertility. The role of these organelles in sperm motility was investigated thoroughly, mainly because the causes of fertility-imparing pathologies linked to a pronounced reduction of sperm motility are largely unknown\textsuperscript{52}. Mitochondria are thought to be the most important organelles for the evaluation of sperm quality, and this may be due to the fact that mitochondria contain their own DNA and membrane potential which can easily be examined \textit{in situ}.
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In vitro to reflect sperm DNA integrity and motility, respectively\textsuperscript{53,54}. Several studies demonstrated the important role of mitochondria in spermatogenesis and fertility, even though a conclusive evidence stating their exact involvement still lacks\textsuperscript{51}. The functionality of sperm mitochondria has been investigated using different technical approaches, such as cytofluorimetric methods and ultrastructural analysis\textsuperscript{55,56}.

In these studies, a positive correlation between the mitochondrial functionality and sperm motility or, in general, the quality of semen samples, was found to be reproducible. Moreover, biochemical analyses showed that inositol ameliorates sperm mitochondrial activities.

MMP directly reflects the energy generation in terms of ATP. Healthy mitochondria have high MMP values and facilitate the adequate synthesis of ATP to sustain the sperm motility\textsuperscript{57}. MMP evaluation, therefore, can be used to evaluate sperm quality. It has been shown that men with normal sperm parameters have a significantly greater percentage of spermatozoa with high MMP compared with patients with abnormal parameters.

Polylol myo-inositol is a compound having an unusually high concentration in the seminiferous tubule fluid. It is known that myo-inositol (MYO) is a compatible osmolyte, and it is postulated that the molecule can help in maintaining an ideal osmotic state when a cell is placed in a hypertonic environment. MYO enters the cell along with sodium to offset the change in osmolarity generated by the loss of water molecules. High concentrations of MYO have been demonstrated to be important for osmoregulation in the seminiferous epithelium\textsuperscript{1}. Condorelli et al\textsuperscript{58} showed that MYO significantly increased the number of spermatozoa with high MMP and significantly decreased the number of those with low MMP in patients suffering oligo-astheno-teratozoospermia. No effect of MYO was observed on phosphatidylserine externalization and chromatin compactness (two parameters of apoptosis induction) in normozoospermic men and patients with OAT\textsuperscript{58}. The results of this study showed that MYO ameliorates sperm mitochondrial function, thereby improving semen motility in patients with altered sperm parameters. In addition, MYO increased the total number of spermatozoa after swim-up in normozoospermic men and, more importantly, in patients with abnormal sperm parameters. These findings suggest that MYO could be used in vitro in assisted reproductive techniques, both to increase the number of spermatozoa to be used for intruterine insemination and to ameliorate the sperm quality to be used for in vitro-assisted reproductive techniques\textsuperscript{58,59}.

**Concluding Remarks**

Male partner contributes to about 30% of case of infertility\textsuperscript{3}. Oxidative stress plays a fundamental role in infertility development\textsuperscript{60,61}. Sperm cells are very susceptible to ROS action as their plasmatic membrane is particularly rich in polyunsaturated fatty acids; in addition, during their maturation process, they lost some of the cytoplasmic antioxidant enzymes. It is well known that high concentrations of ROS in the reproductive apparatus and the seminal fluid are associated with important dysfunctions and cellular damages involving spermatozoa.

Recent scientific evidence indicates that the use of antioxidants, both through the systemic route and as a supplement employed during in vitro techniques for the preparation of seminal fluid, appears to be effective in order to induce a significant reduction of the high ROS levels. Then, MYO represents a promising therapeutic strategy against male infertility.

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**Conflict of interest**

The authors declare no conflicts of interest

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