

Inositol activity in oligoasthenoteratospermia – An *in vitro* study

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Abstract. – **Background:** Oligoasthenoteratospermia, a reduction in motility and number of spermatozoa and a change in their morphology, is one of the most relevant causes of infertility in men. One of the factors, which may influence male infertility is linked to the production of reactive oxygen species (ROS) by morphologically altered spermatozoa. Spermatozoa are more susceptible than other cell species to the detrimental activity of these chemical compounds. In particular ROS can affect motility, morphology and DNA stability of spermatozoa.

Aim: In the present *in vitro* study the role of a natural substance, inositol, has been investigated as a possible antioxidant agent both for the systemic treatment of male infertility and for the improvement in the *in vitro* quality of the sperm used for the fertilization applied to medically assisted reproductive procedures.

Materials and Methods: The collected samples, belonging to subjects suffering from oligoasthenoteratospermia and of healthy subjects were submitted to phase contrast microscopy in order to evaluate spermatozoa motility, treated with inositol 2 mg/ml and then submitted to scanning electron microscopy (SEM) and to transmission electron microscopy (TEM). SEM allowed to study both the surface morphology of the biological samples and the changes induced on them by the treatment with inositol. TEM allowed to study ultrastructural details of the biological samples.

Results: In the samples of subjects suffering from oligoasthenoteratospermia the spermatozoa appear entirely covered with an amorphous fibrous material, that gives an excessive viscosity to the seminal fluid, and reduces or avoids cell mobility. The micrographs of these samples show that the mitochondria, in their intermediate tract, appear to be altered with markedly damaged cristae. After treatment with inositol the pathologic samples clearly shows the absence of the amorphous material, perhaps due to a variation in seminal fluid pH. Furthermore, they show the presence of mitochondria morphologi-

cally more similar to control specimen mitochondria, with less damage involving mitochondrial cristae.

Conclusions: These preliminary data appear to suggest that inositol, on account of its antioxidant activity, could preferentially aim at the mitochondrion. Further studies are requested to the purpose of better defining the combination between ROS values of the samples, inositol *in vitro* treatment and oligoasthenoteratospermia.

Key Words:

Oligoasthenoteratospermia, Reactive oxygen species, Scansion electron microscopy, Transmission electron microscopy.

Introduction

A reduction in number and motility of spermatozoa together with a change in their morphology is called “oligoasthenoteratospermia”. In 30-35% of the infertile couples presenting with it the etiology is unknown. Several are the therapies suggested for counteracting such a disease. However, they are often ineffective. In the past few years the scientific community has addressed the research towards the study of the oxidative stress, since it had been observed that one of the factors, which may influence male infertility, is linked to the production of reactive oxygen species (ROS) generated by morphologically altered spermatozoa¹⁻⁸. In fact, if from a point of view a certain amount of spermatozoa-produced free radicals appears to be important both for the process of their maturation and for their reproductive function⁹, from another point it should be taken into consideration the fact that spermatozoa are cells devoid of those defensive mechanisms character-

izing other cell species^{4,10}. Consequently, spermatozoa are much more susceptible to the negative effects produced by the oxidative reactions. Many of the exceeding radicals are produced by leukocytes of seminal plasma or by immature spermatozoa, prematurely released from the seminiferous tubules. Such radicals generate some stress in male gametes. In certain cases not only the morphological features of such gametes (i.e., head and tail structures) result altered, but, above all, they influence in a negative fashion also the expression and the stability of the spermatozoal core, namely the genetic code¹¹.

Therefore, the oxidative stress affecting male gametes is a condition capable of modifying not only cell motility and morphology but also DNA stability and normal mitochondrial structure. Mitochondria are the organelles producing the energy (ATP) required by spermatozoa for the performance of their function. ROS, in fact, may induce changes in mitochondrial membranes and may thus compromise spermatozoal function¹². This leads to a vicious circle since the damaged membranes, in turn, induce the production of additional ROS¹³ with the following end results: reduced fertilizing capacity of man, early miscarriages and, in certain instances, genetic changes involving the embryo.

Several are the situations capable of inducing an oxidative stress in seminal fluid: morbid conditions involving the reproductive apparatus (varicocele, prostatitis)¹⁴⁻¹⁶, particular life styles (smoking, alcohol abuse, drug addiction)¹⁷⁻²⁰, environmental pollution (radiation, smog, industrial gases)²¹⁻²³ and nutritional errors (unbalanced hyperlipidic diet)²⁴.

The majority of body cells is supplied with endogenous antioxidant systems; some of them are of enzymatic type, such as superoxide desmutase, catalase and glutathione/peroxidase-glutathione/reductase, whereas others are not enzymes type, such as vitamin C, vitamin E, coenzyme Q10, etc. However, when such defensive systems are inadequate, ROS are produced in excess and may lead to pathological damages by oxidizing cell membrane lipids and proteins as well as DNA structure, thus altering cell function.

In the present *in vitro* study the role of a natural substance, inositol, has been investigated as a possible antioxidant agent both for the systemic treatment of male infertility and for the improvement in the *in vitro* quality of the sperm used for the fertilization applied to medically assisted reproductive procedures^{25,26}.

Inositol is a cyclic sugar and a vitaminic factor belonging to the vitamin B₂ complex; it is involved in calcium-dependent intracellular signalling pathways and in cellular signal transduction through the plasmatic membrane²⁷. Inositol regulates several cellular processes, including cell differentiation and proliferation through calcium release²⁸. It can induce stimulant effects both at a motor level and at a central nerve system (CNS) level. It is an organic compound participating in the composition of membrane phospholipids. Inositol is used in the treatment of muscular dystrophy²⁹ and of liver diseases³⁰. In addition, it controls the secretion of certain endocrine glands, including gonads³¹. Its natural sources are untreated whole-meal cereals, citrus fruits, brewer's yeast. Recent studies³² have suggested inositol involvement both in maturation of spermatozoa and in their migration from the epididymis.

Materials and Methods

After subscribing an informed consent, samples of seminal fluid were obtained from healthy subjects and from subjects with oligoasthenoteratospermia. The collected samples were submitted to phase contrast microscopy in order to evaluate spermatozoa motility. The specimens were stained with tripan blue for assessing their vitality and then were treated with inositol 2 mg/ml either for 30 minutes, 1 hour or 2 hours at 37°C under a controlled CO₂ atmosphere. Following the above mentioned procedures, the samples were submitted to scanning electron microscopy (SEM) and to transmission electron microscopy (TEM).

The study was approved by the local Ethical Committee before starting the samples collection.

Scansion Electron Microscopy

SEM allows to study both the surface morphology of properly prepared biological samples and the prospective changes induced on them by the treatment with several types of agents, the development of any pathological situations, and the changes in environmental conditions. The samples were prepared according to the following procedure: the spermatozoa were stuck on glass-slides, 13-mm in diameter, then covered with polylysine and fixed by a 2.5% glutaraldehyde solution in 0.2 M cacodylate buffer (at pH 7.3)

and kept for 60 minutes at room temperature. Following a few washings in the same buffer solution, a post-fixation was carried out by using 1% osmium tetroxide (OsO_4) in 0.2 M cacodylate buffer at pH 7.2 for 60 minutes at room temperature. The samples were then dehydrated by means of increasing scalar solutions of ethanol, exsiccated in the presence of CPD and covered with a 30-nm layer of gold by means of a sputtering technique. Finally the samples were examined through a scanning electron microscope Cambridge Stereoscan 360.

Transmission Electron Microscopy

TEM allows to study ultrastructural details of biological samples. The preparation of samples for TEM examination must aim at achieving an extremely thin specimen, which should be intersected by the electrons originated by the source. The following are the main phases of the procedure: fixation and post-fixation, dehydration, infiltration and polymerization of the resin, specimen sectioning and contrasting. In short, the samples were fixed with 2.5% (v/v) glutaraldehyde solution in 0.1 M (pH 7.4) cacodylate buffer at 4°C temperature overnight. Then the samples were embedded into 2% Agar Noble in water, dehydrated by increasing scalar solutions of ethanol and embedded into epoxidic resin

(Epon 812, Agar Scientific Ltd, Stansted, U.K.). Ultrathin specimen sections were obtained by an ultramicrotome Nova (LKB, Bromma, Sweden). Prior to performing the examinations, the sections were contrasted through incubation in uranyl acetate and lead citrate. The specimens were finally checked with a transmission electron microscope Philips 208 (FEI Company, Eindhoven, The Netherland).

Results

A number of observations by SEM were performed on samples of seminal fluid belonging to normal sperm donors and to donors suffering from oligoasthenoteratospermia (Figure 1). The qualitative analysis of spermatozoal cell surface shows the presence, in the control subjects, of spermatozoa with normal morphology (Figure 1a), whereas in the samples of subjects suffering from oligoasthenoteratospermia the spermatozoa appear entirely covered with an amorphous fibrous material. Such a covering layer could be responsible for the excessive viscosity of seminal fluid and consequently for the reduced or absent cell mobility observed at the phase contrast microscope. Figures 1c and 1d show the micrographies pertinent to the same specimens following

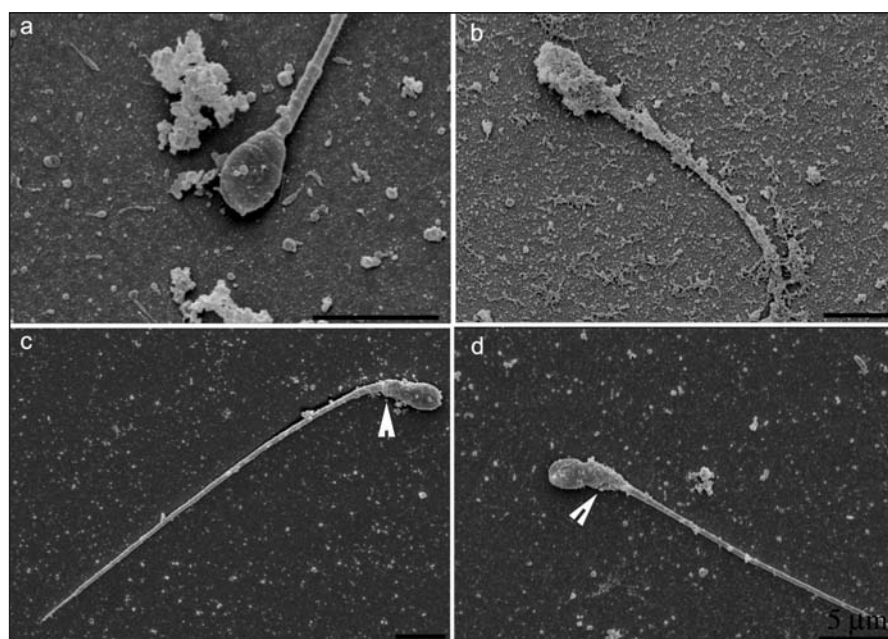


Figure 1. Scanning electron microscopy. **A**, Control specimen. **B**, Oligoasthenoteratospermia specimen covered with amorphous material. **C**, Control spermatic cell following 2-hour treatment with inositol. **D**, Pathological spermatic cell following inositol treatment. Arrow heads point out intermediate tract thickening.

a 2-hour treatment with inositol (2 mg/ml) at 37°C. The spermatic cell of the control (Figure 1c) shows a normal, unaltered surface morphology associated with a slight thickening of the intermediate tract (arrow head) which, as it is well known, contains the mitochondrial sheath. The pathological specimen treated with inositol (Figure 1d) clearly shows the absence of the amorphous material present in the untreated specimen (Figure 1b). In addition, a marked increase in the thickness of spermatozoal intermediate tract is visible even in such a specimen (arrow head). The data found would apparently suggest that inositol treatment might be able to dissolve the initially observed amorphous fibrous material by inducing, perhaps, a variation in seminal fluid pH. Such a hypothesis has been substantiated by the findings obtained on the same specimens through TEM. The observations made on ultrathin sections of the control specimen show a spermatozoon with many intact mitochondria surrounding the axoneme of the spermatic flagellum (Figure 2a). On the contrary, micrographs of seminal fluid specimen belonging to the subject suffering from oligoasthenoteratospermia (Figure 2b) show an impaired spermatozoon with an altered

acrosome (Aa) associated with the presence of marginalized chromatin (Cm). In addition, mitochondria, in their intermediate tract, appear to be altered with markedly damaged cristae (Figure 2b, arrow heads). Following a 2-hour inositol treatment (2 mg/ml) at 37°C, the pathological specimen shows the presence of mitochondria morphologically more similar to control specimen mitochondria (Figures 2c and 2d), with less damage involving mitochondrial cristae.

Discussion

An increase in the number of infertile couples has been observed in the past few decades, with particular reference to an increment of cases ascribable to male factors. Recent data indicate that oxidative stress plays a fundamental role in infertility development³³⁻³⁵. Spermatozoa are especially sensitive to the action of radicals both because of the composition of their plasmatic membrane, which is rich in polyunsaturated fatty acids, and because of the loss of antioxidant enzymes when they lose the cytoplasm during their maturation³⁶.

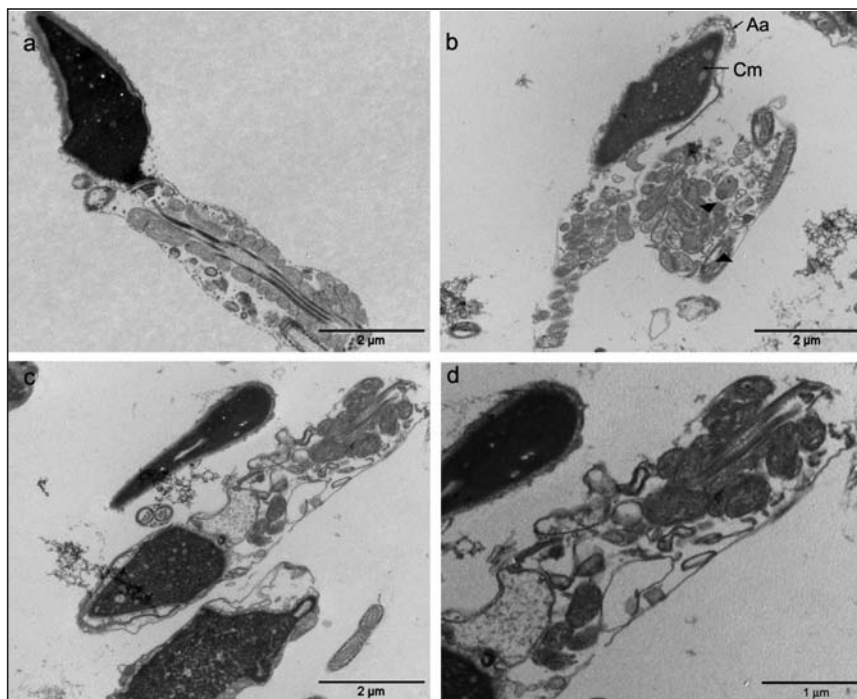


Figure 2. Transmission electron microscopy. **A-B**, Ultrathin section of a control spermatozoon (**A**) and a pathologic spermatozoon (**B**). Marginalized chromatin (Cm) and markedly altered acrosome (Aa) are visible in (**B**). Arrow heads point out altered mitochondrial cristae. **C-D**, Ultrathin sections of pathologic spermatozoa treated with inositol for a 2-hour period. **D**, Magnification of the intermediate tract: some mitochondria are visible with partially restored original morphology.

This is one of the reasons why the medical and scientific interest has been prompted to search for new substances able to improve the expectations in the field of male infertility.

High concentrations of ROS in the reproductive apparatus and in the seminal fluid are associated with important dysfunctions and cellular damages involving spermatozoa^{9,37}. Recent scientific evidence^{24,38-40} 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 in excessively high levels of ROS. This represents a promising therapeutic strategy against male infertility.

The present ultrastructural morphologic study has evidenced that inositol, with its antioxidant activity, may be preferentially aiming at the mitochondrion. Both the altered intramitochondrial metabolism with elevated free iron concentrations and the defective mitochondrial respiratory chain, associated with an increased production of free radicals and a subsequent oxidative damage, may very likely constitute a mechanism capable of compromising the vitality of cells, including spermatozoa. Especially the latter cells, which are devoid of cytoplasm, suffer more heavily the damages due to the high concentrations of ROS produced within themselves.

These preliminary data appear to suggest that inositol, on account of its antioxidant activity, could preferentially aim at the mitochondrion. Several studies⁴¹⁻⁴³ have carried out in man and they have shown a correlation between an altered mitochondrial function (oxidative damage) and a reduction in motility and in fertilizing ability.

Our future research will aim at clarify the inositol's mechanism of action, by correlating the data resulting from the structural analysis (qualitative findings) with the quantitative sensibility tests (flow cytometry), for the purpose of better defining the combination between ROS values of the samples, inositol *in vitro* treatment and oligoasthenoteratospermia.

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