

Effects of myoinositol on sperm mitochondrial function *in-vitro*

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Abstract. – Background and Objectives: Inositol is a component of the vitamin B complex. Myo-inositol (MYO) is the most biologically important form in nature. It is involved in several systemic processes and in mechanisms of signal transduction in the plasma membrane as precursor of second messengers. On the male reproductive function, MYO appears to regulate seminal plasma osmolarity and volume; the expression of proteins essential for embryogenetic development and sperm chemiotaxis; and sperm motility, capacitation, and acrosome reaction. Recently, a seminal antioxidant action has also been suggested.

Aim of the Study: To evaluate the effects of MYO on sperm mitochondrial function and apoptosis.

Materials and Methods: Spermatozoa isolated from 5 normozoospermic men and from 7 patients with oligo-astheno-teratozoospermia (OAT) were incubated *in-vitro* with 2 mg/ml of MYO or placebo (control) for 2 hours. After this incubation period, the following sperm parameters were evaluated by flow cytometry: mitochondrial membrane potential (MMP) by JC-1 staining; phosphatidylserine (PS) externalization by annexin V and propidium iodide double staining; and chromatin compactness following propidium iodide staining.

Results: MYO did not affect the mitochondrial function of spermatozoa isolated from normozoospermic men, whereas it increased significantly the number of spermatozoa with high MMP and decreased significantly the number of those with low MMP in OAT patients. No effect of MYO was observed on PS externalization and chromatin compactness in both normozoospermic men and OAT patients.

Conclusion: The data suggest that MYO is able to ameliorate mitochondrial function in OAT patients. We conclude that this compound may be useful for the treatment of male infertility.

Key Words:

Myoinositol, Male infertility, Sperm mitochondrial membrane potential.

Introduction

Inositol (Ins) is an isomer of a C6 sugar alcohol with nine different stereoisomers. The most important form in nature is myoinositol (MYO) that belongs to the vitamin B complex group¹ (compound B7) and it is produced by the human body. MYO is synthesized from glucose-6-phosphate (first product of glycolysis)² and it is eliminated by the kidney. In the human cells, it is transformed into phosphatidylinositol with multiple functions not yet fully clarified. MYO is a precursor of second messengers in the cellular signal transduction system and it is involved in the regulation of calcium intracellular concentration³, mediator in insulin cascade⁴ and, therefore, it is used for the treatment of women with polycystic ovary syndrome⁵, cardiac regulation⁶, reproduction, etc.

A key human cell enzyme is the phospholipase C (PLC) that hydrolyzed phosphatidylinositol (PIP₂) into two second messengers: diacylglycerol (DAG) and Ins 1,4,5-trisphosphate (InsP₃). DAG activates protein kinase C (PKC) which phosphorylates several intracellular proteins. Instead, InsP₃ binds to a receptor in the endoplasmic reticulum and activates the calcium channels with consequent release of calcium by storage sites⁷. Then, DAG is phosphorylated to phosphatidic acid, while InsP₃ is dephosphorylated to Ins. Ins and phosphatidic acid form phosphatidylinositol (PI) that is phosphorylated again to form PIP₂.

Ins has a variety of clinical applications. In particular, it is involved in several aspect of reproduction: embryogenesis, prevention of neural tube defects, production of surfactant⁸. Recently, an important antioxidant effect of d-chiro-inositol (a metabolite from Arnica) has been shown, mainly against superoxid anion, hydrogen peroxide and hydroxyl radical⁹. The concentration of

MYO in the seminiferous tubules fluid is higher than the levels found in the seminal plasma, but its true role is unknown. MYO is synthesized by two enzymes: MYO-1-phosphate syntase (ISYNA1) and MYO monophosphatase-1 (IMPA1), present in high concentrations within the testis. Testis is the only human organ able to synthesize MYO from glucose-6-phosphate, while serum MYO cannot cross the tight junctions. MYO is then transported into cells by a sodium/myoinositol cotransport protein (SLC5A3) whose expression is sensible to osmolar changes. ISYNA1 and IMPA1 are expressed in germinal and Sertoli cells, while only the latter express SLC5A3. It is well known that MYO is an osmolyte and that Sertoli cells, in hypertonic microenvironment, increase the expression of SLC5A3¹⁰.

These functions suggest a possible role of MYO in the regulation of the vesicular seminal fluid and within the epididymis where lower concentrations of MYO have been associated to reduced fertility¹¹. Protein expression alteration in asthenozoospermic patients has shown an involvement of IMPA1, an enzyme essential for embryonic development. However, high IMPA1 concentrations in asthenozoospermic patients have been related to the sperm motility decrease¹². MYO has also been suggested to play a role in the chemotaxis and human sperm thermotaxis through the activation of PLC, resulting in the production of InsP₃ and calcium channels opening. This mechanism induces Ca⁺⁺ release from internal stores and consequently, increased intracellular concentrations in the flagellum¹³. Ins is an important second messenger which regulates the intracellular Ca⁺⁺ concentrations by acting in the sperm plasma membrane, the mitochondria, the acrosome, and the neck region¹⁵, another intracellular Ca⁺⁺ store¹⁴. In the sperm head, calcium is a key messenger. It modulates the activity of several enzymes including PLC, protein kinase C, phospholipase A2, etc. Each of these molecules is involved in the exocytosis of the acrosomal contents, after binding of spermatozoa to the zona pellucida. An initial effect of Ca⁺⁺ is the activation of PLC that hydrolyzes phosphatidylinositol to DAG and InsP₃. InsP₃ modulates Ca⁺⁺ intracellular variations through the activation of the inositol 1,4,5-trisphosphate receptors (InsP₃-R), located in the intracellular membrane of Ca⁺⁺ stores. InsP₃ binding to receptors induces conformational changes of the channels determining Ca⁺⁺ release. In this process, an important role

could have calreticulin (CRT), present in the rat spermatozoon acrosome. In human spermatozoa, CRT co-localizes with InsP₃-R and this may explain the intracellular Ca⁺⁺ alterations during the acrosome reaction¹⁶. The activation of these intracellular mechanisms induce an increase of cytosolic calcium and consequently the increase of mitochondrial Ca⁺⁺ that stimulates the oxidative metabolism and the ATP production.

A recent study has shown that treating oligoasthenoteratospermic samples with MYO improves sample quality by removing amorphous material being probably the cause of the high viscosity of the seminal fluid¹⁷.

The aim of this study was to evaluate whether MYO has any effect of sperm mitochondrial function, apoptosis, and chromatin compactness *in-vitro*. To address this aim spermatozoa isolated from 5 normozoospermic men and from 7 patients with oligoasthenoteratozoospermia (OAT) were incubated *in vitro* with 2 mg/ml of MYO or placebo (control) for 2 hours. After this incubation period, the following sperm parameters were evaluated by flow cytometry: mitochondrial membrane potential (MMP), phosphatidylserine (PS) externalization, an early marker of apoptosis, and chromatin compactness.

Patients and Methods

Patient Selection

Five healthy fertile men (aged 35-40 years) and 12 patients with OAT (aged 35-40 years) were enrolled in this study. A complete medical history was collected and all of them underwent a careful physical examination and laboratory and ultrasound instrumental evaluation. Men with systemic and endocrine diseases, male accessory gland infection, history positive for cryptorchidism or varicocele, microrchidism, alcohol intake, cigarette smoke and/or drug abuse and recent hormonal treatment were excluded. The protocol was approved by the internal Institutional Ethical Committee and an informed written consent was obtained from each patient.

Sperm Analysis

Semen samples were collected after 3-5 days of sexual abstinence. After liquefaction, they were analyzed according to the World Health Organization criteria¹⁸. The remaining spermatozoa were used for the flow cytometry analysis.

Sperm Flow Cytometry Evaluation

Flow cytometry was performed using the flow cytometer EPICS XL (Coulter Electronics, IL, Milan, Italy), as previously reported¹⁸. Sperm mitochondrial membrane potential (MMP) was evaluated after staining with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine chloride (JC-1), PS externalization following double staining with annexin V and propidium iodide (PrI), and chromatin compactness following PrI staining after incubation with MYO (2 mg/ml) or vehicle (control).

JC-1 Staining

MMP was evaluated as previously reported¹⁸. Briefly, the sperm suspension was adjusted to a density of $0.5-1 \times 10^6$ cells/ml and incubated for 10-15 min at 37°C in the dark with JC-1 (Space Import-Export, Milan, Italy).

Annexin V/PrI Assay

Staining with annexin V/PrI was performed using a commercially available kit (Annexin V-FITC Apoptosis detection kit, Beckman Coulter, IL, Milan, Italy), as previously reported¹⁹. Briefly, an aliquot containing 0.5×10^6 spermatozoa/ml was re-suspended 0.5 ml of binding buffer, labelled with 1 µl of annexin V-FITC plus 5 µl of PrI, incubated for 10 min in the dark, and immediately analyzed. Signals were detected through FL-1 (FITC) and FL-3 (PrI) detectors. The different labelling patterns in the bivariate PrI/annexin V analysis identified the different cell populations, where annexin negative and PrI negative were designated as viable cells and annexin positive and PrI negative as PS externalized spermatozoa (early apoptotic cells).

PrI Staining

Sperm PrI staining was performed as previously reported¹⁹. Briefly, semen samples were centrifuged at 500 g for 10 min at room temperature,

the supernatant removed and spermatozoa collected. An aliquot of about 1×10^6 spermatozoa was incubated in LPR DNA-Prep Reagent containing 0.1% potassium cyanide, 0.1% NaN₃, non ionic detergents, salts and stabilizing (Beckman Coulter, IL) in the dark, at room temperature for 10 minutes and then were incubated in Stein DNA-Prep Reagent containing 50 µg/ml of PrI (<0,5%), RNAsi type A (4 Kunits/ml), <0.1% NaN₃, salts and stabilizing (Beckman Coulter, IL) in the dark, at room temperature for 30 minutes.

Statistical Analysis

Results are reported as mean±SEM. The data were analyzed by Student's *t* test and 1-way analysis of variance (ANOVA) followed by the Duncan Multiple Range test, as appropriate. The software SPSS 9.0 for Windows was used for statistical evaluation (SPSS Inc., Chicago IL, USA). A statistically significant difference was accepted when the *p* value was lower than 0.05.

Results

The main sperm parameters of the 5 normozoospermic men and the patients with OAT are shown in Table I. All the main sperm parameters resulted significantly worse in OAT patients compared to normozoospermic men ($p < 0.05$). Incubation with MYO increased significantly the percentage of spermatozoa with high MMP in patients with OAT ($p < 0.05$, ANOVA followed by Duncan's test), but not in normozoospermic men. Interestingly, after incubation with MYO, the percentage of spermatozoa with high MMP in OAT patients was similar to that found in normozoospermic men (Figure 1, upper panel).

Similarly, the percentage of spermatozoa with low MMP resulted significantly lower in OAT

Table I. Main sperm parameters of the normozoospermic men and the patients with oligo-astheno-teratozoospermia (OAT) enrolled for this study.

Parameters	Normozoospermic men (n = 5)	Patients with OAT (n = 7)
Volume (ml)	3.7 ± 0.48	3.8 ± 0.55
Concentration (× 106/ml)	96.6 ± 31.1	40.7 ± 28.2*
Sperm count (× 106/ejaculate)	322.8 ± 71.4	160.9 ± 113.5*
Progressive motility (%)	50.4 ± 3.3	28.7 ± 4.1*
Normal morphology (%)	18.0 ± 1.8	13.8 ± 1.7*
Seminal leukocytes (× 106/ml)	0.64 ± 0.18	0.59 ± 0.12

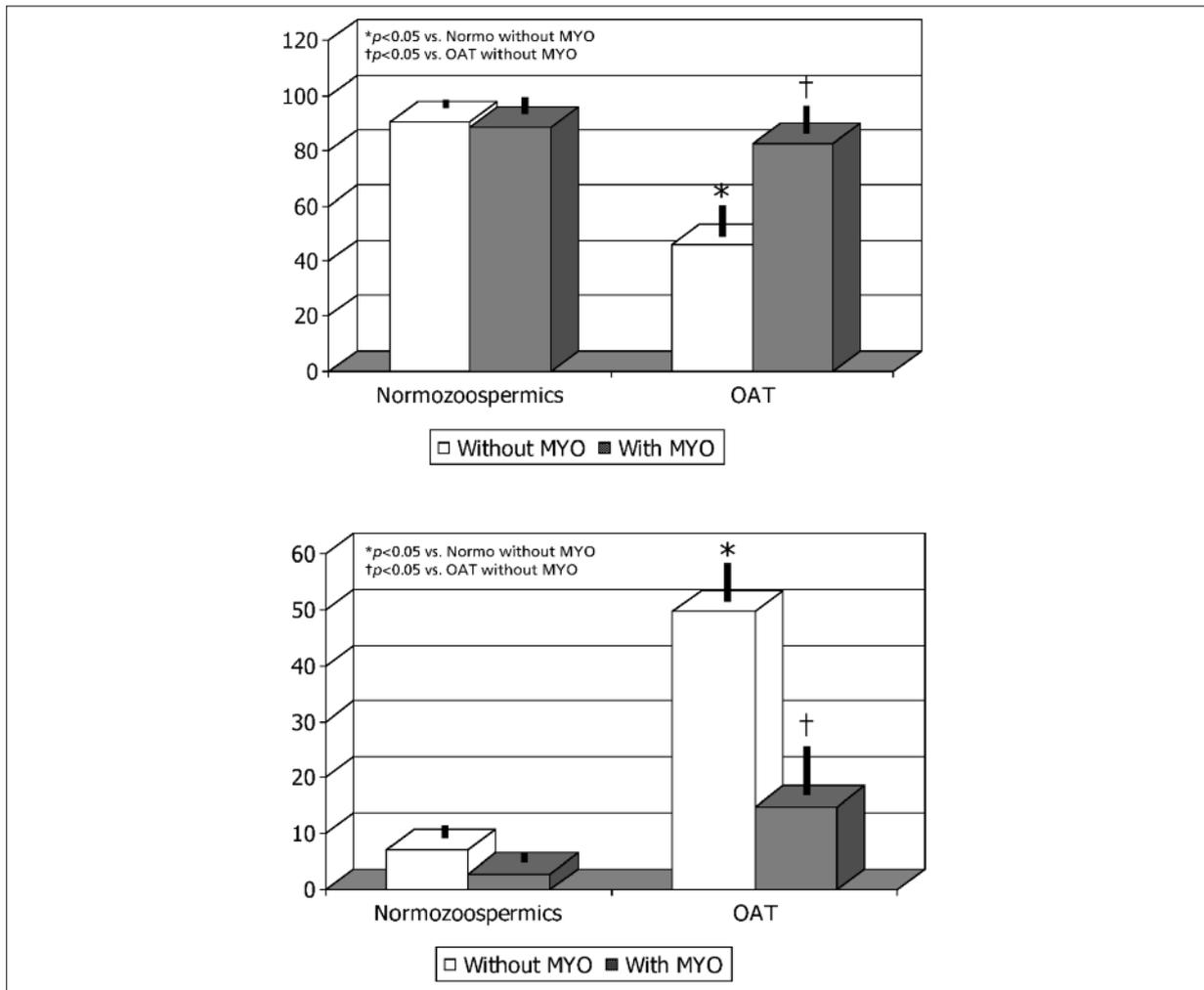


Figure 1. Percentage of spermatozoa with high (upper panel) or low (lower panel) mitochondrial membrane potential (MMP) in normozoospermic men (n=5) and in patients with oligo-astheno-teratozoospermia (OAT) (n=7) following incubation with or without myoinositol (MYO) for 2 hours.

patients following incubation with MYO ($p<0.05$, ANOVA, followed by Duncan's test), whereas no significant difference was detected in normozoospermic men (Figure 1, lower panel).

MYO did not have significant effect on the percentage of spermatozoa with PS externalization or impaired chromatin compactness in both normozoospermic and OAT men (Table II).

Table II. Spermatozoa with phosphatidylserine (PS) externalization or with abnormal chromatin compactness in normozoospermic men and in patients with oligo-astheno-teratozoospermia (OAT) incubated with or without myoinositol (MYO) for 2 hours.

	Normozoospermic men (n = 5)		Patients with OAT (n = 7)	
	Without MYO	With MYO	Without MYO	With MYO
Spermatozoa with PS externalization (%)	2.7 ± 1.1%	7.9 ± 4.1%	4.6 ± 0.8%	8 ± 2.5%
Spermatozoa with abnormal chromatin compactness	19.5 ± 3.6%	17.5 ± 3.1%	27.3 ± 2.9%	23.7 ± 2.9%

Discussion

The results of this study showed that the incubation with MYO increased the percentage of spermatozoa with high MMP and decreased that with low MMP in patients with OAT. This effect was observed after a short incubation time and it seems to be specific because, at the same time, others bio-functional sperm parameters did not show any significant variation. In fact, the percentage of spermatozoa with early molecular signs of apoptosis or with altered chromatin compactness remained unchanged after incubation with MYO. These results suggest that the effects of this molecule are specific on mitochondrial function, at least under the experimental conditions used in this study. A second important aspect is that the improvement observed after incubation with MYO on the sperm mitochondrial function was observed only in patients with OAT, but not in normal men.

MMP is a marker of cellular apoptosis. In fact, an apoptotic stimulus reduced the MMP (early and reversible event) and, consequently, alters the mitochondrial membrane permeability. This leads to the release of c-cytochrome into the cytosol. Viable cells have a high MMP, while low MMP is found in apoptotic cells. MMP determination is a very sensible test to value sperm quality^{20,21}. In fact, it has been shown that the percentage of spermatozoa with high MMP is significantly higher in men with normal sperm parameters than in patients with abnormal parameters. In addition, it has been reported that sperm count with high MMP correlates in a positive manner with the sperm number and motility (including progressive)^{20,21}. Instead, low MMP is associated with reduced sperm motility, because the flagellar movement is ATP-dependent. As sperm motility is one of the most important parameters for natural fertilization, the percentage of spermatozoa with high MMP is an important index of fertility. This highlights the importance of this non conventional sperm parameter for the achievement of fertility²⁰. Furthermore, spermatozoa with high MMP and low DNA fragmentation have a high fertilization rate if used in assisted fertilization programs and the percentage of spermatozoa with high MMP are directly related to the fertilization rate after *in vitro* fertilization-embryo transfer (IVF-ET)²⁰.

In conclusion, this study showed that MYO is able to improve the sperm mitochondrial function in patients with altered sperm parameters. If

these results are confirmed, they suggest a possible use of MYO for the treatment of male infertility both *in-vivo* and *in-vitro*.

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