Myoinositol: Does It Improve Sperm Mitochondrial Function and Sperm Motility?

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OBJECTIVE To evaluate whether an improvement in mitochondrial membrane potential was associated with sperm motility amelioration and greater sperm recovery after the swim-up procedure. A second purpose was to evaluate the effects of myoinositol (MYO) on sperm apoptosis, quality of chromatin compaction, and DNA integrity.

METHODS Spermatozoa from 20 normozoospermic men and 20 patients with oligo-astheno-teratozoospermia were incubated in vitro with 2 mg/mL of MYO or phosphate-buffered saline as a control for 2 hours. After this incubation period, sperm motility was evaluated. Flow cytometry was used to analyze the mitochondrial membrane potential, phosphatidylserine externalization, chromatin compactness, and DNA fragmentation. We also evaluated the total number of motile spermatozoa recovered after swim-up after incubation with MYO or phosphate-buffered saline.

RESULTS MYO significantly increased the percentage of spermatozoa with progressive motility in both normozoospermic men and patients with oligo-astheno-teratozoospermia. Motility improvement in the latter group was associated with a significant increase in the percentage of spermatozoa with high mitochondrial membrane potential. MYO had no effects on mitochondrial function in spermatozoa from normozoospermic men. Sperm phosphatidylserine externalization, chromatin compactness, and DNA fragmentation were unaffected by MYO in both groups. After incubation with MYO, the total number of spermatozoa recovered after swim-up had improved significantly in both groups.

CONCLUSION These data show that MYO increases sperm motility and the number of spermatozoa retrieved after swim-up in both normozoospermic men and patients with abnormal sperm parameters. In patients with oligo-astheno-teratozoospermia, the improvement in these parameters was associated with improved sperm mitochondrial function. These findings support the use of MYO in both in vivo- and in vitro-assisted reproductive techniques. UROLOGY 79: 1290–1295, 2012.

Inositol is a carbocyclic polyol with 9 different stereoisomers synthesized from glucose-6-phosphate, the first product of glycolysis,[1] 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, respectively.[2] The most abundant form of inositol in nature is myoinositol (MYO), which belongs to the vitamin B complex group (compound B_7).[3]

MYO is synthesized by 2 enzymes: myo-1-phosphate synthase and MYO monophosphatase-1, which are present in high concentrations in the testes.[4] The testis is a human organ able to synthesize MYO from glucose-6-phosphate. Serum MYO cannot cross the tight junctions at the testicular level and most of the inositol is synthetized in the kidneys[4] in humans. Accordingly, the concentration of MYO in the seminiferous tubule fluid is greater than in the seminal plasma. MYO is subsequently transported into cells by a sodium/myoinositol cotransport protein, the expression of which is sensitive to osmolar changes. Both myo-1-phosphate synthase and MYO monophosphatase-1 are expressed in germinal and Sertoli cells, and only the latter express sodium/myoinositol cotransport protein. It has been established that MYO is an osmolyte and that, in a hypertonic microenvironment, Sertoli cells increase the expression of sodium/myoinositol cotransport protein.[4]

Previous evidence has suggested a possible role of MYO in spermatogenesis and sperm function. In transgenic mice, a lower concentration of MYO within the epididymis has been associated with reduced fertility,[5] and MYO monophosphatase-1 is present in greater

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amounts in spermatozoa from asthenozoospermic samples.[6] Inositol has also been suggested to play a role in chemotaxis and human sperm thermotaxis through activation of phospholipase C, resulting in the production of inositol triphosphate. Inositol triphosphate binding opens calcium channels, thereby increasing the intracellular concentrations in the flagellum.[7] Inositol regulates intracellular Ca^{2+} concentrations by acting on the sperm plasma membrane, mitochondria, acrosome, and neck region, another intracellular Ca^{2+} store.[8,9] Recently, Colone et al[10] have shown that the spermatozoa of patients with oligo-astheno-teratozoospermia (OAT) are covered by amorphous fibrous material that increases seminal fluid viscosity and reduce sperm motility. In addition, the mitochondria in the intermediate tract of spermatozoa of patients with OAT had damaged cristae. However, after incubation with inositol, the amorphous fibrous material disappeared and cristae damage decreased. Thereafter, spermatozoa from patients with OAT appeared similar to those obtained from normozoospermic men.[10] No studies have examined the effects of MYO on other sperm biofunctional parameters, such as DNA integrity. Moreover, the potential involvement of MYO in oxidative reactions (such as those evaluated in nonanimal models)[11] and the implications of oxidative stress on the quality of these parameters suggest that aspects other than mitochondrial function should be studied.

In a recent publication,[12] we showed that MYO significantly increased the number of spermatozoa with high mitochondrial membrane potential (MMP) and significantly decreased the number of those with low MMP in patients with OAT, and no effect of MYO was observed on phosphatidylserine (PS) externalization and chromatin compactness in normozoospermic men and patients with OAT. The purpose of the present study was to confirm such effects in a greater number of patients and to evaluate the effects of MYO on other biofunctional sperm parameters. Hence, the effects of MYO on sperm motility and the number of spermatozoa recovered after a swim-up procedure from both normozoospermic men and patients with OAT were explored. The effects of MYO on sperm apoptosis, chromatin compactness, and DNA fragmentation were also examined. To accomplish this, spermatozoa from 20 normozoospermic men and 20 patients with OAT were incubated in vitro with 2 mg/mL of MYO or phosphate-buffered saline (PBS) for 2 hours. After this incubation period, sperm motility and the total number of spermatozoa obtained after swim-up were evaluated. MMP, PS externalization (an early marker of apoptosis), chromatin compactness, and DNA fragmentation were investigated using flow cytometry.

MATERIAL AND METHODS

Patient Selection

A total of 40 men, 20 healthy normozoospermic men and 20 patients with OAT, were enrolled in the present study. A complete medical history was collected, and all the subjects underwent a careful physical examination and laboratory evaluation. The exclusion criteria included systemic or endocrine diseases, male accessory gland infection, a clinical history of cryptorchidism or varicocele, micro-orchidism, alcohol intake and/or drug abuse, and recent hormonal treatment. Smokers and/or overweight/obese patients and controls were excluded, because we have previously reported that these conditions have a negative effect on the sperm parameters.[13,14]

The internal institutional review board approved the protocol (number 11/2009), and all subjects provided written informed consent.

MYO Exposure and Sperm Analyses

Semen samples were collected by masturbation after 3-5 days of sexual abstinence. After 30 minutes of liquefaction at 37°C, they were analyzed according to the World Health Organization criteria.[15] The sperm motility was evaluated first by a blinded observer and repeated for quality control by another blinded observer (the mean value of 2 observations was analyzed). The same day, an aliquot of spermatozoa was incubated with MYO (2 mg/mL), and a second aliquot was incubated with PBS as a control, both for 2 hours. Motility and flow cytometric analysis were performed before and at the end of incubation. The total number of spermatozoa obtained after swim-up was determined, as previously reported.[16] In brief, the pellet was overlaid with 0.5 mL of Biggers, Whitten, and Whittingham medium and incubated for 30 minutes, and the supernatant was collected.

Sperm Flow Cytometry Evaluation

Flow cytometry was performed using an EPICS XL (Becker Coulter, Milan, Italy), as previously reported.[17] Sperm MMP was evaluated by staining with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine chloride (JC-1), and PS externalization was assessed after double staining with annexin V and propidium iodide (PI). Chromatin compactness was assessed by PI staining, and DNA fragmentation was determined by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining.

JC-1 Staining

Recently, the lipophilic cationic fluorescent carbocyanine dye, JC-1, has been used to differentially label mitochondria with high and low membrane potential. When JC-1 forms monomers in mitochondria with low potential (altered or pathologic MMP), the JC-1 stain emits a green fluorescence (510-520 nm). JC-1 forms multimers known as J-aggregates after accumulation in mitochondria with high (normal MMP) membrane potential, and the JC-1 stain emits a bright red-orange fluorescence at 590 nm.[18]

MMP was evaluated as previously reported.[17] In brief, the sperm suspension was adjusted to a density of 0.5-1 $\times$ 10^6 cells/mL and incubated for 10-15 minutes at 37°C in the dark with JC-1 (Space Import-Export, Milan, Italy).

Annexin V/PI Assay

Staining with annexin V/PI was performed using a commercially available kit (annexin V-fluorescein isothiocyanate apoptosis detection kit, Beckman Coulter), as previously reported.[17] In brief, an aliquot containing 0.5 $\times$ 10^6 sper-

Table 1. Conventional and flow cytometric sperm parameters before and after incubation with myoinositol (2 mg/mL) for 2 hours at 37°C

Variable	Normozoospermic Men (n = 20)		Patients With OAT (n = 20)	
	Before Incubation With MYO	After Incubation With MYO	Before Incubation With MYO	After Incubation With MYO
Concentration ($\times 10^6$/mL)				
Mean ± SD	93.2 ± 20	91.4 ± 3.2	17.6 ± 4.0*	17.9 ± 5.0*
Range	60-140	55-143	14-19	15-19
Total motility (%)				
Mean ± SD	65.2 ± 2.0	66.5 ± 4.0	28.0 ± 4.0*	42.0 ± 4.0*†
Range	50-80	52-78	20-36	26-46
Progressive motility (%)				
Mean ± SD	44.9 ± 3.0	44.0 ± 3.0	25.0 ± 0.64*	33.0 ± 2.0*†
Range	38-55	37-53	20-30	22-36
Normal morphology (%)				
Mean ± SD	18.2 ± 1.5	18.1 ± 3.0	10.8 ± 1.8*	10.9 ± 2.0*
Range	16-20	16-20	5-13	5-13
Seminal leukocytes ($\times 10^6$/mL)				
Mean ± SD	0.5 ± 0.2	0.4 ± 0.2	0.2 ± 0.2	0.4 ± 0.2
Range	0.1-0.9	0.1-0.9	0.1-0.7	0.1-0.9
Viable spermatozoa (%)				
Mean ± SD	61.0 ± 3.0	63.0 ± 5.14	59.0 ± 4.0	59.1 ± 4.0
Range	50-70	51-73	50-64	50-66
Spermatozoa with PS externalization (%)				
Mean ± SD	3.5 ± 2.0	4.1 ± 1.8	3.9 ± 0.8	4.3 ± 1.47
Range	2-5	2-6	3-9	3-9
Spermatozoa in late apoptosis (%)				
Mean ± SD	6.7 ± 1.2	6.85 ± 1.75	7.0 ± 2.0	7.1 ± 3.0
Range	5-9	5-9	6-12	5-12
Necrotic spermatozoa (%)				
Mean ± SD	23.8 ± 2.0	23.05 ± 2.05	25.0 ± 4.0	24.79 ± 4.23
Range	20-30	19-27	21-29	20-30
Spermatozoa with abnormal chromatin compactness (%)				
Mean ± SD	17.0 ± 3.0	16.75 ± 2.62	18.0 ± 6.0	18.2 ± 3.0
Range	15-20	13-19	16-29	16-28
Spermatozoa with DNA fragmentation (%)				
Mean ± SD	3.0 ± 1.2	2.41 ± 1.22	3.2 ± 1.3	2.70 ± 0.81
Range	1-4	1-4	2-6	2-5
Spermatozoa with high (normal) MMP (%)				
Mean ± SD	90.0 ± 3.0	89.0 ± 4.0	58.0 ± 4.0*	80.0 ± 2.0†
Range	86-94	85-93	53-60	72-86
Spermatozoa with low MMP (%)				
Mean ± SD	5.0 ± 1.0	3.0 ± 2.0	38.0 ± 4.0*	15.0 ± 3.0*†
Range	1-7	1-6	30-45	10-20

OAT, oligo-astheno-teratozoospermia; MYO, myoinositol; MMP, mitochondrial membrane potential.

Normal values according to World Health Organization[15] criteria: concentration ≥20 × 10^6/mL; total motility ≥50%; progressive motility ≥25%; normal forms (Kruger criteria[26]) ≥14%; leukocytes <1 × 10^6/mL.

Data for total and progressive motility determined from mean of 2 observations for each sample.

* $P < .05$ vs normozoospermic men.

† $P < .05$ vs before incubation.

matozoa/mL was resuspended in 0.5 mL of binding buffer, labeled with 1 μL of annexin V-fluorescein isothiocyanate plus 5 μL of PI, incubated for 10-15 minutes in the dark, and immediately analyzed. Signals were detected through FL-1 (fluorescein isothiocyanate) and FL-3 (phycoerythrin) detectors. The different labeling patterns in the bivariate annexin V/PI analysis identified the different cell populations in which annexin-negative and PI-negative staining indicated viable cells, annexin-positive and PI-negative staining indicated PS externalized spermatozoa (early apoptotic cells), annexin-positive and PI-positive staining indicated late apoptotic cells, and annexin-negative and PI-positive cells indicated necrotic cells.

PI Staining

Sperm PI staining was performed as previously reported.[17] In brief, after incubation with MYO or PBS, the 2 semen samples of each normozoospermic man or patient with OAT were centrifuged at 500*g* for 10 minutes at room temperature, the supernatant was removed, and the spermatozoa were collected. An aliquot of about 1 × 10^6 spermatozoa was incubated in LPR DNA-Prep Reagent containing 0.1% potassium cyanide, 0.1% NaN_3, nonionic detergents, salts, and stabilizer (Beckman Coulter) in the dark at room temperature for 10 minutes. The spermatozoa were subsequently incubated in Stein DNA-Prep Reagent containing 50 μg/mL of PI (<0.5%), RNAsi type A (4 kU/mL), <0.1% NaN_3,

salts, and stabilizer (Beckman Coulter) in the dark at room temperature for 30 minutes.

TUNEL Staining

The TUNEL assay was performed using the Apoptosis Mebstain kit (Beckman Coulter), as previously reported.[17] The negative control was obtained by not adding terminal deoxynucleotidyl transferase to the reaction mixture, and the positive control was obtained by pretreating the spermatozoa with 1 μg/mL of RNase-free deoxyribonuclease I (Sigma Chemical, St. Louis, MO) at 37°C for 60 minutes before labeling. Cellular debris was eliminated as described previously.

Statistical Analysis. The results are reported as the mean ± SEM. The data were analyzed using Student's *t* test and 1-way analysis of variance followed by the Duncan multiple-range test, as appropriate. The software program, SPSS, version 9.0, for Windows (SPSS, Chicago, IL) was used for statistical evaluation. A statistically significant difference was determined at $P < .05$.

RESULTS

Age (37.5 ± 2.5 vs 36.8 ± 3.2 years) and body mass index (22.5 ± 2.0 vs 22.0 ± 1.0 kg/m^2) did not significantly differ in the normozoospermic men and patients with OAT. The latter had a significantly lower sperm concentration, total sperm count, sperm motility, and spermatozoa with normal morphology ($P < .05$; Table 1).

A comparison between the patients with OAT and normozoospermic men before and after incubation with MYO is provided in Table 1. MYO significantly improved the total and progressive motility of the sperm from the patients with OAT, and these parameters were unaffected in the spermatozoa of normozoospermic men. No significant differences were found in the percentage of viable spermatozoa, spermatozoa with PS externalization or in late apoptosis, necrotic spermatozoa, spermatozoa with abnormal chromatin compactness, or DNA fragmentation.

A significant increase in the percentage of spermatozoa with high MMP (normal) was found after incubation with MYO in the patients with OAT ($P < .05$). After incubation with MYO, the percentage of spermatozoa with high MMP in those with OAT did not differ significantly from that of the normozoospermic men ($P < .05$). Moreover, in the patients with OAT, the percentage of spermatozoa with altered (low) MMP was significantly reduced after incubation with MYO ($P < .05$; Table 1).

After incubation with MYO, progressive and total motility had increased significantly in both normozoospermic men and patients with OAT ($P < .05$ vs spermatozoa incubated without MYO; Fig. 1).

Finally, the total number of spermatozoa obtained after swim-up had significantly increased in both groups after incubation with MYO ($P < .05$; Fig. 2).

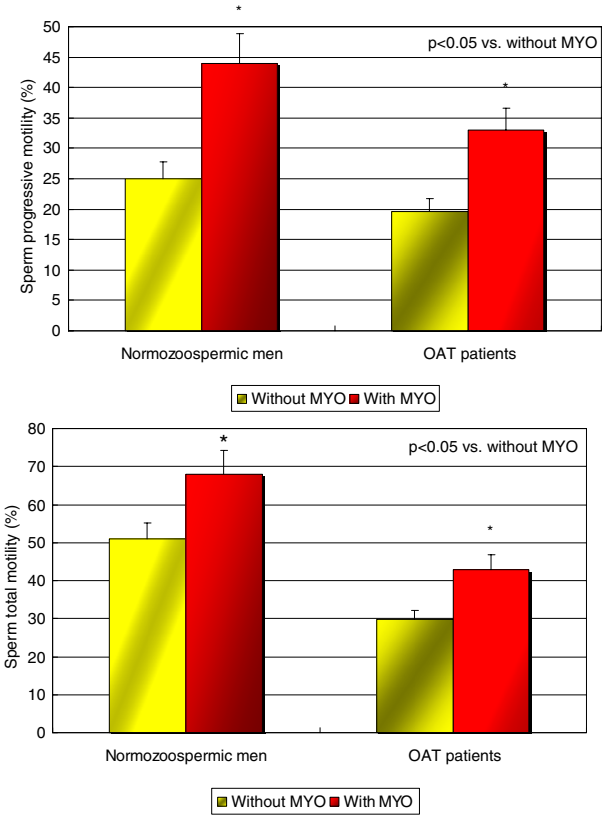

Figure 1. Percentage of spermatozoa with **(Upper)** progressive and **(Lower)** total motility in normozoospermic men (n = 20) and patients with OAT (n = 20) after incubation with or without MYO for 2 hours.

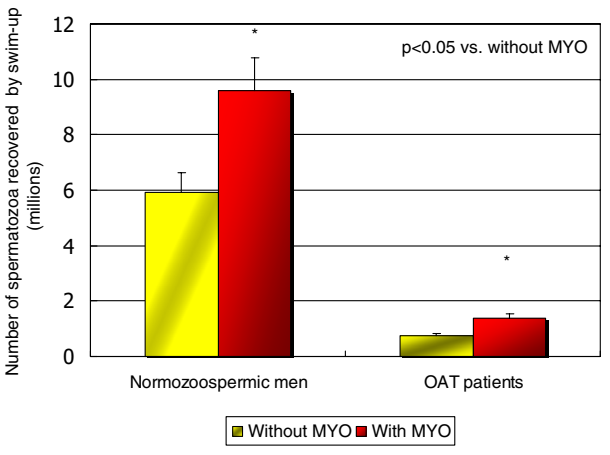

Figure 2. Total number of spermatozoa after swim-up technique from normozoospermia men (n = 20) or patients with OAT (n = 20) after incubation with or without MYO.

COMMENT

The previously described effects of MYO on fertility have focused almost exclusively on ovarian function, particularly in patients with polycystic ovary syndrome, in terms of insulin resistance, ovulation rate, and oocyte quality.[19] The present study investigated sperm function and showed that incubation with MYO improved sperm pro-

gressive motility and doubled the concentration of motile spermatozoa after swim-up in both normozoospermic men and patients with abnormal sperm parameters. An amelioration of sperm mitochondrial function was observed in the OAT group. This effect was observed after a short incubation and seemed to be specific, because no effect was observed on PS externalization and/or chromatin/DNA integrity in sperm from either group of men. Hence, these results suggest that the main site of action of MYO is the sperm mitochondria, where it exerts positive effects on sperm motility, at least under the experimental conditions used in the present study. In particular, a high percentage of sperm with low mytochondrial membrane potential represent a very important index of impaired sperm mytochondrial function, and MYO decreases this percentage. Finally, when comparing patients with OAT and healthy subjects, we found that MYO might have a stimulatory function in OAT sperm and a protective function in normal semen. The present study was the first to show that other functional parameters, such as sperm DNA fragmentation and chromatin compaction, do not improve after MYO treatment. This aspect should be developed further through other study designs that incorporate MYO dose-response studies using different incubation times.

MMP is a marker of cellular apoptosis. An apoptotic stimulus reduces MMP expression (an early and reversible event), thereby altering the permeability of the mitochondrial membrane. This alteration leads to the release of cytochrome c into the cytosol. Viable cells express high levels of MMP, and apoptotic cells express low levels of MMP. MMP expression can be used to evaluate sperm quality.[20,21] It has been shown that men with normal sperm parameters have a significantly greater percentage of spermatozoa that express high levels of MMP compared with patients with abnormal parameters. In addition, it has been reported that the number of spermatozoa with high MMP levels correlates positively with the sperm concentration and progressive and total motility.[20,21] Spermatozoa samples with low MMP are associated with reduced sperm motility because flagellar movement is adenosine triphosphate-dependent.[20] Evidence has been produced that adenosine triphosphate from glycolysis supports sperm motility, and adenosine triphosphate from mitochondrial respiration is used for gluconeogenesis, which supplies glucose in the absence of substrates for glycosylation.[22] Thus, under the experimental condition of the present study, OAT spermatozoa with low MMP might lose motility when incubated in PBS (ie, in the absence of glycosilabile substrates) because dysfunctional mitochondria do not support glycolysis with gluconeogenesis. In contrast, exposure to MYO preserves (or restores) sperm motility by preserving (or restoring) the mitochondrial capability to generate glucose.

Recently, Paoli et al[23] showed a positive correlation between MMP and total motility in 230 semen samples. These results provide additional evidence that sperm motility depends on mitochondrial functional integrity.[23] In addition, the percentage of spermatozoa with high MMP levels directly correlates with sperm viability.[20] This finding disagrees with those from our study, which showed no changes in the percentage of viable spermatozoa after incubation with MYO, despite the increase in the number of sperm cells expressing high MMP levels in patients with abnormal sperm parameters. These discrepancies can be explained by the different methods used to estimate the number of viable spermatozoa and/or the low number of patients we studied. In addition, spermatozoa with high MMP levels and low DNA fragmentation levels have a high fertilization rate when used in assisted reproductive programs. Furthermore, the percentage of spermatozoa with high MMP levels is directly related to the fertilization rate after in vitro fertilization with embryo transfer.[20]

The concentration of motile spermatozoa after swim-up suggests the potential of this molecule in assisted reproduction. Swim-up is the simplest and least expensive procedure currently used as a standard preparation technique to provide sperm samples with good recovery rates, motility, morphology, and fertilization capability.[24,25]

The limitations of the present study included the limited number of samples tested, which slightly reduced the study's statistical power. Therefore, we are expanding this series to obtain more samples. In addition, the evaluation of sperm motility using other systems (eg, computer assisted sperm analyzer) might provide additional data. Finally, although no significant difference in MMP was detected in the sperm of normozoospermic men with and without MYO, motility was significantly increased with MYO. This observation suggests new hypotheses regarding other possible mechanisms through MYO might improve sperm motility.

CONCLUSIONS

The results of the present study have shown that MYO ameliorates sperm mitochondrial function, thereby improving sperm motility in patients with altered sperm parameters. In addition, MYO increases 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 intrauterine insemination and to ameliorate the sperm quality to be used for in vitro-assisted reproductive techniques.

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