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# *In vitro* effects of myo-inositol on normokinetic human semen samples with nonlinear motility

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**Abstract. – OBJECTIVE: As myo-inositol (MI) deficiency has been associated with impaired sperm quality, we aimed to assess its effects on sperm kinetics objectively, using a computer-aided sperm analysis (CASA) system.**

**PATIENTS AND METHODS: We evaluated 59 normokinetic semen samples with nonlinear progressive motility before and after incubation with a solution of MI. The samples were collected from healthy subjects aged 20-40 years who were attending our Laboratory of Seminology for fertility screening.**

**RESULTS: We found a significant increase in linear progressive motility (28.2% ± 10.8 *vs.* 30.9% ± 11.0, T0 *vs.* T1 respectively; $p < 0.001$) and a significant reduction in nonlinear progressive motility (21.0% ± 9.9 *vs.* 18.1% ± 10.2, T0 vs. T1 respectively; $p < 0.001$) after incubation with MI. CASA analysis revealed a significant increase in curvilinear velocity (VCL) (65.0 ± 19.0 *vs.* 67.9 ± 20.4 μm/s, T0 *vs.* T1 respectively; $p = 0.049$). Overall, there was an increase in VCL in 42/59 samples (about 70%), mainly from non-smokers.**

**CONCLUSIONS: These results suggest that MI has a positive in vitro effect on semen samples, but confirmation is needed through further studies taking into account factors capable of modulating MI response, such as smoking and obesity.**

*Key Words:*
Spermatozoa, Sperm motility, Computer-assisted sperm analysis, VCL, Myo-inositol.

## Introduction

Infertility is defined as a failure to conceive after at least 12 months of regular sexual intercourse. It affects more than 100 million couples worldwide, with male factor infertility reported in 20-50% of cases[1]. Several conditions may have a transient or permanent impact on men's reproductive potential. Many of these are characterized by oxidative stress, namely an increase in reactive oxygen species (ROS) that overcomes the spermatozoa's total antioxidant capacity[2]. Spermatozoa have limited antioxidant defenses and hence need the fundamental nutritive support of seminal plasma, which is rich in substances that act as free radical scavengers. A deficiency in these substances is often associated with structural sperm damage such as membrane lipid peroxidation, protein denaturation and DNA fragmentation[3]. The available data indicate that ROS levels in seminal plasma from idiopathic infertile patients are significantly higher than in fertile men[4]. Myo-inositol (MI) is a stereoisomer of inositol, a precursor of phosphatidylinositol 3-phosphate. It is involved in cell signal transduction and thus regulates several cellular functions, such as intracytoplasmic calcium concentrations. The presence of both the enzymes involved in MI synthesis and different concentrations of MI in seminal plasma, the epididymis and the vas deferens compared to the bloodstream suggest it has an important physiological function during the sperm maturation process[5,6]. Its role in infertility has been investigated in several studies. Reduced MI levels are associated with asthenozoospermia and impaired fertility. Moreover, asthenozoospermic patients have an increased activity of the enzymes involved in MI synthesis, suggesting a compensatory effect. *In vitro* studies have shown a significant increase in sperm motility in oligoasthenoteratozoospermic patients after incubation with MI, while data from assisted reproductive technology indicate an *in vitro* improvement in sperm motility after incubation with MI and an increase in ICSI pregnancy rates[7,8]. In light of these data, the aim of our study was to evaluate the *in vitro* effects of MI on sperm kinetics in normozoospermic samples from patients attending a Reproductive Medicine Centre. Normokinetic semen samples with nonlinear progressive motility were incubated with a MI solution to assess its effects on sperm motility using computer-aided sperm analysis (CASA).

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## Patients and Methods

### Patients

This study was approved by our Hospital Institutional Review Board. Informed consent was obtained from all study participants. We studied 59 semen samples from 59 patients (aged 20-40 years) attending the Laboratory of Seminology, Sperm Bank "Loredana Gandini", Department of Experimental Medicine, "Sapienza" University of Rome for analysis of seminal fluid between September 2017 and March 2018. The selected samples were normozoospermic with nonlinear progressive motility. The patients had not been medically or surgically treated in the 3 months before the study and did not have any conditions (fever, etc.) that might interfere with the semen analysis. The exclusion criteria were: hypergonadotropic and hypogonadotropic hypogonadism, cryptorchidism, genitourinary infections, leukocytospermia, previous cancer and antineoplastic treatment and any genetic and/or chromosomal abnormalities.

All semen samples were analysed at T0 (baseline - pre-incubation) and at T1 (after incubation with MI for 30 minutes at 37°C). The following analyses were performed at both time points:

- Semen analysis according to the 2010 WHO Laboratory Manual[9];
- Computer-aided sperm analysis for sperm kinetic parameters (CASA System, Hamilton-Thorne®).

### Myo-inositol Solution

15 µl of concentrated MI solution (133 mg/ml; Andrositol Lab®, Lo.Li. Pharma, Rome, Italy) was added to 1 ml of raw seminal fluid. The mixture was then incubated for 30 minutes at 37°C.

### Semen Analysis and Sperm Kinetics

Semen samples were collected by masturbation after 3-5 days of sexual abstinence. All samples were allowed to liquefy at 37°C for 60 minutes and were then assessed according to the 2010 World Health Organization Laboratory Manual[9]. The following variables were taken into consideration: ejaculate volume (ml), total sperm number (x$10^6$/ejaculate), progressive motility (% linear + % nonlinear) and morphology (% abnormal forms). Automated CASA (Hamilton Thorne® Beverly, MA, USA) for sperm kinetics was carried out on all samples. The variables taken into consideration were curvilinear velocity (VCL m/s), linearity (LIN %), lateral head displacement (ALH µm) and beat cross frequency (BCF Hz).

### Statistical Analysis

Differences in continuous variables were presented as means ± SD and medians. They were evaluated with paired *t*-test or Wilcoxon signed rank test, as appropriate, based on normality of data distribution curves. Categorical variables, expressed as percentages, were compared with $\chi^2$-test. A two tailed *p*-value <0.05 was considered significant. Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) 25.0 (IBM, Armonk, NY, USA).

## Results

The semen samples of 59 patients aged 31.6 ± 7.3 years (median 33 years) were analyzed. The

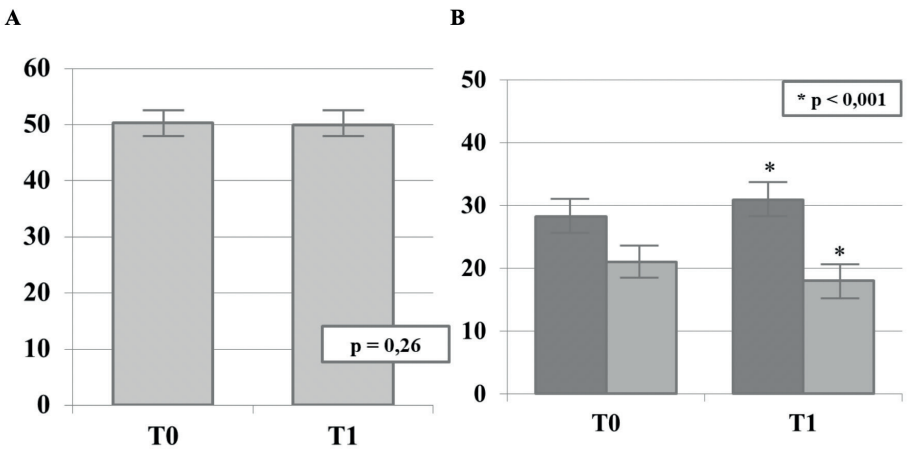


**Figure 1. A,** Progressive motility (%) before and after incubation with MI (means ± SE); **B,** linear progressive motility (dark grey) and non-linear progressive motility (pale grey) before and after incubation with MI (means ± SE).

**Table I.** Sperm parameters of selected semen samples pre- and post-incubation (mean ± SD, medians in brackets).

| | Volume (ml) | Total sperm number (N x10$^6$) | Linear progressive motility (%) | Non-linear progressive motility(%) | Progressive motility (%) | Abnormal forms (%) |
|---|---|---|---|---|---|---|
| T0 (Baseline) | 3.0 ± 1.1 (3.1) | 272.2 ± 100.8 (264.0) | 28.2 ± 10.8 (30) | 21.0 ± 9.9 (20) | 49.2 ± 6.2 (50) | 90.0 ± 2.6 (90.0) |
| T1 (Post incubation) | // | // | 30.9 ± 11.0 (30) | 18.1± 10.2 (15) | 49.0 ± 6.6 (50) | // |
| *p*-value | // | // | <0.001 | <0.001 | ns | // |

means ± SD and medians of the sperm parameters are shown in Table I. Although we found no difference in progressive motility pre- and post-incubation with MI, there was a significant increase in linear progressive motility (28.2 ± 10.8 % *vs.* 30.9 ± 11.0 %, T0 *vs.* T1 respectively; $p$ <0.001) and, consequently, a significant reduction in non-linear progressive motility (21.0 ± 9.9 % *vs.* 18.1± 10.2 %, T0 *vs.* T1 respectively; $p$ <0.001) (Figure 1A and 1B). Post-incubation with MI, CASA revealed a significant increase in curvilinear velocity (VCL) (65.0 ± 19.0 *vs.* 67.9 ± 20.4 µm/s, T0 *vs.* T1, respectively; $p$ = 0.049) – median increase 4.2 µm/s (Table II). This increase was found in 42/59 samples (about 70%). No significant differences were found in the other sperm kinetic parameters (LIN, ALH and BCF).

On the basis of VCL modification, we divided the semen samples into three subgroups (corresponding to terciles): Group A – VCL increase < 1.5 µm/s; Group B - VCL increase between 1.5 and 6.7 µm/s; Group C - VCL increase > 6.7 µm/s.

**Table II.** Means ± SD, medians (in brackets) of sperm kinetic parameters pre- and post-incubation evaluated with CASA (Hamilton-Thorne®).

| | T0 (Baseline) | T1 (Post-incubation) | *p*-value |
|---|---|---|---|
| VCL (µm/s) | 65.0 ± 19.0 (59.6) | 67.9 ± 20.4 (63.0) | <0.05 |
| LIN (%) | 67.7 ± 5.5 (67.0) | 66.7 ± 5.9 (67.0) | ns |
| ALH (µm) | 2.7 ± 0.9 (2.6) | 2.9 ± 1.1 (2.8) | ns |
| BCF (Hz) | 7.7 ± 2.6 (7.5) | 7.9 ± 2.8 (7.5) | ns |

Legend: VCL: curvilinear velocity; LIN: linearity; ALH: lateral head displacement; BCF: beat-cross frequency; ns: not significant.

**Table III.** Means, SD, medians (in brackets) of semen and kinetic parameters (CASA) of groups A, B and C. [a]$p$ <0.01 (Group C *vs.* Group A; Group C *vs.* Group B). [b]$p$ <0.001 T0 vs. T1. [c]p <0.01 T0 *vs.* T1.

| | Group A | | Group B | | Group C | |
|---|---|---|---|---|---|---|
| Smokers (%) | 60%[a] | | 44%[a] | | 10%[a] | |
| Age (years) | 32.5 ± 7.5 (35.0) | | 32.7± 7.0 (35.0) | | 29.5 ± 7.2 (27.0) | |
| | T0 | T1 | T0 | T1 | T0 | T1 |
| VCL (µm/s) | 71.6 ± 22.4[b] (64.3) | 62.7 ± 26.6[b] (55.7) | 63.1 ± 19.6[b] (56.3) | 67.5 ± 19.5[b] (61.4) | 60.3 ± 12.3[b] (58.2) | 73.7 ± 11.6[b] (73.9) |
| LIN (%) | 67.1 ± 6.6 (66.0) | 66.0 ± 6.7 (65.5) | 67.1 ± 4.4 (66.5) | 67.1 ± 5.1 (67.0) | 68.9 ± 5.3 (69.0) | 66.9 ± 6.0 (67.0) |
| ALH (µm) | 3.0 ± 1.0 (3.1) | 2.7 ± 1.2 (2.3) | 2.7 ± 0.8 (2.6) | 2.7 ± 0.9 (2.4) | 2.6 ± 0.9[c] (2.4) | 3.4 ± 1.0[c] (3.2) |
| BCF (Hz) | 7.5 ± 2.8 (6.8) | 7.6 ± 3.1 (7.0) | 7.3 ± 2.4 (6.5) | 7.4 ± 2.2 (7.1) | 8.3 ± 2.6 (8.7) | 8.8 ± 2.9 (9.0) |

Legend: VCL: curvilinear velocity; LIN: linearity; ALH: lateral head displacement; BCF: beat-cross frequency.

**Table III.** Means, SD, medians (in brackets) of semen and kinetic parameters (CASA) of groups A, B and C. [a]$p$ <0.01 (Group C *vs.* Group A; Group C *vs*. Group B). [b]$p$ <0.001 T0 v*s*. T1. [c]p <0.01 T0 *vs.* T1.

| | Group A | | Group B | | Group C | |
|---|---|---|---|---|---|---|
| Smokers (%) | 60%[a] | | 44%[a] | | 10%[a] | |
| Age (years) | 32.5 ± 7.5 (35.0) | | 32.7± 7.0 (35.0) | | 29.5 ± 7.2 (27.0) | |
| | T0 | T1 | T0 | T1 | T0 | T1 |
| VCL (μm/s) | 71.6 ± 22.4[b] (64.3) | 62.7 ± 26.6[b] (55.7) | 63.1 ± 19.6[b] (56.3) | 67.5 ± 19.5[b] (61.4) | 60.3 ± 12.3[b] (58.2) | 73.7 ± 11.6[b] (73.9) |
| LIN (%) | 67.1 ± 6.6 (66.0) | 66.0 ± 6.7 (65.5) | 67.1 ± 4.4 (66.5) | 67.1 ± 5.1 (67.0) | 68.9 ± 5.3 (69.0) | 66.9 ± 6.0 (67.0) |
| ALH (μm) | 3.0 ± 1.0 (3.1) | 2.7 ± 1.2 (2.3) | 2.7 ± 0.8 (2.6) | 2.7 ± 0.9 (2.4) | 2.6 ± 0.9[c] (2.4) | 3.4 ± 1.0[c] (3.2) |
| BCF (Hz) | 7.5 ± 2.8 (6.8) | 7.6 ± 3.1 (7.0) | 7.3 ± 2.4 (6.5) | 7.4 ± 2.2 (7.1) | 8.3 ± 2.6 (8.7) | 8.8 ± 2.9 (9.0) |

Table III summarizes the kinetic parameters of each group.

Notably, the percentage of smokers in groups A and B was significantly higher than in group C. By grouping samples by smoking habit, we found that pre- and post-incubation variations in sperm motility and kinetic parameters were more evident in semen samples from non-smokers. These results are shown in Table IV.

## Discussion

The role of MI in human reproduction has been debated for many years. MI and its various phosphorylated derivatives are present in numerous tissues and biological fluids and undergo strict regulation, carrying out important biological functions[5]. Men with a balanced diet consume about 1 g/day of inositol, which is actively absorbed by the intestinal lumen (sodium-inositol symporter), while Sertoli cells can produce MI from glucose after gonadotropin stimulation[10]. This fact, and the observation of higher concentrations of MI in seminal plasma than in blood, support the hypothesis that MI has an important role in the reproductive process[5].

Spermatogenesis, epididymal maturation and sperm capacitation, important stages of male germ cell maturation from its origin to its acquisition of the capacity to fertilize the oocyte, are regulated in part by the strict control by Sertoli and epididymal epithelial cells of osmolarity and solute concentrations in the luminal environment[11]. As MI, due to its physical and chemical properties, regulates osmotic balance in the kidney, it can be hypothesized that it could do the same in seminal plasma and in the endoluminal environment of the seminiferous tubules[6,12]. MI-derived molecules involved in cell signaling may also influence physiological processes relevant to sperm function, such as $Ca^{2+}$ ion level regulation[13].

Another important role of MI concerns sperm motility. Data from *in vitro* studies suggest that the acquisition of progressive motility during epididymal maturation is closely related to changes in ionic concentrations in the epididymal lumen, especially calcium and bicarbonate ions[11]. *In vivo*, sperm flagellum motion is acquired during epididymal transit through interactions between the dynein and tubulin molecules. However, the relationship between luminal/intracellular ion concentrations and structural cell modifications is still being explored[11].

Evidence for these aspects may be provided by a proteomics study, which identified an overexpression of IMPA1, a protein involved in MI synthesis, in asthenozoospermic subjects. The authors suggested that there may be a post-translational dysregulation of MI synthesis, potentially affecting the osmotic balance of the seminiferous tubule microenvironment[14]. However, these results need to be confirmed in additional studies.

MI also influences the mitochondria, increasing the organelle membrane potential[15-17]. The mitochondria are located in the midpiece region around the axoneme. Although they do not directly possess inositol receptors, the latter have been identified in vesicular areas of "redundant nuclear envelope", also located in the sperm midpiece,

along with calreticulin, a calcium-binding protein expressed in areas of cell deposition. Therefore, the localization of mitochondria near intracellular $Ca^{2+}$ deposits allows the transport of these ions into the mitochondrial matrix[18]. It is known that calcium ions are activators of mitochondrial dehydrogenases, which stimulate oxidative metabolism and the availability of ATP[19]. Coupling between sperm and mitochondrial $Ca^{2+}$ concentrations should ensure the sperm's energy demands[18]. The role of $Ca^{2+}$ ions and the mitochondria has been studied in the induction of hyperactivated sperm motility. However, it is reasonable to suppose that MI signaling pathways may make mitochondrial respiration more efficient and improve sperm flagellar motility.

Daily MI supplementation has shown encouraging results. A randomized placebo-controlled study in infertile patients[20] identified significant improvements in both total sperm number and sperm motility in the subgroup treated with MI supplementation for three months. Another study[7] found an improvement in sperm concentration in a small group of oligoasthenozoospermic patients after daily supplementation with MI. Dinkova et al[21] reported a significant improvement in sperm motility in asthenozoospermic patients. The literature data thus indicate that adequate MI concentrations in the male genital trait may be involved both directly and indirectly in the development and maturation of human spermatozoa.

To our knowledge, this is the first study to analyze kinetic modifications in normozoospermic semen samples after incubation with MI using the CASA system. Our data showed an overall improvement in the quality of sperm motility after incubation with MI, with a decrease in the percentage of nonlinear progressive motility and a corresponding increase in linear progressive motility. Computer-aided kinetic analysis showed an increase in VCL. This underlines how incubation with MI improved flagellar movement, possibly by acting at the mitochondrial level.

Sample stratification as a function of variation in VCL demonstrated that less than one-third of the samples showed no kinetic improvements after incubation. This can be explained at least in part by the higher percentage of smokers among

**Table IV.** Semen and kinetic parameters (CASA) of non-smokers vs. smokers: means ± SD, medians in brackets. [a]$p$ <0.001 (related samples Wilcoxon signed rank test) T0 *vs.* T1. [b]$p$ <0.01 (related samples Wilcoxon signed rank test) T0 *vs.* T1. [c]$p$ <0.05 (related samples Wilcoxon signed rank test) T0 *vs.* T1.

| | **Non-smokers (38 pts)** | | **Smokers (21 pts)** | |
|---|---|---|---|---|
| Age (years) | 34.4 ± 6.8 (36.0) | | 30.1 ± 7.2 (27.5) | |
| | T0 | T1 | T0 | T1 |
| Volume (ml) | 3.2 ± 1.2 (3.2) | // | 2.7 ± 1.1 (2.5) | // |
| Total sperm number (N x $10^6$) | 291.7 ± 102.7 (271.0) | // | 236.9 ± 89.0 (242.5) | // |
| Linear prog. motility (%) | 28.2 ± 10.8[a] (30.0) | 31.7 ± 10.8[a] (35.0) | 28.3 ± 11.0 (30.0) | 29.5 ± 11.6 (30.0) |
| Non-linear prog. motility (%) | 20.9 ± 10.8[a] (20.0) | 17.2 ± 10.8[a] (15.0) | 21.2 ± 8.3[c] (20.0) | 19.5 ± 8.9[c] (20.0) |
| Progressive motility (%) | 49.1 ± 6.4 (50.0) | 48.9 ± 6.9 (50.0) | 49.5 ± 6.1 (50.0) | 49.0 ± 6.2 (50.0) |
| Abnormal forms (%) | 90.1 ± 2.9 (89) | // | 89.7 ± 2.0 (90.0) | // |
| VCL (µm/s) | 63.4 ± 16.6[b] (57.9) | 68.9 ± 17.6[b] (66.6) | 68.0 ± 22.9 (64.2) | 66.1 ± 25.1 (60.3) |
| LIN (%) | 68.3 ± 5.3 (69.0) | 66.4 ± 6.7 (67.0) | 66.6 ± 5.7 (66.0) | 67.1 ± 6.2 (67.0) |
| ALH (µm) | 2.8 ± 0.9 (2.5) | 3.1 ± 1.1 (2.9) | 2.7 ± 0.9[c] (2.8) | 2.6 ± 1.0[c] (2.2) |
| BCF (Hz) | 7.7 ± 2.6 (7.8) | 8.1 ± 2.7 (7.7) | 7.8 ± 2.6 (6.8) | 7.6 ± 2.9 (7.1) |

these samples. Smoking is in fact a known cause of oxidative stress. In recent literature it has often been associated with reduced sperm quality, especially motility[22], with harmful effects that can be exacerbated in the presence of overweight/obesity and/or other common andrological diseases such as varicocele[23]. The oxidative damage induced by smoking may have damaged the kinetic capabilities of the axoneme, cancelling out the action of MI. However, the sample size of this study does not allow an in-depth evaluation of these aspects, which merit exploration in future studies.

## Conclusions

Our study showed that the addition of MI to the semen sample aliquot produced a qualitative improvement in sperm kinetics in about 70% of patients. This improvement consisted of an increase in the percentage of linear progressive motility with a parallel reduction in non-linear progressive motility and an increase in the sperm's curvilinear velocity, without any variation in the overall progressive motility. The most relevant significant result is that incubation with MI induced a change in sperm movement quality even though the percentage of motile sperm was unchanged. Furthermore, we showed that smoking could nullify the improved kinetic parameters induced by MI, as the oxidative stress associated with smoking may prevent it acting on sperm. Investigation of these aspects in future studies could improve the identification of subjects who might benefit from MI supplementation.

### Conflict of Interests

The Authors declare that they have no conflict of interests.

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