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Physiological Intra-Cytoplasmic Sperm Injection (PICSI) outcomes after oral pretreatment and semen incubation with myo-inositol in oligoasthenoteratozoospermic men: results from a prospective, randomized controlled trial

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Abstract. – OBJECTIVE: The aim of the current study was to evaluate the effect of an oral pretreatment with a mix of myo-Inositol (Myo-Ins), folic acid, vitamin E, L-carnitine, L-arginine and selenium (Folandrol, Exeltis, Hungary) and subsequent direct Myo-Ins incubation of spermatozoa before Physiological Intra-Cytoplasmic Sperm Injection (PICSI) procedures in infertile couples due to oligoasthenoteratozoospermia with previous failed PICSI procedures.

PATIENTS AND METHODS: We performed a prospective, randomized controlled trial at the Assisted Reproduction Unit of the Kaáli Institute (Győr, Hungary). The male partners were randomly assigned to two groups: the first one treated with a myo-Inositol-based supplement (Folandrol®, Exeltis, Hungary) for two months; the second one did not undergo any treatment in the same time range (controls). The semen of the treated group was incubated for 2 h with 2 mg/ml of MI (Andrositol Lab, Lo.Li. Pharma, Rome, Italy) for the PICSI protocol.

RESULTS: There was no significant difference for mean female partner age ($p = 0.17$) and mean previous failed PICSI procedures ($p = 0.65$) between the two groups. Although there was no significant difference ($p = 0.85$) regarding the rate of mature oocytes and the fertilization index was significantly higher ($p < 0.001$) in the treatment group than control group. Also, despite the comparable average number of transferred embryos between the two groups ($p = 0.55$), in the treatment group there was a significantly higher rate of good quality embryos at day 3 after fertilization ($p = 0.001$). Finally, 11 pregnancies were obtained only in the treatment group ($p = 0.001$).

CONCLUSIONS: The combination of oral supplementation and semen incubation with MI in oligoasthenoteratozoospermic men could improve PICSI outcomes.

Key Words:
Physiological intra-cytoplasmic sperm injection, Myo-inositol, Oligoasthenoteratozoospermia, Male fertility, Semen.

Introduction

Oligoasthenoteratozoospermia (OAT) is defined as a decreased sperm count, motility and altered morphology. In 30-35% of these cases the etiology is unknown. Oxidative stress (OS) is considered as an important factor, which may influence male infertility and be linked to the production of reactive oxygen species (ROS)[1]. ROS play an important physiological role, modulating sperm proliferation, differentiation, and function. In the semen of fertile men, the amount of ROS generation is controlled by seminal antioxidants[2]. When ROS production exceed the antioxidant capabilities of the male reproductive tract, a damage of spermatozoa may occur, since these cells are highly susceptible to ROS. In particular, ROS induces peroxidative damage in the sperm plasmatic membrane and DNA fragmentation[3]. ROS may arise from different sources: on the one hand, morphologically abnormal spermatozoa (with residual cytoplasm, in particular) and leukocytes[2]; on the other hand, ROS may be due to insufficient antioxidant protection and/or action of redox cycling xenobiotics. Regardless of the cause(s), high levels of ROS are associated with extensive peroxidation of unsaturated fatty acids at membranes level, which subsequently leads to DNA damage in the spermatozoa and reduced fertility rate[4]. Also, ROS may induce changes in mitochondrial

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membranes: the alteration of these ATP-producing organelles impairs sperms motility[3], leading to a reduced fertility rate[5]. It is widely accepted that sperm DNA integrity is directly related to pregnancy outcome during *in vitro* fertilization (IVF). On this regard, accumulating evidence suggests that high sperm DNA fragmentation impair embryo quality and it is associated with spontaneous miscarriage or biochemical pregnancy following assisted reproductive technology (ART)[6]. Furthermore, sperm DNA fragmentation seems to affect also embryo postimplantation development in Intra-Cytoplasmic Sperm Injection (ICSI) procedures[7,8].

Inositol is a polyalcohol classified as an insulin sensitizer and it is naturally occurring as nine stereoisomers. It is synthesized by both prokaryotic and eukaryotic cells, even if in mammals it is mainly obtained from dietary sources (as free inositols, phosphatidyl-inositol or inositol-6-phospahte), and endogenous synthesis from glucose[9]. Myo-Inositol (Myo-Ins), which is the most abundant form of inositol in humans, is converted to D-Chiro-Inositol by an epimerase enzyme[10]. Myo-Ins derivative inositol triphosphate (Ins-1,4,5P3, InsP3) acts as an intracellular second messenger, regulating the activities of several hormones such as insulin, follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH)[11]. In particular, several studies[12,13] have already proved its efficacy in restoring hormonal and metabolic parameters toward homeostasis in polycystic ovary syndrome (PCOS), in reducing metabolic alterations during menopause[14] and even in preventing gestational diabetes mellitus[15].

Recent studies already found that myo-Inositol plays an important role in both the maturation of male gametes and migration from the epididymis[16,17]. It is present in higher concentration in the seminiferous tubules than in the seminal plasma, but the significance of these differences are still unknown. In humans, Inositol plays a role in the regulation of the vescicular seminal solution: natrium/myo-inositol cotransport protein (SLC5A3), which expression is increased in hyperosmotic environment, is required for the passage of inositol through the tissue; also, a low concentration of Inositol in the epididymis is associated to decreased fertility[18].

Accumulating evidence[19] suggests that cell-specific Inositol phosphate signaling regulates organismal responses to ROS. Recent data demonstrated that ROS results in a selective up-regulation of type-2 InsP3 receptors[20]. Reflecting these elements from basic science into the clinical perspective, it was shown that Myo-Ins can reduce oxidative stress also during PCOS[21,22]. Also, several *in vitro* studies[23-25] tested inositol as possible antioxidant agent for the treatment of male infertility, in order to improve spermatozoa quality and subsequently fertilization.

Last but not least, Myo-Ins has also been suggested to play a role in the human sperm chemotaxis and thermotaxis through activation of phospholipase C, resulting in the production of InsP3 and opening of calcium channels; finally, Inositol regulates the intracellular Ca^{2+} concentrations by acting on the sperm plasma membrane, mitochondria, acrosome and neck region, other intracellular Ca^{2+} stores[26]. In this regard, not surprisingly, Inositol causes an increase of cytosolic calcium concentration and consequently an increase of mitochondrial Ca^{2+} that stimulates the oxidative mechanism and the ATP production, improving mitochondrial function of spermatozoa, preventing apoptosis, and facilitating chromatin compactness[27].

Based on available evidence, the aim of the current study was to evaluate the effect of oral pretreatment with a mix of Myo-Ins and selected antioxidant agents such as folic acid, vitamin E, L-carnitine, L-arginine and Selenium (Folandrol, Exeltis, Hungary) and subsequent direct Myo-Ins incubation of spermatozoa before PICSI procedures in infertile couples for andrologic reasons (OAT) with previous failed ICSI procedures.

Patients and Methods

Study Design and Population

This prospective, single-center, randomized controlled trial was performed at the Assisted Reproduction Unit of the Kaáli Institute (Győr, Hungary). We selected 22 couples that had to undergo PICSI procedures for male infertility issues.

In particular, all the couples met the following inclusion criteria: age under 42 years (for both male and female partners); for females: regular menstrual cycles with less than 10 mIU/ml basal FSH level and more than 1 ng/ml Anti-Müllerian Hormone (AMH) concentration in serum; for males: OAT, defined according to World Health Organization (WHO) parameters[28] in the ejaculated semen (in order to exclude obstructive syndromes such as vasectomy, congenital absence of vas deferens, herniorrhaphy, hydrocelectomy, Young's syndrome and ejaculatory duct obstruc-

tion[29]) and previous failed ICSI procedures. We excluded any other type of endocrine, metabolic, autoimmune, neoplastic diseases in both female and male partners.

The male partners were randomly assigned to two groups, using a computer-generated randomization schedule (www.randomization.com): the first group underwent treatment with 1 g of myo-inositol, 30 mg of L-carnitine, L-arginine and Vitamin E, 55 µg of selenium, and 200 µg of folic acid (Folandrol®, Exeltis, Hungary) twice a day, for two months; the second group did not undergo any treatment in the same time range (controls).

Any patient taking less than 80% of the allocated dose of study drug was regarded as non-compliant and excluded from the current analysis. Enrolled patients did not take any other drug, which may modify the analyzed parameters during the previous 3 months, the beginning of the study or during the treatment. As per study design (control group was not on placebo; the evaluation was performed by a single operator), it was not possible to blind the study.

All the design, analysis, interpretation of data, drafting and revisions conform the Helsinki Declaration, the Committee on Publication Ethics (COPE) guidelines (http://publicationethics.org/), the CONSORT (CONsolidated Standards of Reporting Trials)[30,31] and SPIRIT (Standard Protocol Items: Recommendation for Interventional Trials)[32] statements, available through the EQUATOR (enhancing the quality and transparency of health research) network (www.equator-network.org). As standard protocol, each patient was informed about the procedures and signed a consent allowing data collection for research purposes and the study was approved by the Institutional Review Board (IRB) of the setting in which the study was performed.

Controlled Ovarian Stimulation

Patients were evaluated for serum levels of FSH, luteinizing hormone (LH) and estradiol (E_2) levels on day 2 of the cycle, which resulted all in their respective physiologic ranges. Also, antral follicle count (AFC) was performed using transvaginal ultrasound (TVS). All patients underwent flexible gonadotropin-releasing hormone (GnRH) antagonist protocol. The recombinant FSH dose was tailored to patient's response (as described elsewhere[33]) and/or purified menotropin was added to the stimulation protocol. Patients were rechecked after 4 days of stimulation and on day 10 by TVS and serum E_2 and LH. As soon as three follicles ≥ 18 mm were observed by TVS, oocyte maturation was triggered using 250 µg of choriogonadotropin alfa (Ovitrelle, Merck Serono, Germany). Oocyte pickup was performed between 35 and 36 h after choriogonadotropin alfa trigger.

Semen Preparation

The fresh collected samples were analyzed immediately before PICSI procedures. According to the abovementioned WHO criteria[28], semen samples were collected into sterile containers by masturbation after 1 to 3 days of sexual abstinence. After liquefaction, semen was evaluated by light microscopy to evaluate semen's volume, spermatozoa's number and motility. All samples were processed through density gradients centrifuge and swim-up method. Thereafter, the samples of the treated group were incubated for 2 h with 2 mg/ml of MI (Andrositol Lab®, Lo.Li. Pharma, Rome, Italy) dispersed in the *in vitro* fertilization medium (Quinns Advantage™ Fertilization Medium, Origio, Denmark). Spermatozoa were placed in PICSI dish containing samples of hyaluronan hydrogel and selected after binding essay, accounting for their maturity as previously described[34].

Physiological Intra-Cytoplasmic Sperm Injection

Fertilization of the oocytes was carried out by PICSI procedures according to the standard *in vitro* technique described by Palermo et al[35]. Embryos were examined for the number, regularity and granularity of blastomeres and the degree of embryonic fragmentation on day 3 after PICSI, according to criteria proposed by Nagy et al[36]. The five days blastocysts were analyzed by using Gardner et al[37] criteria. Two embryos of good quality were transferred on day 3 or 5 using a dedicated soft catheter (Wallace Classic Embryo Replacement Catheter, Smiths Medical, Brunn am Gebirge, Austria), while supernumerary embryos were vitrified by the Open Pulled Straw method[38]. Lutheal phase supplementation was administered since day 1 after the oocytes retrieval with intravaginal progesterone gel (Crinone 8%, Merck Serono, Darmstadt, Germany). Serum human chorionic gonadotropin (hCG) level was measured 12-14 days after embryo transfer and, if positive, we continued progesterone therapy until 9-10 gestation weeks. Ultrasound examination was scheduled 2 weeks later to assess the number and viability of the implanted embryos.

Statistical Analysis

Statistical analyses were performed using the SPSS 12.0 (SPSS Inc., Chicago, IL, USA) statistical software package. The assumption of normal distribution of continuous variables was tested by Kolmogorov-Smirnov test for goodness of fit. Continuous variables were expressed as means and standard deviations (SD) and compared between the two groups using the Student *t*-test. Categorical variables were expressed as percentages and compared between the two groups using the two-tailed X^2 test. A *p*-value < 0.05 was considered statistically significant.

Results

We included data about PICSI outcomes in 22 couples in which oligoasthenoteratozoospermic male partners underwent oral treatment with 1 g of myo-inositol, 30 mg of L-carnitine, L-arginine and Vitamin E, 55 µg of selenium, and 200 µg of folic acid twice per day. Furthermore, the semen samples of these patients were collected and incubated with 2 mg/ml Myo-Ins for 2 h. Other 13 couples in which oligoasthenoteratozoospermic male partners did not undergo any pharmacologic treatment were considered as control group. As showed in Table I, there was no significant difference for mean female partner age ($p = 0.17$) and mean previous failed ICSI procedures ($p = 0.65$), which allows us to consider the two groups homogeneous for the subsequent analysis.

In the first group, fresh embryo transfer was performed in 18 cases: regarding the remaining four cases, in one patient all embryos were cryopreserved due to her high serum progesterone level; in the other three cases, we used vitrified embryo from previous cycles. In the control group, fresh embryo transfer was performed in 13 cases.

Although there was no significant difference ($p = 0.85$) regarding rate of mature oocytes (as expected, considering the selective enrolment of infertile couples for male factor), the fertilization index was significantly higher ($p < 0.001$) in the treatment group than in the control group.

Also, despite the comparable average number of transferred embryos between the two groups ($p = 0.55$), in the treated group there was a significantly higher rate of good quality embryos at day 3 after fertilization ($p = 0.001$). Finally, 11 pregnancies were obtained only in the treated group ($p = 0.001$). Among these pregnancies, after the end of the study, 2 ended in early spontaneous miscarriages, 5 had already delivered and 4 were still ongoing uncomplicated.

Discussion

Spermatogenesis is essential for human reproduction: this process is finely regulated by multiple hormonal influences, autocrine and paracrine controls. In several cases, male infertility could be due, at least in part, by defective spermatogenesis caused by altered germ cell proliferation and differentiation. As previously summarized, ROS play a pivotal role in male infertility: on the one hand, spermatozoa are particularly sensitive to ROS action for their plasma membrane, which is rich in polyunsaturated fatty acids; on the other hand, the male gametes lose their cytoplasm during their maturation and, consequently, the possibility of antioxidant activity by cytoplasmatic enzymes[39].

Based on these elements, accumulating evidence suggests that antioxidants may improve fertility outcomes both as oral supplement[40] and

Table I. Characteristic of enrolled patients and PICSI outcomes in couples in which oligoasthenoteratozoospermic male partners underwent treatment with Myo-Ins, folic acid, vitamin E, L-carnitine, L-arginine, selenium, and couples in which oligoasthenoteratozoospermic male partners did not undergo any pharmacologic treatment (controls).

	Treatment (n = 22)	Controls (n = 13)	*p*
Female partner age (years)	36.7 ± 2.7	35.2 ± 3.7	0.17
Previous failed ICSI	3.9 ± 1.2	3.7 ± 1.4	0.65
Number of fresh embryo transfers	18	13	–
Rate of the matured oocytes (%)	81.8 (158/194)	80.7 (109/135)	0.85
Fertilization index (%)	84.8 (134/158)	60.5 (66/109)	< 0.001
Number of transferred embryos	1.9 ± 0.4	2.0 ± 0.6	0.55
Good quality embryos at day 3 (%)	54.7	32	0.001
Number of pregnancies	11	0	0.001

Data are expressed as means ± standard deviations, or as percentages.

additive during *in vitro* techniques for the preparation of seminal fluid[41] before ART. Among the possible therapeutic strategies to target the ROS, recent data showed that myo-Inositol improved motility in semen of patients affected by OAT; in particular, an accurate ultrastructural analysis by both scansion and transmission electron microscopy found a reduction of amorphous fibrous material around spermatozoa and improved morphology of mitochondrial cristae[42].

Beside this elegant study, other robust evidence supports the role of Myo-Ins in modulating the molecular and cellular pathways, which orchestrate the physiologic regulation of sperms mitochondrial functions and DNA integrity[16,18,24,25,27], both key determinants of male fertility potential.

In our prospective, randomized controlled trial we found that the oral treatment with Myo-Ins, folic acid, vitamin E, L-carnitine, L-arginine, selenium and incubation of spermatozoa with Myo-Ins significantly improved fertilization index, rate of good quality embryos at day 3 and pregnancy rate in couples undergoing PICSI for male infertility (OAT).

Despite our data analysis, several limitations should be considered: firstly, the study sample is small, although it allowed us to get significant results; secondly, we used a mix of different oral nutraceuticals and subsequent semen incubation with Myo-Ins, thus we can not desume whether the observed results could be due to the action of a single oral nutraceutical, to the mix of several oral nutraceuticals, to the semen incubation with Myo-Ins or to a combination of all these elements. Nevertheless, since previous studies[18,24,25,42] already showed a clear correlation between Myo-Ins action and improvement of male fertility, our data allow us to hypothesize that it plays a pivotal role on investigated parameters. Also, we confirmed the widely accepted favorable safety profile of Myo-Ins.

Conclusions

To the best of our knowledge, this is the first study, which tested the combination of oral supplementation and semen incubation with Myo-Ins in oligoasthenoteratozoospermic men before PICSI. Based on our analysis, we solicit future investigations on the larger cohort of patients to confirm whether the proposed combined therapeutic strategy improve male fertility. Finally, we take the opportunity to suggest the identification of "tailored-dose treatments" of Myo-Ins as a future therapeutic target for male infertility, basing on both personal biophysical characteristics and seminal fluid parameters.

Conflict of Interest

All the authors have no proprietary, financial, professional, or other personal interest of any nature in any product, service, or company. The authors are responsible for all the contents and writing of the paper. No specific funding was obtained.

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