Aim: This study was carried out to assess the outcome of the intracytoplasmic morphologically selected sperm injection (IMSI) technique compared with the previous failed intracytoplasmic sperm injection (ICSI) attempts in oligoasthenoteratozoospermia (OAT)/severe OAT (SOAT)/teratozoospermia patients. Setting: Institution-based, in vitro fertilization center. Study Design: It was a nonrandomized prospective study including 57 couples who had previous one or two ICSI failures (failure due to no implantation as embryos were transferred in these cycles and had no pregnancy) due to male factor. There was no case of total fertilization failure. IMSI was carried out in these couples and the results were compared with their previously failed ICSI attempts. Materials and Methods: Real-time selection of sperms was done using IMSI as it allows the assessment of fine nuclear morphology and vacuoles in the sperm head at a high magnification (>6000×) with differential interference contrast optics. Therefore, IMSI was applied in couples having OAT, SOAT or teratozoospermia as male factor and the results were compared with their previous failed ICSI attempts. Statistical analysis was carried out using GraphPad Prism. Results and Conclusion: Data analysis demonstrated significant difference in the fertilization rate between IMSI and previous ICSI attempts of these patients (30.0% vs. 52.0%; \( P < 0.05 \)). The embryo quality, implantation and pregnancy rates with IMSI were also significantly higher than those of their previous ICSI cycles (32% vs. 56.4%; 30.2% vs. 68.5%; 0.0% vs. 62.4%; \( P < 0.05 \)). Our conclusion is that the IMSI procedure improved embryo development and the clinical outcomes in the same infertile couples with male infertility and poor embryo development over their previous ICSI attempts and can be taken up as the treatment of choice in cases of severe male factor infertility.

Keywords: Intracytoplasmic morphologically selected sperm injection, intracytoplasmic sperm injection, oligoasthenoteratozoospermia, teratozoospermia

**INTRODUCTION**

Assisted reproduction got revolutionized with the inception of intracytoplasmic sperm injection (ICSI) in 1992 to overcome severe male infertility.\(^1\) In ICSI, spermatozoa selection is manually done by an embryologist based on only the sperms’ morphological characteristics visible at ×200 or ×400 magnification and motility and then injected after immobilization. Different types of structural defects in spermatozoa have been shown to be associated with reduced fertilization, and pregnancy rates, and with an increased risk of miscarriage.\(^2-4\) However, the low magnification used in routine ICSI can detect gross structural abnormalities in sperm.
defects such as abnormal sperm head size proportions and midpiece abnormalities but has limitations in detecting sperm organellar malformations, particularly vacuoles in the sperm head, although can be seen.\(^5\) Use of high magnification and resolution for the evaluation of the sperm nuclear morphology is highly recommended.\(^{\text{[6-9]}}\)

Motile sperm organellar morphology examination, is a new technique which evaluates the sperm morphology and motility in real-time without affecting it, made it possible to obtain a perfect sperm with a normal nucleus for ICSI.\(^{\text{[10,11]}}\) This process is a kind of modified ICSI known as intracytoplasmic morphologically selected sperm injection (IMSI). This method helps in the evaluation of detailed morphological features, such as abnormal sperm head proportions, midpiece abnormalities, and the presence of vacuoles in the sperm head. It allows the real-time selection of spermatozoa with morphologically normal nucleus having a symmetrical and oval shape with homogeneous chromatin mass and not more than one or two vacuoles but should be occupying <4% of the nuclear area.\(^{\text{[10]}}\)

The malefactor effect or the paternal influence happens after embryonic genome activation causing poor blastocyst development and in turn affecting the clinical outcome. It has been shown that IMSI could improve the clinical \textit{in vitro} fertilization (IVF) results in patients with previous ICSI failures. The pregnancy rate have been shown to increase in patients who had more than two failed ICSI attempts and also in female infertility patients with unexplained reasons for failure after ICSI with normal sperm. These results also showed that rate of miscarriage was almost halved as compared to that with general ICSI.\(^{\text{[12-15]}}\)

The aim of the present prospective study was to assess the clinical advantages of the IMSI procedure in the treatment of patients with severe malefactors, particularly oligoasthenoteratozoospermia (OAT) and teratozoospermia by comparing the outcomes of their previous failed ICSI cycles.

**Materials and Methods**

**Patients**

This study was performed in 57 couples who had undergone ICSI treatment in the previous cycle for male factor infertility, i.e., OAT/severe OAT (SOAT) or teratozoospermia. The study period was from May 2014 to June 2015 at our IVF center. The patients provided informed consent for IMSI and the study was approved by Institutional Ethical Committee.

**Semen evaluation and preparation**

Semen collection was done in the sperm wash medium to get good viable sperms to avoid the damage caused by the seminal fluid factors in cases of poor semen parameters.\(^{\text{[16]}}\) The World Health Organization (2010) criteria of OAT were followed: sperm concentration <10 × 10^6/mL, motility <40%, and strict morphology <4%. The semen was prepared by centrifugation on a density gradient (80% and 40% sage density gradient and sperm wash medium) and followed by simple wash or swim up depending on the sperm count.

**Ovarian stimulation and oocyte retrieval**

Ovarian stimulation in women was carried out with gonadotropin-releasing hormone antagonist protocol and recombinant follicle stimulating hormone (Gonal F, Merck Serono,). When more than two follicles reached 18 mm in diameter, a dose of 10,000 IU of human chorionic gonadotropin (hCG) (Ovitrelle, Merck Serono) was given and oocyte retrieval was performed 36 h after hCG trigger. Oocyte cumulus complexes retrieved were washed in GMOPS Plus medium and were incubated in GIVF Plus medium for 2–3 h before proceeding to denudation, i.e., removal of cumulus cells attached to eggs. Denudation was carried out in 80 IU hyaluronidase (Sage).

**Conventional intracytoplasmic sperm injection**

After removing the cumulus cell attached to the eggs in, the collected eggs were cultured in G-1 Plus medium (Vitrolife) for 15–30 min. The preparation of ICSI dish was done as shown in Figure 1. An inverted Olympus microscope (I × 71) equipped with a Narishige micromanipulator (Narishige, Tokyo, Japan) and heated stage was used for the ICSI procedure. Sperm selection was performed under ×200–×400 magnification and sperms with near normal morphology were injected into the oocyte.

![Figure 1: Preparation of intracytoplasmic sperm injection Dish: one 5 µl drop of polyvinylpyrrolidone for priming the injection pipette; one polyvinylpyrrolidone streak made with 5 µl. 5 µl GMOPS Plus drops for keeping the oocytes during intracytoplasmic sperm injection. The number of GMOPS drops depends on the number of oocytes to be injected. Everything covered under oil.](image-url)
Intracytoplasmic morphologically selected sperm injection

Oocyte preparation was same as done in ICSI procedure. IMSI dish preparation was done as shown in Figure 2. All motile spermatozoa were analyzed under high-magnification (×6600) microscopy (IX 71 Olympus) incorporating ×60 air (oil-free) objectives with differential interference contrast (DIC) illumination. Sperm selection was performed in elongated drops of polyvinylpyrrolidone (PVP) (Sage), and the preselected sperm of specific choice at ×6600 in the given sample were accumulated in small PVP drops which were covered with sterile ovoil (Vitrolife) in a glass bottom dish (Will Co.). Almost 3 times the number of sperms were selected at × 6600 and accumulated in the PVP drop for ICSI. ICSI was then carried out at ×400 in the same dish. Cassuto et al’s “HAVBIC” criteria were used for the selection of top quality sperms.[4] In this criteria, a detailed classification scoring scale was established ranging between 6 and 0 points. It added 2 points for the normalcy of head, 1 point for the normal base symmetry, and 3 points for complete absence of vacuoles. Therefore, Class 1 spermatozoa had a score of 4–6, Class 2 or medium quality spermatozoa had a score of 1–3, and Class 3, poor quality spermatozoa showed a score of 0.

In cases of very poor samples such as teratozoospermia or SOAT, the sperm selection under DIC optics was performed almost immediately after oocyte pickup thereby avoiding oocyte aging. All the cases had teratozoospermia factor and morphology was found to be 0%–2%. There were six couples who had 0% normal morphology and had vacuoles in almost all the sperms. In such cases, no Class I sperm was found. The effort was made to select at least Class II sperms with utmost care to avoid sperms having vacuoles in the nuclear region and 1–2 small vacuoles in the acrosomal region were allowed. This procedure sometimes takes as long as 3–4 h.

Embryo culture and transfer

Fertilization was assessed 17 h postinsemination by the appearance of two distinct pronuclei and two polar bodies. The zygotes were cultured in G1 Plus medium (Vitrolife) at 37°C in Minc incubator (6% CO₂, 5% O₂, and 89% N₂) for further 2 days. The embryos transfer was carried out on day 3.

Confirmation of pregnancy

Pregnancy confirmation was done by estimating the β-hCG concentration (>80 mIU/ml) in the blood 14 days after embryo transfer (ET) and doubling of the β-hCG after 48 h. The clinical pregnancy was established by the presence of gestational sac by ultrasonography performed at 7–8 weeks of pregnancy. The number of embryonic sacs seen in the uterus out of the number of embryos transferred during ET was taken as the implantation rate. The number of cycles having at least one live birth divided by the total number of cycles was taken as live birth rate. Fetal loss before 12 weeks’ gestation despite the presence of a gestational sac in the first-trimester ultrasound was taken as miscarriage.

Statistical analysis

Statistical analysis was performed with Graph Pad Prism Instat (GraphPad Software Inc, San Diego, USA). A P < 0.05 was accepted as statistically significant.

RESULTS

In this study, the outcome of 57 IMSI cycles was compared with their previous ICSI cycles’ outcomes. A comparison of patient-specific variables showed no difference with respect to retrieved oocytes, number of mature oocytes or the endometrial thickness during ET [Table 1]. Comparisons of the clinical outcomes between the two groups are presented in Table 2. Significant differences were observed between the two groups with regard to all the aspects, i.e., fertilization rate, high-quality embryo rate per embryo on day 3, clinical pregnancy rate and live birth rate. No difference was observed in the miscarriage rate.

DISCUSSION

It is a well-known fact that evaluation of sperm morphology in ICSI procedure is very crude due to low magnification (200×/400×) which also depends on the individual embryologists’ prerogative and thus has its
own limitations. This kind of evaluation becomes even more limiting in severe cases of malefactors such as OAT and teratozoospermia.

No correlation has been observed between high magnification sperm selection and early embryo development in terms of fertilization, or the rate of top quality embryos.[12,13] No significant difference was found in the fertilization rate when sperm selection was performed at a high magnification before ICSI compared with conventional ICSI in a sibling oocyte study.[3] Moreover, IMSI provided no significant difference in embryo quality up to day 3, but blastocyst formation and quality were correlated with the different grades of sperm. In our experience also, when outcome results of ICSI and IMSI were compared in normozoospermic cases, there was an increasing trend toward fertilization rate, good quality embryos and implantation rate when IMSI was performed (unpublished). However, when t-test was applied to these data, it was found to be statistically nonsignificant.

IMSI was first used in patients who had repeated implantation failures[10,11,13] or in patients who displayed high sperm DNA fragmentation.[15] Studies have shown that there is a strong relationship between the sperm head vacuoles and the aberrant chromatin packaging of sperm DNA.[8,9,12,15] It has been shown that the integrity of sperm DNA and the chromosome complement is compromised in sperms with vacuoles compared to the sperms lacking vacuoles.[9] Correlation has been observed between DNA fragmentation and sperms having vacuoles and morphological abnormalities.[17] DNA fragmentation was found to be higher in sperms with large or multiple vacuoles as compared to the sperms lacking vacuoles.[18]

It has been postulated that vacuoles might be associated with chromatin condensation failure. Studies have shown that embryo development was slow when abnormal sperm carrying vacuoles was used in ICSI. Aneuploidy of the embryo or DNA damage in sperm leads to delays in embryonic development.[19] Use of IMSI gave better embryo development and the clinical outcomes in the infertile couples with male infertility and poor embryo development in their previous ICSI attempts.[19] More viable blastocysts without chromosomal abnormalities are produced, and as a consequence, the chances of having genetically normal embryos are higher with the use of IMSI procedure.[20]

In our study, involving cases with severe male factor, IMSI increased the fertilization rate which in turn was associated with higher implantation and live birth rates selectively in these OAT patients. Our results also showed that IMSI improved fertilization rate (52% vs. 30%, P < 0.05), clinical pregnancy rate (68.5% vs. 30.2%), and implantation rate (53.7% vs. 17%, P < 0.05) compared to their previous ICSI cycles in cases of OAT patients as opposed to normozoospermic patients. Live birth rate was 62.4% in IMSI cycles of OAT patients. We saw miscarriages in only those patients where we got only class 3 sperms. These results are in accordance with the study by Setti et al., 2011,[21] where analysis was specifically conducted on the patients with OAT divided into two subgroups according to the criteria used for the diagnosis (WHO reference values of 2010 or WHO reference values of 1999). IMSI increased the fertilization rate in both subgroups and was associated with higher implantation and pregnancy rates selectively in the OAT2010 subgroup. These findings highlight the inadequacy of the new reference values for the detection of patients with a high degree of sperm dysfunction. Therefore, patients with OAT selected in accordance with the previous reference limits (which provide too high threshold values for sperm morphology, motility, and concentration) do not significantly benefit from IMSI treatment when compared to conventional ICSI. Similar results have been obtained by Kim et al., 2014.[22]

**CONCLUSION**

IMSI performed at high magnification such as ×6600 has the advantage of enabling the sorting of sperm
with vacuoles in their heads or other defects that cannot be observed during conventional ICSI (200×/400× magnification) and gives good outcome in cases of a severe male factor. Clinical IMSI is an effective technique for IVF-ET with positive clinical outcomes such as better implantation and pregnancy rates than conventional ICSI and can be used as a frontline treatment for OAT patients without having their first ICSI attempt as a failure.

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Conflicts of interest
There are no conflicts of interest.

REFERENCES
1. Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancy after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet 1992;340:17-8.
2. De Vos A, Van De Velde H, Joris H, Verheyen G, Devroey P, Van Steirteghem A, et al. Influence of individual sperm morphology on fertilization, embryo morphology, and pregnancy outcome of intracytoplasmic sperm injection. Fertil Steril 2003;79:42-8.
3. Vanderzwalmen P, Hiemer A, Rubner P, Bach M, Neyer A, Stecher A, et al. Blastocyst development after sperm selection at high magnification is associated with size and number of nuclear vacuoles. Reprod Biomed Online 2008;17:617-27.
4. Cassuto NG, Bouret D, Plouchart JM, Jellad S, Vanderzwalmen P, Balet R, et al. A new real-time morphology classification for human spermatozoa: A link for fertilization and improved embryo quality. Fertil Steril 2009;92:1616-25.
5. Berkovitz A, Eltes F, Soffer Y, Zabludovsky N, Beyth Y, Farhi J, et al. ART success and in vivo sperm cell selection depend on the ultramorphological status of spermatozoa. Andrologia 1999;31:1-8.
6. Tesarik J. Paternal effects on cell division in the human preimplantation embryo. Reprod Biomed Online 2005;10:370-5.
7. Berkovitz A, Eltes F, Lederman H, Peer S, Ellenbogen A, Feldberg B, et al. How to improve IVF-ICSI outcome by sperm selection. Reprod Biomed Online 2006;12:634-8.
8. Franco JG Jr., Baruffi RL, Maudi AL, Petersen CG, Oliveira JB, Vagnini L, et al. Significance of large nuclear vacuoles in human spermatozoa: Implications for ICSI. Reprod Biomed Online 2008;17:42-5.
9. Garolla A, Fortini D, Menegazzo M, De Toni L, Nicoletti V, Moretti A, et al. High-power microscopy for selecting spermatozoa for ICSI by physiological status. Reprod Biomed Online 2008;17:610-6.
10. Bartoov B, Berkovitz A, Eltes F, Kogosowski A, Menezes Y, Barak Y, et al. Real-time fine morphology of motile human sperm cells is associated with IVF-ICSI outcome. J Androl 2002;23:1-8.
11. Bartoov B, Berkovitz A, Eltes F, Kosogovsky A, Yagoda A, Lederman H, et al. Pregnancy rates are higher with intracytoplasmic morphologically selected sperm injection than with conventional intracytoplasmic injection. Fertil Steril 2003;80:1413-9.
12. Berkovitz A, Eltes F, Yaari S, Katz N, Barr I, Fishman A, et al. The morphological normalcy of the sperm nucleus and pregnancy rate of intracytoplasmic injection with morphologically selected sperm. Hum Reprod 2005;20:185-90.
13. Berkovitz A, Eltes F, Ellenbogen A, Peer S, Feldberg D, Bartoov B, et al. Does the presence of nuclear vacuoles in human sperm selected for ICSI affect pregnancy outcome? Hum Reprod 2006;21:1787-90.
14. Hazout A, Cohen Bacrie P, Junca A, Tesarik J. Outcomes of high magnification ICSI in cases with low, moderate and high degrees of sperm DNA fragmentation. Fertil Steril 2005;84:578-9.
15. Hazout A, Dumont-Hassan M, Junca AM, Cohen Bacrie P, Tesarik J. High-magnification ICSI overcomes paternal effect resistant to conventional ICSI. Reprod Biomed Online 2006;12:19-25.
16. Zollner U, Zollner KP, Dietl J, Steck T. Semen sample collection in medium enhances the implantation rate following ICSI in patients with severe oligoasthenoteratozoospermia. Hum Reprod 2001;16:1110-4.
17. Wilding M, Copolla G, di Matteo L, Palagiano A, Fusco E, Dale B, et al. Intracytoplasmic injection of morphologically selected spermatozoa (IMSI) improves outcome after assisted reproduction by deselecting physiologically poor quality spermatozoa. J Assist Reprod Genet 2011;28:253-62.
18. Hammoud I, Boitrelle F, Ferfouri F, Vialard F, Bergere M, Wainer B, et al. Selection of normal spermatozoa with a vacuole-free head (x6300) improves selection of spermatozoa with intact DNA in patients with high sperm DNA fragmentation rates. Andrologia 2013;45:163-70.
19. Knez K, Zorn B, Tomazevic T, Vrtačnik-Bokal E, Virant-Klun I. The IMSI procedure improves poor embryo development in the same infertile couples with poor semen quality: A comparative prospective randomized study. Reprod Biol Endocrinol 2011;9:123.
20. Luna D, Hilario R, Dueñas-Chacón J, Romero R, Zavala P, Villegas L, et al. The IMSI procedure improves laboratory and clinical outcomes without compromising the aneuploidy rate when compared to the classical ICSI procedure. Clin Med Insights Reprod Health 2015;9:29-37.
21. Setti AS, Figueira Rde C, Baraga DP, Iaconelli A Jr., Borges E Jr. Intracytoplasmic morphologically selected sperm injection benefits for patients with oligoasthenoteratozoospermia according to the 2010 World Health Organization reference values. Fertil Steril 2011;95:2711-4.
22. Kim HJ, Yoon HJ, Jung JM, Oh HS, Lee YJ, Lee WD, et al. Comparison between intracytoplasmic sperm injection and intracytoplasmic morphologically selected sperm injection in oligo-astheno-teratozoospermia patients. Clin Exp Reprod Med 2014;41:9-14.