MOLECULAR AND EXPERIMENTAL BIOLOGY IN MEDICINE

CAN WE MAKE INTRACYTOPLASMIC SPERM INJECTION (ICSI) SAFER AND MORE EFFECTIVE?

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Abstract: Intracytoplasmic sperm injection (ICSI) is an in vitro assisted fertilization procedure in which a single sperm is injected directly into the oocyte. Since its introduction in the early 90s, ICSI has revolutionized the treatment of male infertility, becoming a rapidly accepted technique worldwide. Today it is the most commonly used assisted reproductive procedure that has resulted in the birth of millions of babies. ICSI is a multistep, invasive micromanipulation technique that bypasses all the initial steps of natural fertilization providing the most reliable and consistent chances of oocyte fertilization, but at the same time it brings potential risks, primarily of oocyte degeneration, but also of transmission of chromosomal abnormalities that are more frequently associated with male infertility. Many steps are not fully understood and because there is no optimal ICSI protocol, we will discuss different approaches and current knowledge that can potentially improve ICSI outcome.

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INTRODUCTION

Infertility is defined as the inability of a couple to conceive naturally after one year of regular unprotected intercourse. It is estimated that more than 186 million people worldwide are affected. In Croatia, one in every six couples has difficulty conceiving. Infertility can affect one or both partners, and is commonly caused by ovulation disorder and blocked or damaged fallopian tubes in female infertility, and low sperm count, motility or even absence of sperm in male infertility. Some of the many factors that can contribute to infertility are age, weight, genetics, lifestyle and exposure to environmental toxins.[1]

Many couples with fertility problems turn to assisted reproductive technology (ART). Two most commonly used techniques to achieve fertilization *in vitro* are conventional *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI).[2] In conventional IVF, an adequate amount of prepared sperm is incubated with oocytes in a culture dish facilitating the natural process of fertilization that follows. For some couples, primarily those with female infertility, it is an efficient method with 50-70% of oocytes being fertilized, but almost half of the couples fail to achieve any fertilization at all. That is often attributed to male infertility and compromised sperm quality (e.g. low sperm number and motility) or the inability of spermatozoa to successfully fertilize an oocyte (e.g. sperm defects in acrosome reaction and interaction with the oolemma). Conventional IVF failure and severe male infertility have contributed to the rise and development of a new assisted fertilization technique named intracytoplasmic sperm injection (ICSI). Soon after ICSI was successfully performed on animal models, the first human pregnancy was achieved in 1992.[3] Currently, ICSI is the most commonly used assisted reproductive technology, used not only for severe male factor infertility, but also for

mild and borderline male factor, for women of advanced age, previous fertilization failure after conventional IVF and for unexplained infertility.

The ICSI technique is carried out on the heated stage of an inverted microscope with micromanipulation equipment. For ICSI to be performed, oocytes that are collected by follicle aspiration are further processed because they are surrounded by granulose cells that must be removed prior to sperm injection. Granulose cells are removed using enzymatic digestion with hyaluronidase followed by mechanical denudation through pipetting. After denudation, the oocyte nuclear maturation status can be determined. Only mature oocytes with an extruded first polar body (PBI) are used for further procedures. Sperm is prepared by one of the standard techniques for sperm preparation used in ART. Purified sperm is then added to a solution of polyvinylpyrrolidone (PVP) used to slow down the motile sperm. An embryologist selects the sperm based on its morphology and motility. Selected sperm is immobilized by crushing its tail with the injection micropipette and then aspirated tail first. The oocyte is fixed by a holding micropipette with PBI usually oriented at the 12 or 6 o'clock position (Figure 1a). The injection micropipette with the sperm at the tip is then positioned at 3 o'clock and pushed through the zona pellucida and the oolemma into the ooplasm (Figure 1b). The ooplasm is aspirated into the injection pipette until the breakage of oolemma is observed. The sperm is then injected back into the oocyte and the needle is slowly withdrawn from the oocyte (Figure 1c). Fertilization is checked 16-18 hours after injection.[4]

It is important to consider that ICSI is an invasive technique that bypasses all the initial steps of natural fertilization bringing potential risks, primarily of oocyte degeneration but also of transmission of chromosomal abnormalities associated with male infertility to the offspring. Many steps are not fully understood and because there is no optimal ICSI protocol we will discuss different approaches and current knowledge that can potentially improve ICSI outcome.

DISCUSSION

Oocyte maturity

In standard laboratory practice numerous oocytes are isolated, often at various stages of maturation. Mature oocytes (MII oocytes) are arrested in metaphase II and ready to be fertilized. Approximately 15% of retrieved oocytes are immature, and while some laboratories discard them, others use them to increase the number of embryos. Most studies with in vitro rescued immature oocytes that progressed to MII oocytes after prolonged short-term cultivation reported reduced fertilization rates and poor clinical outcomes.[6] Until recently, it was not known that a high proportion of embryos that derived from immature oocytes are genetically abnormal, with a higher aneuploidy rate for longer rescue cultivation time.[7] Accordingly, it is recommended to avoid usage of immature oocytes. In case no other embryos are available for transfer, the potential risk must be considered and discussed with patients.

Orientation of the first polar body

Oocytes are classified as mature by the presence of extruded PBI as a result of completed meiotic division I. Mature oocytes are arrested in metaphase II with the meiotic spindle (MS) assumed to be in close proximity to the PBI. Inserting the injection micropipette into or close to the MS can cause spindle disruption and lead to subsequent chromosome missegregation, aneuploidy or cell death. Therefore, to avoid MS disruption, injection is traditionally made at the 3 o'clock position with the PBI oriented at the 12 or 6 o'clock position, but optimal orientation is not clear yet. One retrospective study found a significantly higher quality embryo after 7 and 11 o'clock PBI orientation compared with the 6 and 12 o'clock, whereas the 9 o'clock orientation was the most deleterious in terms of fertilization rates.[8] Other recent study showed that the 11 o'clock position may be the preferred position of PBI during ICSI.[9] It is suggested that the optimal injection may be when the opening of the needle is facing away from the PBI and the cytoplasm far from the spindle is aspirated. Also, the fertilization rate seems to be affected by the distance between the injected sperm and the spindle. Additional research is needed, but the traditional PBI positioning may not be the optimal.

Timing of oocyte denudation and injection

For normal fertilization to occur, nuclear and cytoplasmic maturation are required. In natural cycles these processes are highly coordinated, but in stimulated cycles they can be asynchronous. Therefore, the presence of PBI as a sign of nuclear maturity does not have to imply cytoplasmic maturity and the presence of the required maternal mRNA, proteins, reorganization of the cytoplasmic organelles etc. If not fertilized on time, oocytes undergo a process of aging. The optimal time for oocyte denudation and injection is still unclear, mostly due to different strategies in short/long pre-incubation time and presence/absence of oocyte surrounding cumulus cells that also play an important role in oocyte maturation and aging. Patrat et al. showed that denudation should be performed at least 2h and up to 3h following oocyte retrieval, with the best results achieved around 1.5-2h for high fertilization and implantation rates without negatively affecting the percentage of MII oocytes.[10] Garor et al. also included the timing of human chorionic gonadotropin administration (hCG) and ovum retrieval in their study, showing that late ovum retrieval (>36h) is associated with higher fertilization and clinical

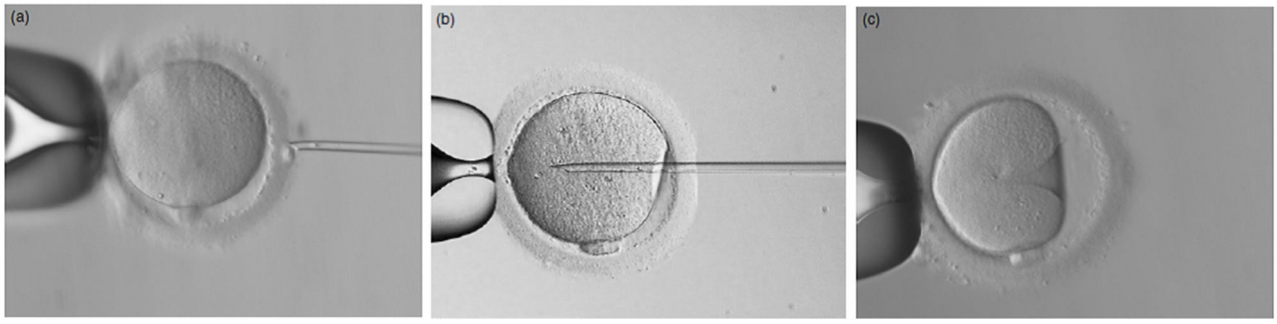

Figure 1. The ICSI technique. (a) Mature oocyte with PBI at the 6 o'clock position. Sperm at the tip of a micropipette at the 3 o'clock position. (b) Sperm injection into the cytoplasm. (c) Post-injection with typical track left. Adapted from "In Vitro Fertilization. Third Edition."[5]

pregnancy rates. [11] Some studies indicated that there is an optimal time frame for ICSI that is relatively narrow. Based on examination of MS over time, it is concluded that the optimal time is probably between 39-40.5 h post hCG administration.[12] Other study reported the highest level of implantation when ICSI is performed between 37-39 h after hCG.[13] Existing results are often contrary and not fully conclusive, but the majority of studies suggest pre-incubating the oocyte Ó2-3h with cumulus-corona cells that may have a positive effect and performing the injection straight after denudation.

Sperm immobilization

To achieve successful fertilization under normal circumstances in vivo, sperm must first undergo capacitation and the acrosome reaction. Capacitation involves modifications in the sperm plasma membrane, which lead to hyperactivation and permit the acrosome reaction. The acrosome reaction involves multiple fusions between the outer acrosome membrane and the overlying sperm plasma membrane, enabling the soluble contents of the acrosome to leak out, allowing sperm to penetrate and fuse with the oocyte membrane. ICSI bypasses all the events involved in physiological sperm penetration and fusion and requires no specific pretreatment of sperm other than immobilization.[14] It was shown that mechanical immobilization of sperm with the ICSI needle by compressing and rolling the sperm tails causes alterations in the acrosomal region including disruption of the plasma membrane, and disruption, vesiculation or even loss of the acrosome which facilitates fertilization.[15] Techniques used for sperm immobilization vary greatly and include conventional immobilization by compressing the tail of the spermatozoon against the bottom of a dish with a micro injection pipette until a clear bend is visible, and more aggressive techniques like permanently crimping the tail in the mid-piece region and cutting the tail at different places. Also, immobilization can be induced by lasers or piezo-pulses to sperm tails. Traditionally more aggressive mechanical sperm immobilization is suggested for higher ICSI fertilization rates. In contrast, one recent RCT compared triple touch with single touch sperm immobilization and showed that more aggressive techniques have no advantages for fertilization rates and resulted in a significantly lower number of good quality embryos on day 3.[16] There are no optimal immobilization techniques to date as the amount of damage, the number of strokes and the region of strokes remaining unknown.

Sperm selection

In the natural fertilization process there are anatomical and physiological barriers in the female reproductive tract that eliminate poor quality sperm, allowing only the most competent sperm to participate in fertilization. The next selection takes place on the level of oocyte ï sperm interaction, ensuring that only one mature, structurally normal sperm with intact chromatin can fertilize the oocyte. During ICSI, all natural barriers are bypassed and selection is performed by an embryologist based on their subjective evaluation of sperm motility and morphology without knowing anything about the sperm chromosome status. Since sperm contribute half of the genome to offspring, and male infertility is more frequently associated with chromosomal abnormalities, further improvements in sperm selection techniques are needed.

One of the more advanced strategies is hyaluronic acid (HA) binding sperm selection. HA is incorporated in the extracellular matrix that surrounds the oocyte and acts as a natural selector. Only mature spermatozoa that have extruded their receptors can bind to and digest HA, and reach and fertilize the oocyte. It is shown that sperm that binds to HA have a normal shape, minimal DNA fragmentation and low frequency of chromosomal aneuploidies.[17] Therefore, HA is used as a natural and safe alternative to synthetic plastic PVP in so called ñphysiologic ICSIò.[18] It is currently available in a viscous medium or attached to the bottom of a plastic culture dish. Some studies with HA-bound spermatozoa used for ICSI reported a higher fertilization rate and better embryo quality, while pregnancy rates were comparable or only moderately increased.[19, 20] According to meta-analysis, routine use of hyaluronic acid binding assays in all ICSI cycles is

not supported. Identification of patients that might benefit from this technique needs further study.[21]

Intracytoplasmic morphologically selected sperm injection (IMSI) is a technique that utilizes high-power microscopy enhanced with digital imaging to analyze the ultra-structures of the sperm. The light microscope used for IMSI has a greater magnifying power of x6000-12000, compared to x400 of conventional ICSI microscopy. It has been reported that IMSI is associated with significantly higher clinical pregnancy rates and a reduction in abortion rates.[22, 23] IMSI has its limitations because it is a time-consuming technique prolonging the exposure of sperm to heated stage that can cause sperm damage. Besides, its high cost makes it difficult for every laboratory to practice this technique. Currently, the only clinical indication of IMSI is elevated levels of DNA fragmentation, several miscarriages in the past and previous unsuccessful IVF/ICSI procedures.

Health outcomes of children born after ICSI

Since the introduction of ICSI there has been major concern about its safety related to the technique itself, but also related to intrinsic parental characteristics that can affect gamete quality and fertility. It is of great importance to determine and compare the incidence and nature of health risks between children born after ICSI, conventional IVF and naturally conceived children.

According to studies, there is a significantly higher rate of de-novo chromosomal anomalies, about a 3-fold increase of sex chromosome abnormalities and also an increase of structural autosomal abnormalities in ICSI versus naturally conceived children.[24] These elevated rates have been more associated with low quality of the sperm than with the ICSI procedure itself. Even infertile men with normal karyotypes have increased levels of aneuploid and diploid spermatozoa.[25] Before an IVF/ICSI programme, it is highly recommended to perform chromosomal analysis, followed by genetic counseling in case of abnormal karyotypes.

Some studies indicate an increased incidence of imprinting disorders (Beckwith - Wiedemann syndrome, Angelman's syndrome) in children conceived by IVF/ICSI as a result of imprinting defects.[26] Current evidence shows no direct effect of IVF/ICSI on imprinting disorder rate. Relative risk is estimated to be less than 1% and routine screening for imprinting disorders is not necessary.

Studies investigating congenital malformations reported increased risks of birth defects in children after ICSI or IVF compared with those conceived naturally. Most common defects are hypospadias, omphalocele and neural tube defects. Some studies reported a decrease in the number of birth defects over time. In recent meta-analysis, after taking into account the contribution of subfertility as a risk factor, the risk of malformations diminished substantially.[27] Further prospective studies are required to determine whether congenital malformations are related to techniques or to parental genetic defects.

Most pregnancies after ART result in normal healthy outcomes, but compared to naturally conceived pregnancies there is an increased risk of obstetric and neonatal complications (preeclampsia, preterm delivery, lower average birth weight). Several studies have demonstrated that children born after ICSI do not have poorer neonatal outcome than children born after IVF. Authors concluded that the pathology of the infertility had a greater impact on fetal growth than ART techniques.[28]

Further development of children conceived with ICSI and IVF is comparable in growth and physical health.[29]

Some studies indicate that children conceived with ICSI may be at an increased risk of autism and intellectual impairments. First studies of males conceived after ICSI showed high risks of reduced sperm concentration and total sperm count.[30] Further long- term studies are needed.

A direct link between ART and health-related outcomes in assisted conception children could not be established. When interpreting results, it must be taken into account that the ART population may be different from the general population. Further large-scale, multicentre, prospective, epidemiological studies are needed to elucidate the health outcomes in children born after ART.

CONCLUSION

ICSI has revolutionized the treatment of male infertility, requiring only a single viable sperm which can be isolated from ejaculate, but also from the epididymis and testis in the case of azoospermia. ICSI bypasses all the initial steps of natural fertilization and brings potential risks of genetic disease transmission to the offspring; therefore it should not be performed in the presence of normal sperm parameters (sperm count and motility). ICSI introduction has not been achieved by following the classic steps of looking for new technologies to be applied to humans; it implies so many critical phases potentially affecting epigenetic, genetic or chromosomal errors that follow-up studies in the human embryo and pediatric follow-ups are needed for each of them.

This article focused on potential improvements in daily ICSI practice. Less common cases like absolute immotile sperm, oocyte degeneration and fertilization failure are not discussed. There are rare cases (1-3%) of complete fertilization failure even after ICSI when additional physical, mechanical or chemical treatments are applied.

For the success of the ICSI, high technical skills are required, but attention must also be paid to biological details of each step of the procedure. As described, many steps of the ICSI procedure are still debatable. Even many years after its introduction, the improvements and technical developments in ICSI still

lack sufficient well-designed studies. There are various options for performing ICSI and embryologists should be encouraged to share their experiences with different ICSI approaches used. There is a need for sufficiently powered RCTs to evaluate the safety and clinical benefit of latest technological advances, indicating the way to perform ICSI in a safer and more effective way.

REFERENCES

1. Carell D, Peterson CM. Reproductive endocrinology and infertility. Springer, New York, NY; 2010.
2. Gardner DK, Rizk BR, Falcone T. Human assisted reproduction technology. Cambridge, UK; 2011.
3. Palermo G, Joris H, Devroey P, van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet. 1992;340:17-18.
4. Neri QV, Monahan D, Palermo GD, Rosenwaks Z. Intracytoplasmic Sperm Injection: Technical Aspects. United Kingdom: Informa UK Ltd; 2012.
5. Kay E, Brian D. In Vitro Fertilization. Third Edition. Cambridge, UK/New York: Cambridge University Press, 2011.
6. Shin SB, Cho JW, Lee SH, Yang KM, Lim CK, Lee HS. Fertilization and pregnancy potential of immature oocytes from stimulated intracytoplasmic sperm injection cycles. Clin Exp Reprod Med. 2013;40:7-11.
7. Strassburger D, Goldstein A, Friedler S, Raziel A, Kasterstein E, Mashevich M, Schachter M, Ron-El R, Reish O. The cytogenetic constitution of embryos derived from immature (metaphase I) oocytes obtained after ovarian hyperstimulation. Fertil Steril. 2010;94:971-978.
8. Blake M, Garrisi J, Tomkin G, Cohen J. Sperm deposition site during ICSI affects fertilization and development. Fertil Steril. 2000;73:31-37.
9. Anifandis G, Dafopoulos K, Messini CI, Chalvatzas N, Messinis IE. Effect of the position of the polar body during ICSI on fertilization rate and embryo development. Reprod Sci. 2010;17:849-853.
10. Patrat C, Kaffel A, Delaroche L, Guibert J, Jouannet P, Epelboin S, De Ziegler D, Wolf JP, Fauque P. Optimal timing for oocyte denudation and intracytoplasmic sperm injection. Obstet Gynecol Int. 2012:403531, 2012.
11. Garor R, Shufaro Y, Kotler N, Shefer D, Krasilnikov N, Ben-Haroush A, Pinkas H, Fisch B, Sapir O. Prolonging oocyte in vitro culture and handling time does not compensate for a shorter interval from human chorionic gonadotropin administration to oocyte pickup. Fertil Steril. 2015;103:72-75.
12. Kilani S, Cooke S, Chapman M. Time course of meiotic spindle development in MII oocytes. Zygote. 2011;19:55-62.
13. Dozortsev D, Nagy P, Abdelmassih S, Oliveira F, Brasil A, Abdelmassih V, Diamond M, Abdelmassih R. The optimal time for intracytoplasmic sperm injection in the human is from 37 to 41 hours after administration of human chorionic gonadotropin. Fertil Steril. 2004;82:1492-1496.
14. Palermo GD, Cohen J, Alikani M, Adler A, Rosenwaks Z. Intracytoplasmic sperm injection: a novel treatment for all forms of male factor infertility. Fertil. Steril. 1995;63:1231-1240.
15. Takeuchi T, Colombero LT, Neri QV, Rosenwaks Z, Palermo GD. Does ICSI require acrosomal disruption? An ultrastructural study. Hum Reprod. 2004;19:114-117.
16. Velaers A, Paternot G, Debrock S, D'Hooghe T, Spiessens C. Triple touch sperm immobilization vs. single touch sperm immobilization in ICSI - a randomised trial. Reprod Biol Endocrinol. 2012;29;10:65. doi: 10.1186/1477-7827-10-65.
17. Huszar G, Ozenci CC, Cayli S, Zavaczki Z, Hansch E, Vigue L. Hyaluronic acid binding by human sperm indicates cellular maturity, viability, and unreacted acrosomal status.Fertil Steril. 2003;79:1616-1624.
18. Parmegiani L, Cognigni GE, Bernardi S, Troilo E, Ciampaglia W, Filicori M. "Physiologic ICSI": hyaluronic acid (HA) favors selection of spermatozoa without DNA fragmentation and with normal nucleus, resulting in improvement of embryo quality. Fertil Steril. 2010;93:598-604.
19. Nasr-Esfahani MH, Razavi S, Vahdati AA, Fathi F, Tavalaee M. Evaluation of sperm selection procedure based on hyaluronic acid binding ability on ICSI outcome. J Assist Reprod Genet. 2008;25:197-203.
20. Parmegiani L, Cognigni GE, Ciampaglia W, Pocognoli P, Marchi F, Filicori M. Efficiency of hyaluronic acid (HA) sperm selection. J Assist Reprod Genet. 2010;27:13-16.
21. Beck-Fruchter R, Shalev E, Weiss A. Clinical benefit using sperm hyaluronic acid binding technique in ICSI cycles: a systematic review and meta-analysis. Reprod Biomed Online. 2016;32(3):286-298.
22. Souza Setti A, Ferreira RC, Paes de Almeida Ferreira Braga D, de Cássia Sávio Figueira R, Iaconelli AJr, Borges EJr. Intracytoplasmic sperm injection outcome versus intracytoplasmic morphologically selected sperm injection outcome: a meta-analysis. Reprod Biomed Online. 2010;21:450-455.
23. Teixeira DM, Barbosa MA, Ferriani RA, Navarro PA, Raine-Fenning N, Nastri CO, Martins WP. Regular (ICSI) versus ultra-high magnification (IMSI) sperm selection for assisted reproduction. Cochrane Database Syst Rev. 2013;7:CD010167. doi:10.1002/14651858.CD010167.
24. Bonduelle M, Van Assche E, Joris H, Keymolen K, Devroey P, Van Steirteghem A, Liebaers I. Prenatal testing in ICSI pregnancies: incidence of chromosomal anomalies in 1586 karyotypes and relation to sperm parameters. Hum Reprod. 2002;17:2600-2614.
25. Calogero AE, De Palma A, Grazioso C, Barone N, Romeo R, Rappazzo G, D'Agata R. Aneuploidy rate in spermatozoa of selected men with abnormal semen parameters. Hum Reprod. 2001;16:1172-1179.
26. Manipalviratn S, DeCherney A, Segars J. Imprinting disorders and assisted reproductive technology. Fertil Steril. 2009;91:305-315.
27. Rimm AA, Katayama AC, Katayama KP. A meta-analysis of the impact of IVF and ICSI on major malformations after adjusting for the effect of subfertility. J Assist Reprod Genet. 2011;28:699-705.
28. Cooper AR, O'Neill KE, Allsworth JE, Jungheim ES, Odibo AO, Gray DL, Ratts VS, Moley KH, Odem RR. Smaller fetal size in singletons after infertility therapies: The influence of technology and the underlying infertility. Fertil Steril. 2011;96:1100-1106.
29. Belva F, Henriet S, Liebaers I, Van Steirteghem A, Celestin-Westreich S, Bonduelle M. Medical outcome of 8-year-old singleton ICSI children (born ?32 weeks' gestation) and a spontaneously conceived comparison group. Hum Reprod. 2007;22:506-515.
30. Belva F, Bonduelle M, Roelants M, Michielsen D, Van Steirteghem A, Verheyen G, Tournaye H. Semen quality of young adult ICSI offspring: the first results. Hum Reprod. 2016;31:2811-2820.