

Intracytoplasmic sperm injection: method and application in veterinary science

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Introduction

Intracytoplasmic sperm injection is the newest and the most successful micromanipulation technique for treating male factor infertility. It entails the mechanical insertion of a chosen spermatozoon directly in to the cytoplasm of an oocyte. The technique was used in veterinary science for the purpose of clarifying the different steps of fertilization by investigating the fusion and penetration of animal gametes. First mammalian egg injection procedure was reported by Lin (1966). Later Uehra and Yanagimachi (1976) described the microinjection of human and golden hamster spermatozoa into hamster eggs. Hosoi *et al* (1998) obtained live offspring after transfer of micro fertilized rabbit eggs into the oviduct of pseudo pregnant female rabbits. Based on the successful fertilization of normal animal gametes using ICSI, the procedure was experimentally applied to human gametes.

In the veterinary field micromanipulation of murine and domestic animal species has been used for the past two decades both as an experimental tool and in the commercial field. The focus of the commercial field has largely been on developing and refining the cloning technology and production of transgenic animals. The use of micromanipulation technique to enhance fertilization in domestic animals has not been developed because fertilization is rarely a problem. In humans, however one in six couples has fertility problems and male factor components are responsible for one third of infertile couple.

Requirements

The manufacture and use of precision glass instruments is critical for the success with ICSI. Specialized instruments like holding pipette, Injection pipette of borosilicate plastic and a micro analyzer are require for the technique. The pipettes can be straight for almost horizontal side entry into drops of medium on depression well slides, or they can have one or more bend in them so that they can

be brought in from above petridish. Z shaped pipette scan also be used which enter a shallow angle relative to horizontal, but still clear the lip of a glass petridish. lid. The important point is that the tips of the holding and injection pipettes must be horizontal so that minimum amount of pressure need to be exerted on the oocyte during injection.

Holding pipettes of outside diameter (OD) 60-120 mm are found suitable. The injection pipette should be sharp and should have an OD at the tip of 6-8 mm. Providing that this criteria are met the bevel angle can vary from 28-30 (Hamberger *et al.*, 1995; Palermo *et al.* 1992) to about 45-50 (Van Steirteghem *et al.* 1993), and the pipettes can either be fitted with a sharp spike or they can be used without a spike.

Technique

The general set up includes inverted microscope equipped with differential interference contrast (DIC) optics a Perspex environmental chamber, heater and a CO₂ controller. The chamber should be maintained at 37° C and a constant humidified atmosphere of 5% CO₂ in air. Prior to and after microinjection, oocytes are handled in converted premature infant incubators which are similarly gassed with 5% CO₂ in air, humidified and heated to 37.0 il borosilicate glass capillaries.

Prepartation of oocytes and sperm for injection

Oocyte preparation: Ovaries should be placed in saline 0.89 M NaCl containing 40 ig/ml gentamycin sulphate) within 20-30 minutes of slaughter and should be transported in thermal containers at 25 - 30° C. Aspirate the follicular fluid from follicles of less than 3 cm diameter through an 18 gauge ´ 1 ½" needle and collect into sterile tubes which are maintained in water bath 38 °C. During aspiration a scraping motion within the follicle dislodge the oocyte from the follicular wall. The collected oocytes are then examined under stereomicroscope equipped with a heating stage set. Oocyte classification and

selection are to be done as per the same procedure followed in IVF.

Semen preparation: Frozen or fresh semen samples can be used for ICSI. Sperm cells for IVF/ICSI are to be prepared using the swim up procedure in Tyrodes lactate medium modified for sperm treatment supplemented with 0.4% human serum albumin and antibiotics. Then centrifuge them. Remove the supernatant as much as possible leaving only pellet and resuspend it in about 100 ml of fresh medium.

Immobilization of spermatozoon: Examine the sperm in PVP drop at 200x magnification and select morphologically acceptable sperm, which are swimming slowly along the base of the petridish lid. After selecting the sperm the injection pipette is raised and positioned over the mid-piece of the sperm tail and while the spermatozoon is still moving slowly, the injection pipette should bring down onto the mid-piece. If pipette is in contact with the tail and the tail is in the bottom of the petridish, the spermatozoon will stop moving forward. The injection pipette is then drawn sharply across the tail, which immediately immobilizes the spermatozoa. If it retains motility the procedure should be repeated until complete immobilization occurs. Then aspirate the immobilized sperm into the injection pipette tail first and position close to pipette opening. Transfer the injection pipette to a 5 μ l drop containing oocyte.

Intracytoplasmic sperm injection

The oocyte is held flat against the holding pipette using gentle suction. The oocyte should be in contact with the bottom of the dish to provide an extra axis of support during injection. The injection pipette should be positioned such that the oocyte plasma membrane and the tip of the injection pipette are both in sharp focus. This will ensure injection through middle of the oocyte. The polar body is oriented at 12 O Clock and the level of injection pipette faces 6 O Clock. Then push the injection pipette steadily against the zona pellucida until it pierces the ZP and is situated well within the oocyte, surrounded by a shallow furrow. Sometime the oocyte plasma membrane doesn't rupture by this process. To ensure the rupture, the oocyte cytoplasm is aspirated back into the injection pipette, which indicates whether the membrane is ruptured. Push the oocyte cytoplasm back into the oocyte along with the spermatozoon and a minimal volume of PVP solution and withdraw the injection pipette leaving behind the spermatozoon in the oocyte. The disturbance on the oocyte at the

time of the injection should be minimal. A large amount of Cytoplasmic disturbance indicates that the diameter of the injection pipette is too large or due to hurried replacement of oocyte cytoplasm. The actual injection of spermatozoa in to the oocyte usually takes 5s.

The oocyte plasma membrane slowly assumes its original conformation. Once all the oocytes in the petridish have been injected each oocyte should be checked to ensure the spermatozoon is indeed located inside the cytoplasm. Then the oocytes are to be washed through a change of HTF medium and then culture overnight in 10 ml drops of HTF under mineral oil. Examine the oocyte under 200x approximately 17 hour post injection for evidence of fertilization (2 pronuclei, 2 polar bodies) and then culture the zygotes further for 48 hours in fresh drops of HTF under mineral oil.

Problems encountered during Injection

1. Failed injection: If the oocyte plasma membrane is not ruptured the sperm will not be deposited in the cytoplasm when the needle is withdrawn even though it may look as though it is. During the next 5 min the sperm will be drawn back with the membrane as oocyte resumes its original shape and it will eventually be ejected into the PVS.

2. Oocyte lysis during injection: An injection pipette with a long sharp spike can cause immediate rupture of the oolemma and the lysis of oocyte. If plasma membrane is at the equator of the oocyte and the injection pipette are both in sharp focus during injection then the minimum area of plasma membrane will be pulled back into the injection pipette as the membrane is broken.

Scope of ICSI in Veterinary Science

1. Availability of material: Ovaries in animals are primarily obtained from the abattoir where they are essentially waste material, hence the oocyte recovery is economical and their use is ethical.

2. Research: Virtually nothing is known about what happens to the spermatozoon and the oocyte within few hours following sperm injection. Research with human material is necessarily limited, so the use of domestic species should rapidly promote research in to the fundamental mechanism of ICSI.

3. Training: Technical competency with ICSI takes time and experience. ICSI with domestic species is technically similar to human and thus its suitable as a training tool.

4. The potentiality of ICSI in exotic species for

conservation is yet to be exploited: There are two areas where ICSI is already having a direct impact. One is direct propagation. For example, a premium bull might have excellent characteristics with the exception of poor quality of semen. In extreme cases the semen can be unsuitable for artificial insemination (AI) or even IVF, but ICSI can be successfully used to generate embryos. In such cases, an embryo may be worth much, that the initial investment can be commercially viable.

Another use of ICSI in domestic species is in the testing of X and Y sperm separation technique. The ability to produce offspring of a defined sex has obvious implication in Animal husbandry. To date the only established and economically viable method of separating X-chromosome and Y-chromosome bearing spermatozoa is flow cytometric method. In animals especially ovine, sperms are particularly susceptible to the stress of flow cytometry and ICSI can be used to circumvent this limitation.

Ovine ICSI

In a study conducted by Catt et al (1995) out of 50 oocytes where ICSI was conducted there was penetration rate of 70%, two third of which (34) showed activation of the oocyte. Sham injection of ovine oocyte caused activation of 40% giving rise to random polar body abstractions and pronuclear formation. Ovine oocytes have a tendency to activate after injection procedure regardless of the presence or absence of sperm.

Bovine ICSI

Keefetr et al (1990) demonstrated that bovine oocytes could be successfully injected and subsequently undergo early development..The study revealed that the success of bovine ICSI also depends on proper activation of the oocyte [Lacham-Kaplan O1994]. Mechanical stimulation by the injection pipette alone can only occasionally bring this about in cattle; in more than 95% of cases this stimulus is insufficient (Keefer, 1990).

Approximately 70% of the bovine oocytes matured *in vitro* had a visible first polar body and uniformly dense ooplasm, which were considered to make them suitable for ICSI. Out of a total of 761 injected oocytes, 57 (8%) were mechanically damaged by the micromanipulation procedure and

degenerated within 3-24 h. A further 32 (4%) expelled the injected spermatozoon into the perivitelline space.

Equine ICSI

In equines ICSI is especially important because of the low fertilization rate obtained with IVF and for commercial reasons such as the high cost of valuable stallions. In the comparisons between standard IVF and ICSI procedures reported the oocytes and sperm preparation technique used for IVF were inevitably different from the ones used for ICSI based on the fact that in equines IVF has yielded very poor results to date.

Conclusion

ICSI is powerful micromanipulation tool for achieving fertilization and development independent of semen characteristics, collection methods and storage. Sub fertile spermatozoa, epididymal sperm ,testicular sperm and even only sperm head can be successfully used for ICSI. There must be a good routine IVF programme in the lab before introducing ICSI All aspects of ICSI should be carried with rigid attention and top cleanliness. The factors which reduce sperm and oocyte survival considered vigilantly and should be avoided to the maximum extend. High quality glass wares are also important for the success of ICSI. This technique has wide scope in the Veterinary field from experiment to conservation level.

References

1. Catt JW, Rhodes SL. (1995): *Reprod Fertil Dev* . **7**:161-167
2. Hamberger, L., Sjoogren, A., Lundin, K., Soderlund, B., Nilsson, L., Bergh., (1995). *Reprod. Fertil. Dev.* **7**, 263-8.
3. Hosoi Y, Iritani A *Mol Reprod Dev* 1993; **36**:282-284
4. Keefer CL, Younis AI, Brackett BG. (1990); *Mol Reprod Dev* **25**:281-285.
5. Lacham-Kaplan O, Trounson A. In: Tesarik J (ed.), *Male Factor in Human Infertility*. Rome, Italy: Ares-Serono Symposia Publications; 1994:287-304.
6. Uehara, T., and Yanagimachi, R (1976). *Biol. Reprod.* **15**. 467-70
7. VanSteirteghem AC, Liu J, Joris H, Nagy Z, Janssenswillen C, Tournaye H, Derde MP, Van Assche E and Devroey P (1993): *Hum Reprod* **8**:1055-1060.

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