

# Factors influencing the intracytoplasmic sperm injection (ICSI) efficacy in goat and sheep

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Journal of Livestock Science (ISSN online 2277-6214) 15: 102-110  
Received on 29/12/23; Accepted on 18/3/24; Published on 25/3/24  
doi. 10.33259/JLivestSci.2024.102-110

## Abstract

Intracytoplasmic sperm injection is performed by microscopically injecting a sperm cell into an egg cell's ooplasm effectively bypassing usual steps taken in natural reproduction. ICSI in goats and sheep provides opportunities for better genetic selection to improve small ruminant production potentially. It has also shown promise for wildlife conservation by preserving spermatozoa from endangered species. Different factors that affect the success rate of ICSI in sheep and goats have been categorized into external and internal factors. Reproductive seasonality suggested that oocyte harvest can be done year-round but efficient oocyte development occurs during breeding (autumn) season. Superovulation is induced in goats and sheep via administration of follicle stimulating hormone (FSH), luteinizing hormone (LH), equine chorionic gonadotropin (eCG), and pregnant mare serum gonadotropin (PMSG). Toxins accumulating around the ICSI-produced embryo such as free radicals and ammonium ions has led to embryonic arrest. Female animals cannot be juvenile nor geriatric to be able to produce viable oocytes for ICSI procedures. Donor animals with proper nutrition and ideal weight produced high quality oocytes. Boer goats were found to be polyestrous and sensitive to hormonal treatment making them the ideal breed for ICSI. Proper sourcing and management of the oocyte has greatly affected success rates of ICSI. Fresh sperm from male donor animals produced more blastocysts than frozen-thawed sperm. Frozen-thawed sperm however has shown excellent membrane integrity important for ICSI-embryo development. Immobilizing sperm by breaking the tail before ICSI has increased blastocyst yield. ICSI success rates are affected by external and internal factors responsible for sex gamete quality.

**Keywords:** assisted reproductive technology (ART), goats, intracytoplasmic sperm injection (ICSI), sheep, small ruminants

## Background

In livestock, semen samples are valuable genetic material sources and open endless possibilities in livestock management and breeding practices. It is therefore important not to waste these resources and use them to their full potential. In livestock, death of a genetically superior animal or the animal's inability to mate is detrimental to the industry (Parmar *et al.*, 2013). Genetically superior but biologically inferior males are not only unsuccessful in natural mating but are also less successful in in vitro fertilization (IVF) and most artificial insemination (AI) procedures as well. However, In ruminants, future improvements are required for ICSI to be more widely used (Briski and Salamone, 2022).

Intracytoplasmic Sperm Injection (ICSI) is a type of Assisted Reproductive Technology (ART) where a microscopic needle injects the sperm cell or sperm head / nucleus directly into the egg cell's ooplasm. It is a type of micromanipulation that circumvents all the usual steps in a typical mating procedure and this technique represents an alternative to improve the reproductive capacity of the male (Quispe *et al.*, 2024). ICSI can utilize sperm not only from semen samples but also from epididymal and testicular samples as well (Lopez-Saucedo *et al.*, 2012). The steps encompassed in an ICSI procedure include sperm immobilization, mechanical insertion of the sperm into the oolemma, and oocyte activation (Neri *et al.*, 2014). It also shows potential in sex-sorted spermatozoa that is crucial in goat milk production (Parmar *et al.*, 2013). Goat sperm is also being used as DNA carriers in livestock. sperm quality traits have low heritability values which make traditional genetic selection little efficient to its improvement (Serrano *et al.*, 2021). ICSI in wildlife is playing a major role in the conservation of endangered species and other usual method that was used somatic cell nuclear transfer (SCNT) (Mastromonaco and Songsasen, 2020). ICSI not only conserves entire species of animals but also preserves superior genetics in livestock (Khan *et al.*, 2016).

ICSI births in sheep and goat are limited to only a few although success rates are dependent on both external (environmental) and internal (sex gametes, breed, and age) factors (Lopez-Saucedo *et al.*, 2012). The scope of this review article will be factors affecting the success rate of ICSI procedure in goats and sheep.

## Discussion

### History of Intracytoplasmic Sperm Injection (ICSI)

The successful birth of Louise Brown spearheaded by Steptoe and Edwards opened the door for other countries worldwide to utilize ART to tackle infertility. Lopata *et al.* (1980) produced the first in vitro fertilization (IVF) birth in Australia and then four years later in 1984, the first IVF birth in the United States was successfully performed by Jones *et al.* (1984). By 1988, the first few developments included creating a hole or a crack in the zona pellucida itself to facilitate fertilization by the sperm (O'Neill *et al.*, 2018). The history of the development of ICSI in animals not only has been reported in livestock such as rabbits, cattle, dogs, cats, pigs, goats, sheeps but also wild animal such as monkeys, lions, gorillas and wallabies (Lopez-Saucedo *et al.*, 2012)

By the end of the 1980s, subzonal injection (SUZI) was developed to avoid going through the zona pellucida itself. This method takes the spermatozoon and places it directly in the perivitelline space, thus bypassing the zona pellucida (O'Neill *et al.*, 2018). When Gianpiero Palermo accidentally pokes a hole in the egg cell membrane and into the ooplasm while performing SUZI experiments, he inadvertently lets a spermatozoon enter the egg's cytoplasm thus discovering a new ART technique called Intracytoplasmic Sperm Injection (ICSI). ICSI is injecting a single acrosome and sperm membrane intact spermatozoon, sourced from the testicles or epididymis, directly into the ooplasm where it results in a fertilized metaphase II (MII) oocyte (Palermo *et al.*, 2015; Dyer *et al.*, 2016). This implies that the ICSI technique bypasses the typical fertilization process such as plasma membrane and cortical region interactions. It has been stated in a research conducted by Lemseffer *et al.* (2022) that oocyte quality has reduced disparity caused by sperm penetration. Moreover, high quality MII oocytes injected via ICSI have better chances at fertilization and embryo development.

Although Steptoe and Edwards were considered the pioneers in IVF and ICSI, it was Lillie (1914) and Hiramoto (1962) who were the first to dabble in ART that led to ICSI development in humans. Specifically, Hiramoto microinjected live spermatozoa in echinoderms *Psammechinus microtuberculatus*, *Paracentrotus lividus*, and *Clypeaster japonicus* in their research. In amphibians, Graham (1966) and Brun (1974) experimented on the *Xenopus laevis* commonly known as African clawed frog. Graham's experiment included inducing ovulation via injection of gonadotropic hormones and microinjection of sperm in the egg cells where the vegetal pole of the frog eggs. Uehara and Yanagimachi in 1976 performed mammalian fertilization via ICSI in hamsters. These experiments, although crude, gave a solid foundation for ICSI's refinement as an ART.

Kimura and Yanagimachi (1995) performed ICSI experiments on mice and discovered that a piezo-driven micropipette was a better tool rather than the traditional pipette. The first live births via ICSI were reported in rabbits (Hosoi *et al.*, 1988) and in cows (Goto *et al.*, 1990). Wang *et al.* (2003) reported one live kid was successfully born from four goat recipients who received six ICSI-derived two-cell embryos.

Widjiati *et al.* (2020) conducted an experiment on Kacang goats to differentiate conventional IVF from ICSI and discovered that cleavage process was significantly higher in ICSI embryos partly due to the latter's ability to resist fertilization failure. Another study revealed that the rate of live goat embryos born via ICSI was significantly higher than conventional IVF (Speyer *et al.*, 2019). The same results were obtained by Pereyra-Bonnet *et al.* (2011) in sheep where ICSI was the only method efficient enough to produce exogenous gene-expressing embryos.

### **Benefits of ICSI in research and production**

The different indications of ICSI in livestock species are mostly production focused. ICSI is the recommended ART especially when conventional methods such as IVF fail (Briski and Salamone, 2022). It is also indicated in cases where there are limited sources of sperm available. ICSI grants scientists the ability to obtain epididymal sperm even from deceased animals, especially from genetically superior males. Hammer *et al.* (1998) have also discussed the scientific importance and economic promise of transgenic livestock with higher-grade genetics. Their research first tackled transgenesis in mice and since that gave promising results, furthered their research to larger animals like rabbits, pigs, and sheep.

In the grand scheme of things, ICSI's applications have a much wider impact in the field of conservation. ICSI research has also provided options for wildlife species at the brink of extinction where sperm of these endangered animals could be preserved and microinjected in a donor oocyte. Livestock species have also been utilized in experiments instead of the valuable endangered species. Overall, ICSI sparked interest for possible better production parameters and an option if conventional IVF fails (Parmar *et al.*, 2013).

## **Factors affecting success of ICSI in goat and sheep**

### **Season**

Multiple studies (Pierson *et al.* 2004; Mara *et al.*, 2014; Souza-Fabjan *et al.*, 2021) have garnered evidence that laparoscopic oocyte pick-up (LOPU) was performed multiple times at different intervals in goats, all in different seasons, and influenced the oocyte's development. Specifically, Pierson *et al.* (2004) investigated the four different seasons; Autumn (September-December), Winter (January-March), Spring (April-June), Summer (July-August); and their possible effect on LOPU-derived oocytes. The recovery rate of the pooled oocytes from each of the seasons were 92%, 90%, 88%, and 90% respectively. The results of the experiments revealed that sufficient oocytes were obtained regardless of the seasons they were harvested in as long as proper care and management of the donor animals have been taken into consideration. However, the study was limited to the number of oocytes obtained rather than the quality and competence of the oocytes to advance to the next stages of fertilization.

Souza-Fabjan *et al.* (2021) suggested that perhaps reproductive seasonality in adult goats can improve the retrieved oocyte's ability to forge ahead in the fertilization process. The study mentioned that the caprine species is a seasonal breeder, specifically autumnal breeders, thus hormones and systemic processes, both important in reproduction, can be influenced by seasonal change. The study reported that cleavage rates were 72% in autumn, 71% in summer, 66% in winter and 51% in spring. Additionally, cumulus-oocyte complexes produced higher blastocyst rates in autumn (52%) compared to the remaining seasons (approximately 40%). These findings can be corroborated by Mara *et al.* (2014) where Sarda sheep have shown to have increased blastocyst rate during the breeding season. We can therefore deduce that although oocytes can be harvested throughout the year, the breeding season (autumn) can significantly improve oocyte developmental efficiency by increasing cleavage and blastocyst rates (Souza-Fabjan, 2021).

### **Medications given**

Superovulation gives ICSI procedures better chances of increasing fertilization and embryo development. Two frequently administered gonadotropin treatments are Follicle Stimulating Hormone (FSH) and equine chorionic gonadotropin (eCG) (Lonergan and Boland, 2011). However, FSH needed veterinary guidance more since it requires more doses while eCG needed only one dose or injection therefore also making the eCG cheaper in cost compared to FSH (Shiple et al., 2009). FSH with suitable levels of Luteinizing Hormone (LH), although more expensive, has proven to be more effective than eCG in inducing superovulation in goats (Goel and Agrawal, 2005; Lehloenya, 2013). This just denotes that eCG acts less like LH, and behaves more like FSH instead. After all, exorbitant amounts of the LH causes inactivity of the LH receptors that then causes premature follicles to luteinize and ovulate prematurely and may cause significant superovulatory response reduction in the ovaries (Lehloenya, 2013).

For the past years, goats have now developed eCG antibodies in their immune system as a result of continuous exogenous hormonal treatment. Donor goats have displayed estrus postponement and decrease in fertility due to their immune response (Baldassarre and Karatzas, 2004). Inciting superovulation in goats by utilization of a hormone called Pregnant Mare Serum Gonadotropin (PMSG) without using eCG has shown promising results in improving genetic selection and increasing goat population when done hand in hand with ICSI (Holtz, 2005). Nevertheless, further investigation and refinement on proper dosage and drug intervals on PMSG has to be done in order to maximize the drug's benefits.

Both FSH and LH were found to be successful in inducing superovulation in sheep. In under 24 hours, the oocytes obtained from sheep and cattle supplemented with the hormones underwent maturation (Çizmeçi and Güler, 2018). Pereyra-Bonnet et al. (2011) reported that superovulation treatments for *in vivo* embryo production in sheep prior to performing ICSI. The medications they chose were FSH and eCG, and had similar indications and purpose in goats.

### **Environmental conditions surrounding the embryo**

The environment surrounding the goat or sheep embryo that underwent an ICSI procedure was found to influence its embryonic development. Approximately 80% of sheep embryos (Gomez et al., 1997) while 100% of goat embryos (Jimenez-Macedo et al., 2005) produced via ICSI all sustained embryonic arrest on the 16-cell to morula stage of development (Rahman, 2012). Further investigation of the possible causes documented metabolic activity products and by-products. These artifacts were accumulating to toxic concentrations detrimental to the embryo's growth and development (Phua, 2006). Ruminant embryological development has been hindered by oxygen-derived free radical formation and accumulation causing decreased oxygen levels have negatively affected intracellular hydrogen peroxide levels (Agarwal et al., 2022). This could possibly be due to a component *in vivo* that is actively scavenging free radicals or it could be insufficient oxygen levels *in vivo*.

Gardner in 1994 observed that an accumulation of ammonium ions was linked to incorporating amino acids in IVC medium. It was recommended to replace the medium before the ammonium ions reach toxic levels or to enzymatically extract ammonium from the culture medium (Kleijkers et al., 2016). Comparably, *in vitro* ovine embryo development is discouraged by L-lactic acid accumulation. This accumulation has been previously associated with utilizing excessive sodium DL-lactate solution (Gardner, 2015).

### **Female factors: age**

Age of donor nannies and ewes has affected superovulation and other ovarian responses in goats and sheep. Jainudeen et al. (2000) stated that different age groups also require different physiological needs that also affect reproductive responses of the donor goat. Lehloenya et al. (2005) revealed that young donor goats have a longer timespan between removed controlled internal drug release (CIDR) until the onset of estrus. Evidence of these negative effects from steroid use include longer response time and resistance of young goats to the exogenous hormonal treatment. Both stimulus and hormonal treatment therefore must be increased in young goats to elicit an ovarian response (Baril et al., 2000). Nanny goats and ewes 3 years old and below have been reported to have poor superovulatory responses. Immature ovaries have a dominant follicle causing a suppressed total follicular recruitment and a decreased response to exogenous gonadotropin treatment. Immature goats compared to mature goats are more affected by the negative inhibitory effect of steroids and inhibin (Driancourt, 2001; Senger, 2003; Lopes et al., 2006). Oocytes produced by prepubertal goat donors are less likely to develop into viable embryos because of cytoplasmic insufficiency, metabolic, and cell architecture abnormalities (Armstrong, 2001; Cogne et al., 2003). Abnormal fertilization from prepubertal oocytes was also observed and these include prominent maternal chromatin, deficiency in the formation of sperm aster, inability of the sperm head to undergo decondensation (Salamone et al., 2017; Villamediana et al., 2001).

Simon et al. (2020) stated that the decreasing reproductive competence that comes with a donor goat's old age is caused by smaller pre-ovulatory follicles failing to achieve final meiotic maturation before undergoing ovulation. Impaired insulin-like growth factors (IGF) secretion, decreased gonadotropin levels both in circulation and production, and higher susceptibility to inhibin's feedback mechanism have also been reported. All these factors result in the failure of antral follicle growth, crucial in increasing oocyte viability, to develop in older donor goats fully and properly. Old donor goats are also less likely to produce viable oocytes (Morel et al., 2010).

A study by Jimenez-Macedo et al. (2007) revealed that prepubertal goats had a blastocyst yield from ICSI oocytes at 13% in 2006. In 2007 they conducted the same study and produced a higher yield of 35%. Comparatively, adult goats produced a yield of 18% in 1997 while a higher yield of 35% was obtained in 2003 (Keskinetepe et al., 1997; Wang et al., 2003).

### **Female factors: weight**

Mwaanga and Janowski (2001) highlighted how anoestrus was positively correlated to severe weight loss in all kinds of domestic species. Studies have shown that nutritional intake of the donor animal affected different aspects of the ovarian activity. Changes in important metabolic hormones such as insulin and insulin-like growth factor-I (IGF-I) as well as change in plasma metabolites were observed. Decrease in growth factors and hormones obtained from follicular fluid were also noted in the study. All these cause a significant decrease in follicular growth in the animals' ovaries (Ferguson et al., 2003; Mihm and Bleach, 2003).

### **Female factors: breed**

Boer (occasionally crossbred with Jamnapari) goats are known to be highly fertile, polyestrous, and are capable of producing viable oocytes all throughout the year (Braga Lobo et al., 2010). At the peak of their fertility, this breed of goats also exhibited increased sensitivity to hormonal stimulation making them susceptible to exogenous hormonal

treatment, produce higher oestrus response, exhibit oestrus faster than other breeds, and have a reduced duration between CIDR removal and the start of the oestrus cycle (Lehloeny et al., 2005). Ammoun et al. (2006) reported that all animals injected with ovine Follicle Stimulating Hormone (oFSH) have shown higher plasma FSH levels. Inconsistency among breeds within animal groups however may be due to route of administration, the specific preparation for the hormone utilized, or simply by how different breeds eliminate the hormone mutable ways as well. Moreover, differences among breeds can also be attributed to the increase or decrease of FSH receptors expressed on the ovaries (Kumar, 2023).

#### **Female factors: oocyte**

A crucial factor in increasing success rates of ICSI in goats and sheep is the donor oocyte as one of two sources of gamete cells (Merchant et al., 2011). Falchi et al. (2022) has revealed that goat and sheep embryos undergo embryonic arrest during the 8-16 cell stages which is its supposed genome activation stage as well. Jimenez-Macedo et al. (2005) stated that 100% of goat embryos underwent embryonic arrest on the 16-cell morula stage. While a study by Gomez et al. (1997) observed that 80% of sheep embryos met the same fate. Villamediana et al. (2001) reported that chromosomal abnormalities were observed in 50% of goat embryos. Rahman (2012) stated that poor oocyte quality and inadequate enzymes produced by the embryo have been known to cause embryonic arrests. Moreover, evidence of genetically rooted debilitated embryo regulation as well as oocyte nondisjunction were also observed. Goats need artificial activation to activate their oocytes while sheep need a sham injection. This helps the oocyte achieve the 16-cell stage however it is important to note as well that those treated by ICSI that developed to blastocyst stage were only at 8% compared to the 18% when IVF was utilized.

#### **Male factors: fresh vs frozen-thawed sperm**

Livestock semen stock has practically been stored frozen and thawed only when needed in the future. Hashida et al. (2005) remarked that fresh goat spermatozoa produced a distinction from frozen-thawed spermatozoa in terms of mitochondria as well as plasma and acrosomal membranes where the membranes in the frozen-thawed semen samples were observed to have loose and expanded properties. The diminished IVF rate in frozen-thawed samples compared to fresh samples can be attributed to the aforementioned observations in plasma and acrosomal membranes. Menendez-Blanco et al. (2019) utilized pre-pubertal goat oocytes and produced 18.46% blastocysts from freshly collected semen compared to the 12.12% from frozen-thawed semen samples.

Morphology, DNA fragmentation, membrane integrity and vitality are the four parameters established in a 2019 study by Gonzalez-Castro and Carnevale to help predict frozen-thawed sperm samples' performance in ICSI procedures. The study revealed that embryo development in ICSI procedures had higher chances of survival in frozen-thawed semen samples that had excellent membrane integrity and vitality.

In 2001, Bartoov initiated the Intracytoplasmic sperm injection or IMSI in order to aid in this selection. IMSI includes a more sophisticated sperm selection method where the sperm is evaluated under high magnification of up to x6600. Other techniques were the ones used by Gianaroli et al. (2010) by utilizing polarized light in the sperm selection process. The polarized light was able to distinguish the acrosome-reacted spermatozoa via their birefringence. Stanger et al. (2010) discussed the hypo-osmotic swelling test (HOST) and how, prior to microinjection, specific spermatozoa with even minute traces of DNA fragmentation can be distinguished.

#### **Male factors: sperm cell membrane permeability**

Improving ooplasm-sperm sub-membrane interaction is a crucial step in ICSI procedures (Javed and Michael, 2012). The microneedle utilized in ICSI procedures as well as the piezo-pulse manually breaks the sperm's tail and has yielded 35% blastocysts (Wang et al., 2003). Once the sperm is immobilized, the sperm plasma membrane is damaged as well, thus instigating oscillation of intracellular Ca<sup>2+</sup>. Nuclear decondensation of the sperm, a key step in proper fertilization, is induced by the prior oscillation. If done correctly, next steps of fertilization occur and will all lead up to the first mitotic cleavage and most importantly, DNA synthesis.

There is also a non-chemical option to oocyte activation in ICSI. Mechanically breaking a hole in the oolema via a Piezo-driven needle has shown promising developmental rates in both cows and goats. Bori (2021) utilized crippled goat sperm samples in an ICSI procedure and concluded that sperm mobility was not necessary to successfully perform ICSI. On the contrary, utilizing motile spermatozoa that show signs of motion even after being injected resulted in 17.78% oocyte degeneration. Similarly, the sperm plasma membrane in sheep is damaged to improve blastocyst development by 15.5%. However, in contrast to the goat where the tail is broken first, the mechanical damage is directly performed on the acrosome and plasma membrane (Anzalone et al., 2016). Rahman (2012) has stated that sperm with scanty chromatin fail to undergo decondensation and have resulted in embryonic arrest

#### **Practical utility of ICSI in context of developing countries**

One of the available techniques for improving the reproduction of animals is ICSI. In small ruminants the development of production system is important due to the component of meat and wool production. ICSI has potentially important application use with semen of valued animals, with epididymal sperm, in the fertilization of prepubertal or cryopreserved oocytes (López-Saucedo et al., 2012). ICSI also has an essential role in pre-implantation genetic

diagnosis, which avoids the contamination of oocytes with multiple spermatozoa (Feldman et al., 2017). The ICSI technique can be implemented from experiment to conservation level in animal sector (Jacobson et al., 2010; Parmar et al., 2013).

## Conclusions

ICSI in goats and sheep have a production-centered history. This field of animal production and medicine is still an untapped resource having the potential to improve production parameters and using them to our advantage. They can be used to acquire superior hybridization of small ruminants through transgenesis. This can also be utilized in other species especially endangered animals where sperm count is limited to those who remain alive. Both the external and internal factors work hand in hand to produce the best possible outcome for ICSI as an ART. Further studies need to be done to improve factors that either encourage or discourage embryo development. In spite of this, its potential is boundless, going by the information gathered across various experiments.

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