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Intracytoplasmic Sperm Injection-Revolution in Human and Animal Assisted Reproduction: A Review

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Abstract: The Intracytoplasmic Sperm Injection (ICSI) is a kind of micromanipulative technique in which a single sperm is directly injected into the ooplasm of a matured oocyte using a microscopic needle. It is a type of *in vitro* fertilization which is mainly used to treat severe male factor infertility in human. This procedure can bypass many barriers of fertilization including the process of sperm penetration through cumulus-corona cells, zona pellucida and oolemma during fertilization by directly depositing the sperm into the ooplasm. The introduction of ICSI in assisted reproduction has helped to investigate and understand the early events of fertilization such as sperm capacitation, the acrosome reaction and pronucleus formation. Like humans, domestic animals including goats, suffer from infertility or sub-fertility, which lowers their lifetime productivity and reduces the number of offspring that could be obtained from a sire or dam. The prevalence of this problem coupled with the desire of people to understand and subsequently control the reproductive processes has led to the development of novel assisted reproductive technologies (ARTs) like ICSI. It is one of the modern ART which can be used in livestock for the improvement and preservation of livestock genetics and the enhancement of reproductive efficiency. Till now, ICSI technique - one of the modern ARTs - has been successfully used in humans, rodents and other animals, especially in the cattle. The present study describes different aspect of ICSI including history, types of micromanipulative techniques, remodeling of fertilization process, oocyte activation and factors affecting outcomes of ICSI etc.

Key words: ICSI, sperm, oocyte, micromanipulation, MFI, IVF, ZD, PZD, SUZI, embryo

INTRODUCTION

During normal fertilization, the sperm nucleus is incorporated into an oocyte through membrane fusion between the two gametes. The ICSI is the mechanical insertion of a single sperm directly into the ooplasm of a matured oocyte using a microscopic needle. It is a variation of *in vitro* fertilization (IVF) that has been a standard clinical technique for many years (Committee, 2004). Its efficacy and short-term safety made the technique reliable to use in the clinical setting to alleviate severe Male Factor Infertility (MFI) in human. The MFI is a term used to describe an individual with one or more sperm abnormalities that may impair their fertilizing capability (Gordon and Talansky, 1986; Ng *et al.*, 1988; Palermo *et al.*, 1992). Problems with the production and maturation of sperm are the most common causes of MFI. Sperm may be immature, abnormally shaped, or unable to move properly. Or, normal sperm may be produced in abnormally low numbers (oligospermia) or seemingly not at all (azoospermia) due to various reasons. Therefore, the Practice Committee of the American Society for Reproductive Medicine has approved ICSI as a clinical

technique rather than experimental in human medicine (Committee, 2004). This procedure can bypass many barriers of fertilization including the process of sperm penetration through cumulus-corona cells, zona pellucida (ZP) and oolemma during fertilization by directly depositing the sperm into the ooplasm. The introduction of ICSI in assisted reproduction has helped to investigate and understand the early events of fertilization such as sperm capacitation, the Acrosome Reaction (AR) and pronucleus (PN) formation (Keskintepe *et al.*, 1997).

In natural mating, genetically important but biologically inferior (e.g., low sperm production, poor sperm motility and sperm abnormalities) males cannot be used. In IVF, severe MFI could not be treated. However, ICSI is a technique by which these situations can be overcome. Therefore, ICSI would be an effective technique to use on genetically important but biologically inferior male gametes for procreating domestic and wild livestock species (Keskintepe *et al.*, 1997) and thereby facilitating the production of large number of embryos and offspring from a single genetically valuable animal. Development of an ideal ART like ICSI revolutionizes the management of infertile or sub-fertile males. Nowadays

the ICSI became an efficient and reliable clinical technique in human. Because of its higher success rate, this technique is expanding very rapidly in clinical as well as research fields both in human and animal reproduction. In animals, especially in livestock, the ICSI technique could be an alternative approach to generate transgenic livestock, for example goats, using sperm as the DNA carrier (Perry *et al.*, 1999, 2001), for propagation of useful genetics (Wang *et al.*, 2003) or gender pre-selection (Hamano *et al.*, 1999). The progeny produced by this technique could be used as founder animals for the production of recombinant protein in their milk such as pharmaceutical proteins for the treatment or prevention of human diseases or biomaterials for medical use (Niemann and Kues, 2003; Keefer, 2004). Therefore, engaging in ICSI appears to be profitable and beneficial to livestock industry. The present study will discuss the ICSI technique with special reference to history and gradual development of micromanipulation, ICSI success, remodeling of reproductive process, oocyte activation and various factors affecting the outcomes of ICSI.

HISTORY AND GRADUAL DEVELOPMENT OF MICROMANIPULATION AND ICSI

Micromanipulation is comparatively a recent term of biological micro-dissection that replaced the old term micrurgy. As cited in Timson and McDermott (1994), the term micrurgy was first coined by Peterfi (1923) for biological micro-dissection, which was also known as micrurgy. The term micromanipulation is used to include all operations performed in the microscopic field of vision with the aid of mechanical devices that guide the operating tools (Timson and McDermott, 1994). Although, the invention of microscope made it possible to perform all sorts of micro-dissection under the microscopic field, however, the invention of mechanical devices which guide the operating tools proper that heralded the advent of micromanipulators. Micromanipulation techniques are being used to obtain fertilization and pregnancies in cases, which would have been considered hopeless only one and a half decades ago. The gradual developments of micromanipulation and ICSI techniques have been reviewed elsewhere (Hiramoto, 1962; Iritani, 1991, 1994; Timson and McDermott, 1994; Goto, 1997; Payne, 1995; Mansour, 1998; Horiuchi *et al.*, 2002; Horiuchi, 2006).

Development of micromanipulation tools and techniques: The ICSI has been the major breakthrough in the field of assisted reproduction among the all micromanipulation techniques developed in the eve of twentieth century. All

the micromanipulation techniques now used for assisted reproduction basically originated from a pre-existing technology of micromanipulation, which was developed for various purposes other than assisted fertilization (Mansour, 1998). It is to be mentioned here that the micromanipulative techniques were first conceived and applied by the microbiologist (Timson and McDermott, 1994). As cited in Timson and McDermott (1994) and Schmidt (1859) seemed to have been the first to describe the use of a microscopic dissector. However, the use of micromanipulation tools was first described by Chabry (1987) and Timson and McDermott (1994) in the study of the embryos of marine animals. Chabry (1987) developed modified equipment and was one of the earliest to comment on the properties of micromanipulated embryonic blastomeres. As mentioned by Timson and McDermott (1994), Hiramoto (1962) that Barber (1904, 1911) and G.L. Kite (never published cited in Lillie, 1934), two early microbiologists, extended the use of micromanipulation, respectively, in bacteriology and basic cell biology. Subsequent scientists broadened and fine-tuned the tool-making technology and micromanipulation-dissection developed with enormous potential and considerable accuracy (Timson and McDermott, 1994). The micromanipulative techniques have attained a high degree of perfection since that time. In 1928, Emmerson (El Badry, 1963) described the first micromanipulator with a joystick design that could directly transmit the movements required by the operator. The development of the varied types of exceedingly delicate microtools went hand in hand with the development of the micromanipulators and the significant advances in microscopy aided greatly in that direction (Timson and McDermott, 1994). As mentioned in (Mansour, 1998), hydraulic principles that are now used for the joystick manipulators had been described by De Fonbrune (1934) who also invented the first microforge and microtool-making techniques that are still in use today. The timeline of early developmental history of micromanipulation tools and techniques has been depicted in the Table 1.

History and gradual development of sperm injection experiments: Although ICSI in the field of assisted reproduction is a recent innovation, research on sperm injection into the oocyte in animals had already started a century ago. For more than half a century, sperm injection experiments had been conducted with lower animals especially with marine echinoderms. Scientists were devoted themselves to develop various techniques for direct micromanipulation of living cells from the beginning of the twentieth century. As mentioned by Hiramoto (1962), the first sperm injection experiment was attempted

Table 1: Timeline of development of micromanipulation tools and techniques

Year	Author	Discovery/development
1859	Schmidt (1859)	First described the use of a microscopic dissector
1887	Chabry (1987)	First described a modified micromanipulation equipment to study the embryos of marine animals
1904, 1991	Barber (1904, 1991)	Extended the use of micromanipulator to study bacteria
1911	G.L. Kite (Lillie, 1914)	Extended the use of micromanipulator in the study of basic cell biology
1928	Emmerson (El Badry, 1963)	First micromanipulator with a joystick design
1934	de Fonbrune (1934)	Described hydraulic principles that could be used for the joystick manipulators
1934	de Fonbrune (1934)	Developed the first microforge and microtool-making techniques

in 1911 by G.L. Kite (Lillie, 1914), one of the pioneers in the field of microsurgery or micrurgy. He used micro-needles and microinjected starfish sperm into the starfish egg. With the aid of micro-needles G.L. Kite in his nuclear dissection experiment was able to separate the PN in newly fertilized zygotes (Chambers, 1940). However, due to interruption of his experiment, unfortunately the complete result of his work was never been published and the study became inconclusive (Lillie, 1914). In the 1940s and 1950s, micromanipulative or microsurgical procedures were first attempted in several species of mammals to study the living eggs of the rat, the human, the mouse and the rabbit although the results of these studies were not found (Timson and McDermott, 1994). In his book on fertilization Rothschild (1956) mentioned that live sperm injection into the cytoplasm of sea urchin was possible although no fertilization could be found, however, he did not cite any reference for his comment. It is to be mentioned here that T.C. Lin (Fishel and Symonds, 1993), a pioneer mammalian embryologist, performed numerous studies on the technical and experimental nature of egg micrurgy or micromanipulation during the 1950s and 1960s.

The first detailed and successful sperm injection experiment was conducted by Hiramoto (1962) who injected live sea urchin sperm into the protoplasm of an unfertilized egg of the same species that had been activated and the injected sperm nuclei participated in the meiotic process. At that time, it was not performed for the purpose of assisted fertilization, but to prove that sperm nuclear decondensation and male pronucleus (MPN) formation did not require prior interaction between the sperm and the oolemma. This work inspired the scientist to study the technique in higher species of animals. In amphibians, the technique was first documented in frogs by Graham (1966) followed by Brun (1974).

After these achievements in lower animals, sperm injection experiments were conducted in mammals. Uehara and Yanagimachi (1976) investigated the possibility of hamster and human sperm to develop into MPN when injected into hamster oocytes. It was found that isolated hamster sperm nuclei developed into MPN in hamster oocytes. The nuclei of fresh, frozen-dried and frozen-thawed human sperm were also able to develop

into MPN in hamster oocytes. In another experiment, hamster, mouse, rabbit and fish sperm nuclei, or hamster hepatocyte nuclei were microinjected into hamster oocytes were able to develop into MPN for each type of nuclei and began DNA synthesis (Naish *et al.*, 1987). These findings suggested that the ooplasmic factors controlling the transformation of sperm nuclei into MPN are not species-specific. Injection of sperm head, detached from tail, induced normal fertilization of unfertilized mouse oocyte (Markert, 1983). Injection of grossly defective and immotile mouse sperm into the oocyte exhibited the same reactions found in the fertilization with healthy sperm.

Among domestic animals, sperm injection was first attempted in bovine oocytes with MPN formation (Westhusin *et al.*, 1984). Laws-King *et al.* (1987) reported the insertion of a single sperm under the ZP of human oocytes with a high rate of fertilization. However, improvements in microinjection using a rabbit model not only led to MPN formation following ICSI-induced fertilization, but also resulted in further embryonic cleavage and the first live birth of normal offspring (Hosoi *et al.*, 1988). A historical picture of sperm injection attempts (first report only) has been illustrated in Table 2 before the first successful live birth.

Development of other micromanipulation techniques related to ICSI:

In most of the early research activities on micro-fertilization, ooplasmic sperm injections or ICSI were exclusively conducted. However, ooplasmic microinjection bypasses all natural barriers to sperm penetration and removes all biological selectivity from the process, such as sperm binding to the ZP and fusion of the sperm with the oolemma (Gordon and Laufer, 1988). Because of this reason, clinicians preferred other micromanipulation methods without disruption of normal fertilization process. Therefore, attempts were made to develop other micromanipulation techniques to assist fertilization which include Zona Drilling (ZD), Partial Zona Dissection (PZD) and Sub-Zonal Sperm Injection (SUZI). Although these techniques were successful in producing live offspring in laboratory animals as well as in human, however, they were not able to become the method of choice due to a number of reasons. Therefore, the

Table 2: History of sperm injection attempts in different animals and human

Year	Author	Species	Results/development
1911	Lillie (1914)	Starfish	First sperm-egg injection experiment by G. L. Kite with inconclusive result
1911	Chambers (1940)	Starfish	Separation of the PN in newly fertilized zygote by G.L. Kite
1962	Hiramoto (1962)	Sea urchin	Decondensation
1966	Graham (1966)	Frog	MPN formation
1976	Uehara and Yanagimachi (1976)	Hamster	MPN formation
1983	Markert (1983)	Mouse	Blastocyst formation
1984	Westhusin <i>et al.</i> (1984)	Cattle	MPN formation
1987	Laws-King <i>et al.</i> (1987)	Human	High rate of fertilization

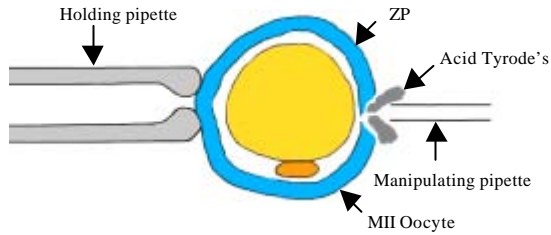


Fig. 1: Diagrammatic representation of Zona Drilling (ZD). Acid Tyrodes solution is used to make a hole in the ZP to allow sperm access to the oocyte. Drawn by the Author from Catt (1996)

principal breakthrough and method of choice in the field of assisted fertilization was ICSI. The salient features of other micromanipulative techniques are described briefly in the following subsections.

Zona drilling: The ZD technique was first described by Gordon and Talansky (1986). This technique involves the use of a fine stream of acid Tyrodes solution (pH 2-3) to dissolve a small hole in the ZP of a cumulus-free and mature oocyte held with a micromanipulator (Fig. 1). The piercing of ZP can also be done with a fine needle after prior softening with chymotrypsin (Iritani, 1991, 1994). Like IVF, the sperm must be capacitated, acrosome reacted and progressively motile in ZD. The fact that fertilization occurred at increased rates after ZD suggests that the ZP is a significant barrier to the fertilizing sperm and that sperm do not necessarily have to come in contact with the outer surface of the ZP for the acrosome reaction to occur. Although, ZD could be utilized for oligospermia with weak sperm motility, this cannot be applied to immotile sperm and the oocytes are also damaged to certain extent (Iritani, 1991, 1994). Fertilization rates with ZD in human ranged from 6 to 35% compared with 3 to 13% in control oocytes (Payne, 1995). However, polyspermy and immature hatching due to big hole (approximately 15.2 μm in diameter) (Payne, 1995) are major problems. Live births after ZD were reported in mouse (Gordon and Talansky, 1986) and in human (Jean *et al.*, 1992). In clinical practice ZD method was discontinued when it was discovered that the human oocyte is particularly sensitive to the low pH

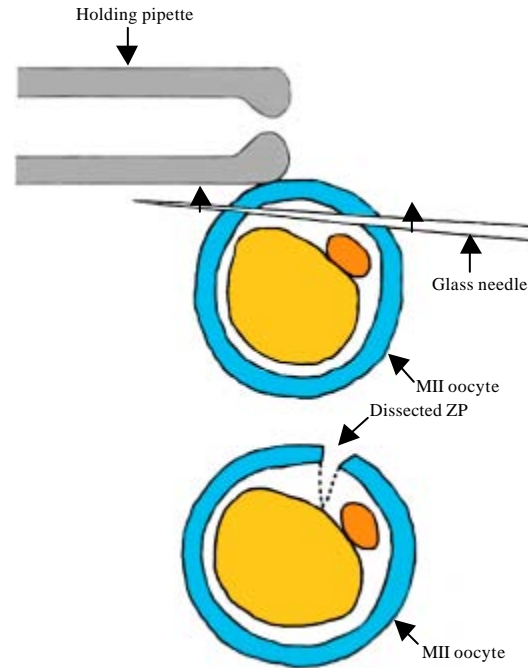


Fig. 2: Diagrammatic representation of Partial Zona Dissection (PZD). A cumulus-free oocyte is held by suction onto a holding pipette and a fine glass needle is pushed through both sides of the ZP; the oocyte is then released and the glass needle is pushed against the holding pipette until a slit in the ZP is produced. Drawn by the Author from Payne (1995)

of the acidified Tyrodes and that high oocyte degeneration and poor embryonic development occurred as a consequence of its use (Payne, 1995).

Partial zona dissection: The PZD technique is also known as zona cutting or zona opening. The oocyte is held by a holding pipette and a fine glass pipette or metal microprobe is pushed through the ZP and the oocyte is released from the holding pipette and rubbed against the cutting pipette until a slit is produced (Fig. 2). The hole produced in PZD is much smaller (approximately 8.9 μm in diameter) (Payne, 1995) than that produced in ZD. Like

ZD, the sperm must be capacitated and acrosome reacted for fusion with oolemma in PZD. Instead of using glass microneedles or metal microprobe, laser technology has been investigated as an alternative method for creating opening in the ZP (Feichtinger *et al.*, 1992; El-Danasouri *et al.*, 1993). Using this technique, fertilization and live birth had been reported in human (Malter and Cohen, 1989; Feichtinger *et al.*, 1992). However, the technique has not been widely used because it requires relatively high number of progressively motile sperm and moreover, the incidence of polyspermy was high even when few sperm were used (Goto, 1997). Compared with IVF, the results of PZD were also found to be unsatisfactory and were associated with erratic but generally low normal fertilization rates (Cohen, 1992).

Sub-zonal injection: In the next step after ZD and PZD, the microinjection of sperm into the perivitelline space (PVS) or under the ZP was developed which is known as Sub-Zonal Injection (SZI) or SUZI. In SUZI, 5 to 8 sperm are generally injected into the PVS of cumulus-free and matured oocytes (Fig. 3), so that selection of biologically normal sperm is expected to a certain extent during the process of membrane fusion (Iritani, 1991, 1994; Goto, 1997). Although the technology for SUZI had been in existence before the clinical use of ZD and PZD (Metka *et al.*, 1985; Laws-King *et al.*, 1987), it was not used clinically until the limited application and success of ZD and PZD were demonstrated (Payne, 1995). The technique circumvents the process of sperm penetration through the ZP, so that sperm with extremely poor motility can be injected. However, fertilization rates after SUZI were similar to those using the previous techniques, yet polyspermy was a particular problem, with some IVF units reporting polyspermy in more than 30% of the fertilized oocytes (Payne, 1995). Although SUZI led to the birth of live offspring in the mouse (Mann, 1988) and human (Ng *et al.*, 1988); the incidence of polyspermy limits its use and thus, like ZD and PZD, SUZI could not be popularized (Goto, 1997).

In general, the greatest weakness of ZD, PZD and SUZI methods was their inefficiency, as they fail to achieve normal fertilization rates beyond 20% and naturally produce high rate of polyspermic fertilization (Cohen, 1992). Whereas using only one sperm achievements in ICSI are far better than the other micromanipulative techniques. The advantage of ICSI over other micromanipulative techniques are that any type of sperm (sperm head, immotile, dead or grossly defective sperm) can be injected and both capacitation and acrosome reaction appear to be unnecessary for fertilization (Iritani, 1991, 1994). Mechanical pricking of the

oocytes and the infusion of reagents such as polyvinylpyrrolidone (PVP) in the injection medium appear to be effective for oocyte activation (Markert, 1983; Iritani and Hosoi, 1989).

REPORTS OF LIVE BIRTHS THROUGH ICSI

The first report of live birth through ICSI came from Japan with rabbit (Hosoi *et al.*, 1988). Since then, many reports have been published on live birth in a number of mammalian species, even in insects and birds. However, ICSI technique had revolutionized in 1992 when human babies born in Belgium using this technique (Palermo *et al.*, 1992). The ICSI proved its efficacy and successfulness to overcome MFI in human and well accepted by the clinicians due its higher success rates. Since its first success in 1992, thousands of human babies had born using ICSI technique that represented a major breakthrough in the field of assisted fertilization. This way, micromanipulation techniques have gradually developed over the century and have reached such a precision that skilled scientists are able to micro-dissect one single chromosome. The ICSI procedure is illustrated in the Fig. 4. The chronological report (first report only) of

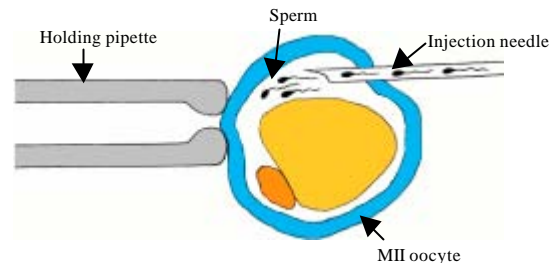


Fig. 3: Diagrammatic representation of sub-zonal injection (SUZI). Several sperm are selected and injected under the ZP in SUZI. Drawn by the Author from Payne (1995)

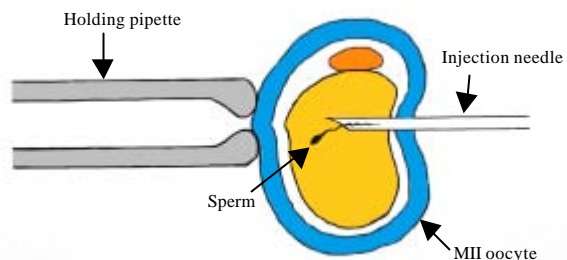


Fig. 4: Diagrammatic representation of ICSI. The ICSI involves the injection of a single, live, immobilized sperm into the ooplasm of the oocyte. Drawn by Author

Table 3: Chronological reports of live offspring production through ICSI in animals and human

Author	Authors	Species
1988	Hosoi <i>et al.</i> (1988)	Rabbit
1990	Goto <i>et al.</i> (1990)	Cattle
1992	Palermo <i>et al.</i> (1992)	Human
1994	Hatakeyama <i>et al.</i> (1994)	Sawfly (<i>Athalia rosae</i>), an insect
1995	Ahmadi <i>et al.</i> , 1995)	Mouse
1996	Catt (1996)	Sheep
1998	Cochran <i>et al.</i> (1998)	Horse
1998	Pope <i>et al.</i> (1998)	Cat
1999	Hewitson <i>et al.</i> (1999)	Monkey
2000	Martin (2000)	Pig
2001	Poleo <i>et al.</i> (2001)	Zebrafish
2002	Hirabayash <i>et al.</i> (2002)	Rat
2002	Yamauchi <i>et al.</i> (2002)	Hamster
2002	Ogonuki <i>et al.</i> (2002)	Mastomys (a rodent species)
2003	Wang <i>et al.</i> (2003)	Goat
2003	Magarey and Mate (2003)	Tammar wallaby (a kangaroo species)
2003	Hrabia <i>et al.</i> (2003)	Japanese quail (bird)

live offspring production through ICSI in different species of animals and human has been depicted in Table 3.

REMODELING OF FERTILIZATION PROCESS AND OOCYTE ACTIVATION IN ICSI

The ICSI is the latest and by far the most efficient, variant of micromanipulated-assisted fertilization, whereby a single sperm is selected, aspirated into a microinjection needle and injected into ooplasm of a MII oocyte. The development of this technique is mainly linked to application in human assisted reproduction for which it enables fertilization with defective sperm that would not otherwise be able to penetrate an oocyte by proper means. Because ICSI bypasses many steps of natural fertilization process, therefore, remodeling of fertilization process that occurs in ICSI is different from that of fertilization *in vivo* or *in vitro*. The ICSI has contributed greatly to the knowledge concerning the different stages of fertilization, such as sperm capacitation, AR, membrane fusion, oocyte activation and ooplasmic factors involved in the transformation of sperm nucleus into a MPN. The factors involved in sperm pronuclear development are not species-specific. It was demonstrated that sperm of some species (e.g., human sperm) could develop into normal MPN in another species (e.g., hamster oocyte) and form a normal chromosome complement (Elder and Dale, 2000). Sperm injection from a number of species, for example, frog, hamster and human was able to activate hamster oocyte and subsequently developed to MPN (Perreault *et al.*, 1988). This result suggests that sperm interaction with the ooplasm is less species specific than the interaction with the oolemma (Perreault *et al.*, 1988). The ooplasmic factors controlling the transformation of sperm nucleus to MPN are also not species specific (Iritani, 1991).

Sperm capacitation might not be necessary to attain fertilization through ICSI as oocyte activation was induced with injection of both capacitated and non-capacitated sperm (Thadani, 1979). Although, in goat ICSI, generally capacitated sperm are used (Keskin-tepe *et al.*, 1997; Jimenez-Macedo *et al.*, 2005; Rahman *et al.*, 2007, 2009; Abdullah *et al.*, 2008), a higher fertilization rate was also obtained with un-capacitated washed sperm (Wang *et al.*, 2003). During fertilization *in vivo* (or *in vitro*) sperm penetrates the ZP without its plasma membrane over the acrosomal cap, together with the underlying outer acrosomal membrane and acrosomal contents disperse. The remaining plasma membrane over the equatorial segment fuses with the oolemma. Thus, all the remaining portions of the sperm including the nucleus, perinuclear material, centriole and tail are engulfed by the ooplasm in a phagocytic manner (Yanagimachi, 1994, 1997). In contrast, during ICSI whole sperm is incorporated into the ooplasm including the acrosomal content and membranes. In this case, although sperm binding to ZP is bypassed, the AR still could occur in the ooplasm of a mature Metaphase II (MII) oocyte (Ben-Yosef and Shalgi, 1998, 2001). The AR is preceded by acrosomal swelling followed by vesiculation of surface membranes, exposing the inner acrosomal membrane, similar to that observed on the surface of the ZP during IVF (Sathananthan *et al.*, 1997). It is demonstrated in the ICSI studies that barriers penetrated by sperm during fertilization *in vivo* or *in vitro* are probably not biologically necessary and can be bypassed by direct sperm injection into the ooplasm.

Other important features of *in vivo* fertilization or IVF, for example capacitation and AR - the functional changes that the sperm undergoes in order to acquire the fertilization potential - are also not a prerequisite to ICSI (Yanagimachi, 1994, 1997). From different ICSI studies, it is now known that all the sperm components are not required for successful oocyte activation at fertilization. In mouse, injection of isolated sperm head is enough to activate oocyte (Kimura *et al.*, 1998) and sperm centriole is not necessary for fertilization (Schatten, 1994). However, in other mammals, for example human, cattle, sheep or goat sperm centriole, a structure later serving as an organizer of zygotic centrosome, is very important (Le Guen and Crozet, 1989; Crozet, 1990; Navara *et al.*, 1994; Schatten, 1994; Sathananthan *et al.*, 1997). The sperm centriole plays a key role in the formation of microtubule within the zygote. A radial array of microtubules, called the sperm aster, is formed, which emanates from the sperm centrioles following its exposure to the ooplasm. The sperm aster is responsible for

bringing the MPN and female pronucleus (FPN) into close contact to enable syngamy (Yanagimachi, 1994). Therefore, in mammals excluding rodent species sperm head and neck where centrioles are located, must be injected. In goat, high fertilization and embryo development rates were achieved after injection of tail-cut sperm near the mid-piece (Wang *et al.*, 2003).

The ability of the sperm to induce oocyte activation following injection is a crucial requirement for successful fertilization. Two hypotheses have been proposed to explain the mechanisms by which the sperm induces the activation of the mammalian oocyte: the receptor hypothesis model and the sperm factor hypothesis. In the receptor hypothesis, an oocyte surface receptor is coupled to a G-protein (Miyazaki *et al.*, 1993) or a tyrosine-kinase-mediated (Ben-Yosef and Shalgi, 1998) signaling pathway and when activated by a sperm, leads to the release of Ca^{+2} from intracellular stores. These sperm induced Ca^{+2} oscillations usually last several hours after the initial Ca^{+2} increases and the frequency shows considerable variation between species (Jones, 1998). The sperm factor hypothesis, on the other hand, suggests that egg activation is initiated only after sperm-oocyte fusion, by introducing a sperm-borne-oocyte-activating-factor (SOAF) or Sperm Factor (SF) into the ooplasm (Stice and Robl, 1990; Swann, 1990). When homogenized rabbit sperm was microinjected into rabbit oocytes, activation rates were higher than in oocytes injected with medium alone; activated oocytes exhibited cortical granule exocytosis, MPN formation and cleavage (Stice and Robl, 1990). Additionally, sperm extracts do not appear to be species-specific as rabbit sperm fractions activated both rabbit and mouse oocytes following injection into the ooplasm (Stice and Robl, 1990). Finally, the ICSI procedure itself bypasses sperm-oocyte binding and fusion and its success also supports the soluble sperm factor hypothesis. Recently, a hypothesis combining the two major hypotheses was proposed (Tesarik, 1998). In this trigger and oscillator hypothesis, the role of the trigger or initiation of Ca^{+2} oscillations, normally released by sperm-oocyte cell surface interactions in normal fertilization, is substituted for ICSI by an artificial Ca^{+2} influx generated by the ICSI procedure itself. In this hypothesis, the sperm cytosolic factors play the role of the oscillator.

It is generally accepted that intracellular increase in Ca^{2+} level, which mimic fertilization very closely (Jones *et al.*, 1995), is the universal signal for triggering oocyte activation and subsequent embryo development in all mammals studied so far (Vitullo and Ozil, 1992; Homa *et al.*, 1993; Tesarik, 1995). Moreover, it is a histone kinase inhibitor and prevents the re-accumulation of

M-phase or maturation promoting factor and this yields an improvement in the efficiency of oocyte activation (Susko-Parrish *et al.*, 1994). As observed in fertilization *in vivo* or *in vitro*, Ca^{2+} oscillations are characteristics of activated ICSI oocytes which continue for several hours after the ICSI procedure (Sousa *et al.*, 1996), but the dynamics of the response is different from IVF. In contrast, no Ca^{2+} oscillations are observed after sham injection of oocytes with sperm-free culture medium (Tesarik *et al.*, 1994), demonstrating the decisive role of SF in this process (Tesarik and Mendoza, 1999). Although, no studies related to Ca^{2+} oscillations pattern after ICSI in goat was reported, one study reported that a typical oscillatory Ca^{2+} release pattern does occur following IVF in the goat, as previously been shown in other mammalian species (Jellerette *et al.*, 2006). Analyses of human unfertilized oocytes by ICSI indicated that this caused by a failure of oocyte activation (Sousa and Tesarik, 1994; Tesarik, 1995). In fact, a high fertilization rate is reported in human oocytes after ICSI and artificial induction activation with calcium ionophore (Ca^{2+} ionophore) or electric pulse (Tesarik, 1995; Abe *et al.*, 1996). Furthermore, normal offspring were born after the artificial activation of ICSI oocytes in cattle (Goto *et al.*, 1990), sawfly (Hatakeyama *et al.*, 1994) and human (Hoshi *et al.*, 1994). However, abnormalities of Ca^{+2} signals driving oocyte activation after ICSI are suspected to be at the origin of various abnormalities in the resulting embryos including chromosomal abnormalities (Tesarik, 1995). Therefore, a proper method of oocyte activation that mimics a sperm must be developed.

The exact timing of fertilization events *in vivo* or the IVF procedure is difficult to evaluate, as the exact moment of gamete fusion cannot be determined. Several studies have monitored the ultrastructure of sperm-oocyte interaction and the timing of the fertilization events after ICSI in human (Nagy *et al.*, 1994; Bourgain *et al.*, 1998). The sign of AR observed 15 minutes after ICSI procedure, whereas in IVF or *in vivo* fertilization it occurs before gamete fusion. Cortical Reaction (CR) that occurs in IVF within a second after sperm entry takes 90 min to occur after ICSI. Decondensation of sperm head chromatin occurred from 30 min to 3 h after ICSI. Relocation of MII spindle with a paired set of chromosome near the oolemma in close apposition to the polar body-1 (PB-1) occurs up to 3 h of ICSI. Extrusion of PB-2 started after 4 h of ICSI. The MPN is formed before the FPN and around 8 h after ICSI approximately 80% PN formation is completed; 100% PN formation complete by 16 h (Nagy *et al.*, 1994). Although, timing of extrusion of PB-2 and PN formation are similar in both IVF and ICSI procedure, but ICSI embryos start to cleave earlier (approximately 20 h) than

IVF. Therefore, all the events which are bypassed by ICSI procedure occur in the first few hours after sperm injection within the oocyte (Ben-Yosef and Shalgi, 1998, 2001). However, chromatin remodeling is more asynchronous in ICSI than in IVF. In their study, Ajduk *et al.* (2006) compared sperm chromatin remodeling in IVF and ICSI in the mouse. Their results demonstrated that sperm capacitation prior to injection enhanced remodeling asynchrony and resulted in delayed PN formation and DNA synthesis. They also found that injection of Ca^{2+} ionophore into the mouse oocyte after removal of acrosome allowed more synchronous chromatin remodeling, timely DNA synthesis and good embryo development than that of detergent Triton X-100. Although the dynamics and ultrastructure of sperm interaction within ooplasm of an oocyte occur somewhat differently in ICSI than fertilization *in vivo* or *in vitro*, most of the cellular events responsible for mediating oocyte activation are stimulated in the same manner (Ben-Yosef and Shalgi, 2001).

FACTORS AFFECTING THE OUTCOMES OF ICSI

As in all micromanipulation techniques, ICSI involves intricate and precise maneuvers of microtools on the oocytes and sperm under an inverted microscope. Although the skill and experience of the embryologist in performing ICSI is important, the outcome of the procedure depends largely on the quality of the microtools such as the microinjection needles, sperm immobilization and injection technique. Other factors such as oolemma characteristic, orientation of PB, use of PVP, oocyte *in vitro* maturation (IVM) conditions, oocyte morphology and IVC conditions are also important. Although, ICSI does not have strict requirements of sperm number, motility, morphology and quality to achieve pregnancies; some of the sperm parameters may affect the outcome of ICSI. Some of the important factors affecting ICSI outcomes are describes in the following subsections mostly based on human ICSI.

Injection needle: Injection of sperm into the ooplasm is the most invasive micromanipulation technique in assisted reproduction. Hence, the design and quality of the microtools especially the injection needle is of utmost important as the size and shape of the ICSI needle will determine the success or failure of a micromanipulation procedure. Generally, thick- and thin-walled borosilicate glass capillaries are used to make holding pipettes and ICSI needles, respectively (Payne, 1995). There are two features of the injection needle which are important for ICSI. First, the needle must be beveled and sharp, enough to perforate both the ZP and the oolemma. Second, the outer and inner diameter (OD and ID) of the needle should

be perfect, just large enough to draw in a sperm head. Although no reports were published in goat, fertilization and embryo development from bovine oocytes after ICSI was reported to be affected by the OD and ID of injection needles (Tocharus *et al.*, 1996). The percentage of embryo development was statistically higher with narrower diameter injection needles. The additional mechanical force and increased volume of injected solution with the wider diameter needles cause too much disruption of the ooplasm and arrest development (Tocharus *et al.*, 1996). However, if too narrow needle is used it will be difficult to move sperm in and out of the needle (Payne, 1995). Providing that these criteria are met, the bevel angle can vary from 28 to 30° (Hamberger *et al.*, 1995; Palermo *et al.*, 1995) to about 45 to 50° (Van Steirteghem *et al.*, 1993) and the needle can either be fitted with a sharp spike or they can be used without a spike (Catt *et al.*, 1995). To achieve better results in goat ICSI, the ID of ICSI needle should be between 6 and 7 μm with a sharp spiked tip (Wang *et al.*, 2003; Abdullah *et al.*, 2008; Rahman *et al.*, 2009).

Sperm immobilization prior to ICSI: Immobilization of motile sperm prior to ICSI, by touching the tail with the needle is mandatory even with immotile sperm (Fishel *et al.*, 1995; Van den Bergh *et al.*, 1995; Vanderzwalmen *et al.*, 1996). Sperm immobilization induces permeabilization of the sperm membrane which may result in the release of a cytosolic factor that diffuses into the ooplasm with subsequent intracellular Ca^{2+} oscillations and hyperpolarization of the oocyte (Homa and Swann, 1994; Dale *et al.*, 1999). This phenomenon is known as oocyte activation. As already mentioned earlier, the activation of the oocyte triggers a series of biochemical processes in the ooplasm that would eventually lead to sperm nuclear decondensation (Tesarik and Kopečný, 1989; Montag *et al.*, 1992), PB-2 emission, PN formation and exocytosis (Stice and Robl, 1990; Swann, 1990). At the edge of the sperm-PVP droplet, a morphologically normal motile sperm is immobilized by touching its tail with the needle. The sperm tail is then crushed by sliding the needle on the mid-piece of the tail until a kink is seen. It is then aspirated tail-first into the needle. The sperm neck contains the centriole that contributes a major role in fertilization in mammals other than rodents; therefore, care should be taken not to damage the neck. Proper immobilization technique is necessary to ensure leakage of SOAF or SF into the ooplasm which have significant function in the fertilization process.

The role of polyvinylpyrrolidone in ICSI: The PVP, a large molecular weight polymer (molecular weight 360,000), has been used since the beginning of assisted fertilization

programs (Lanzendorf *et al.*, 1988). The PVP when dissolved in a solution becomes very viscous which allows better manipulation of sperm, control of the ooplasmic aspiration and sperm injection procedures. It also prevents adhesion of sperm to the inner wall of the injection needle (Uehara and Yanagimachi, 1976). The use of PVP also allows the three-dimensional motility patterns of the sperm to be carefully observed (Cohen *et al.*, 1994). Control of the volume of ICSI medium injected into the oocyte is more difficult without using PVP (Joris *et al.*, 1998). The concentration of PVP is an important factor affecting the outcome of ICSI and usually 10% (w/v) PVP is used (Palermo *et al.*, 1992). Gordon (1997) examined the effect of different PVP concentrations as a sperm retardant for ICSI in bovine oocytes matured *in vitro*, as measured by cleavage rate and early embryo development. Although the damage rate in oocytes following ICSI was affected significantly by the level of PVP in the injection medium, there was no difference in the cleavage rates in all groups studied. However, the efficacy and safety of sperm immobilization process and use of PVP has been questioned as it is accused of having a potential harmful, mutagenic or carcinogenic effect (Ashwood-Smith, 1971; Feichtinger *et al.*, 1995; Jean *et al.*, 1996) and may also interfere with sperm nuclear decondensation (Dozortsev *et al.*, 1995b). Using PVP, mouse sperm are found susceptible to alterations that affect both fertilization and development (Mizuno *et al.*, 2002). In their study, Motoishi *et al.* (1996) evaluated the safety of ICSI procedures on bovine zygotes by using PVP and concluded that the ICSI procedures with PVP currently used for animal and human are neither detrimental to embryo development nor to embryo quality. There are reports of better fertilization rates with PVP-free medium (Butler and Mason, 1996; Jean *et al.*, 1996), even though embryo quality and implantation rates remain the same (Hlinka *et al.*, 1998; Tsai *et al.*, 2000). In goats, no reports have been published on the effect of sperm immobilization with PVP or use of PVP in ICSI medium on the embryo development. Until now all the researchers dealing with goat ICSI used PVP (Wang *et al.*, 2003; Jimenez-Macedo *et al.*, 2006; Keskinetepe *et al.*, 1997; Abdullah *et al.*, 2008; Rahman *et al.*, 2008c, 2009). Therefore, PVP will still be the substance of choice in ICSI unless its potential harmful effects are proven to be true or a safer and better alternative is found.

Polar body orientations of the oocyte for ICSI: During ICSI, the MII oocyte is held under minimal suction by the holding pipette with orientation of the PB-1 at either 6 or 12 o'clock. This orientation has been used in the first ICSI pregnancy in human (Palermo *et al.*, 1992) and thereafter

continued to be used in human and animal ICSI because, damage to the MII spindle could be avoided. Although there was no significant difference in the survival or normal fertilization rates when injection of oocytes were compared between 6 and 12 o'clock PB orientation, injection of oocytes at 6 o'clock PB orientation resulted in higher number of good quality embryos (Nagy *et al.*, 1995) and possible pregnancy rates (Van Der Westerlaken *et al.*, 1999). Except for the injection of the oocyte at 9 o'clock, normal fertilization can occur irrespective of the other PB orientations. However, the PB orientation that could result in the sperm deposited near the MII spindle subsequently could result in better development of the embryo. In a retrospective analysis of oocyte fertilization and embryo development with respect to sperm deposition during ICSI (Blake *et al.*, 2000) demonstrated that injection of human oocytes with the PB oriented at 7 or 11 o'clock position resulted in higher number of high quality embryos, significantly more than the conventional 6 or 12 o'clock PB orientation. However, till now, goat oocytes are injected either at 6 or 12 o'clock PB orientation (Keskinetepe *et al.*, 1997; Wang *et al.*, 2003; Jimenez-Macedo *et al.*, 2007; Rahman *et al.*, 2007; Abdullah *et al.*, 2008).

General ICSI technique: Although the timing of sperm injection does not significantly affect the survival and fertilization rates of the oocytes and embryo quality (Van de Velde *et al.*, 1998), higher number of good quality human embryos can be obtained when ICSI is performed between 1 and 9 h after oocyte recovery (Yanagida *et al.*, 1998). However, unlike human, in goats when oocytes collected by LOPU 36 h (Baldassarre *et al.*, 2003), which is a general practice for OR from live donors, or even 60 h (Abdullah *et al.*, 2008; Rahman *et al.*, 2008b) after eCG treatment are still at the immature stage and require IVM for 27 h before being able to acquire meiotic competence. The ICSI technique performed by most human IVF centers require that the oolemma is broken at the point of injection before the sperm is delivered into the ooplasm (Dozortsev *et al.*, 1995a; Palermo *et al.*, 1996; Vanderzwalmen *et al.*, 1996; Joris *et al.*, 1998). Ooplasmic aspiration to induce oolemma breakage has been considered as an integral part of the ICSI procedure as it ensures that the sperm is in the oocyte and in intimate contact with the ooplasm. Factors that are present in the ooplasm promote the formation of MPN (Tesarik and Kopečný, 1989; Montag *et al.*, 1992). Ooplasmic aspiration is also proposed to trigger the initial steps in inducing oocyte activation by an artificial Ca^{+2} influx generated by ICSI Tesarik *et al.* (1994) and Tesarik (1998). It is to be remembered that ooplasmic aspiration is a

delicate part of the technique. Gentle ooplasmic aspiration within the needle would result in higher survival rate of the oocytes and embryo quality (Vanderzwalmen *et al.*, 1996; Carrillo *et al.*, 1998). Vigorous ooplasmic aspiration as advocated by Tesarik and Sousa (1995) as a crucial step to the success of ICSI would instead increase the rate of oocyte damage and did not improve fertilization rate. The amount of ooplasm aspirated depends on the size of the needle and the speed in which it is performed. Minimal amount of ooplasm aspirated with a slow and gentle aspiration ensures that the oocyte is not unduly traumatized. On the other hand, large amount of ooplasm aspirated with or without a sudden aspiration will alter the integrity of the oocyte resulting in degeneration, failure to fertilize or poor embryo quality. Likewise, gentle re-injection of sperm with minimal amount of PVP is preferred. This can be achieved by placing the sperm at the tip of the needle that has already been positioned close to the ZP prior to injection. The sperm should be injected through the oocyte at 9 o'clock position. Similar procedure is also followed during ICSI of farm animal oocytes including goat (Keskinetepe *et al.*, 1997; Wang *et al.*, 2003; Jiménez-Macedo *et al.*, 2007; Abdullah *et al.*, 2008; Rahman *et al.*, 2009). The average time taken to inject an oocyte is about one minute. It is to be remembered that oocytes are not recommended to expose on the micromanipulation microscope for more than 10 minutes (Keskinetepe *et al.*, 1997); less exposure time will provide better results. To maintain proper temperature, a transparent heating plate or stage warmer is generally used on the ICSI microscope. The temperature of the stage should correspond to the temperature that is maintained inside the CO₂ incubator and must be specific for species. For goat, 38.5 to 39°C temperatures is generally maintained (Keskinetepe *et al.*, 1997; Wang *et al.*, 2003; Jiménez-Macedo *et al.*, 2007; Abdullah *et al.*, 2008; Rahman *et al.*, 2009). To keep the pH of the ICSI medium at 7.2 to 7.4, HEPES is supplemented in the ICSI medium (Keskinetepe *et al.*, 1997; Wang *et al.*, 2003; Jiménez-Macedo *et al.*, 2007; Abdullah *et al.*, 2008; Rahman *et al.*, 2009).

ICSI technique in relation to flexibility of the oolemma:

The oolemma characteristic is also important for efficiency in ICSI. The oolemma of oocyte is flexible or elastic; however, in some cases flexibility or elasticity of oolemma is very high or low. As ICSI begins with the insertion of a needle, damage to the oocyte, especially those having very high or low elasticity, cannot be avoided. Although advances in ICSI technology have improved oocyte survival rates of 50 to 70% to current level of 85 to 90% or even better (Tucker *et al.*, 1996; Van Steirteghem *et al.*,

1996; Devroey, 1998; Yanagida *et al.*, 1999), but ICSI technicians acknowledge the high probability of oocyte damage sometimes, even if the ICSI is performed carefully and properly with oolemma of normal elasticity. When the oolemma is less elastic, the ICSI needle can penetrate easily as soon as inserted into oolemma and when it is more elastic become very difficult to penetrate. Therefore, needle with a sharp spiked tip is generally used to minimize damage to the oolemma during ICSI (Nagy *et al.*, 1995; Palermo *et al.*, 1996). However, the processing of needle tips requires skill and it is difficult to obtain tips of consistent sharpness (Yanagida *et al.*, 2001). The size of the sperm head of buck is bigger than that of human and as a result the pore size of the injection needle in goat ICSI is larger than that used for human ICSI. Therefore, like in bovine ICSI, breakage of oolemma by aspiration results in remarkable damage in goat (Rahman *et al.*, 2007). Also, oolemma of goat oocyte is elastic like that of cattle and mouse and therefore difficult to puncture them by conventional ICSI (Horiuchi and Numabe, 1999). To overcome this difficulty, avoid damage and improve the efficiency of ICSI, a Piezo-driven procedure has been developed (Kimura and Yanagimachi, 1995) by which oolemma breakage can be done using completely flattened tips. Using a Piezo-driven micromanipulator, ICSI efficiencies were dramatically improved in mice (Kimura and Yanagimachi, 1995), cattle (Katayose *et al.*, 1999), human (Huang *et al.*, 1996), rats (Hirabayash *et al.*, 2002), horses (Choi *et al.*, 2002) and goat (Wang *et al.*, 2003). This technique is far less traumatic to mouse oocytes than the conventional method using a mechanically-driven pipette when performing ICSI (Kimura and Yanagimachi, 1995). The Piezo-electric effect is used to produce a stabbing, punctuate movement to puncture the oolemma with minimal distortion of the oocyte. In Piezo-ICSI aspiration of ooplasm is not necessary which can reduce the chance of oolemma damage. The incidence of normal fertilization was about 10 times higher in Piezo-operated oocyte than in oocytes injected using the conventional method (Kimura and Yanagimachi, 1995). This technique is now extensively used in human and, in some extent, in animal ICSI. In goat, using Piezo-driven ICSI with a sharp spiked needle tip, high fertilization and embryo developmental rates were obtained without any activation treatment (Wang *et al.*, 2003). However, this technique has not been popularized in goat ICSI yet. Therefore, goat ICSI is still done conventionally without the used of a Piezo-driven micromanipulator and usually sperm or oocytes are treated to get better fertilization and embryo developmental rates (Keskinetepe *et al.*, 1997; Zhou *et al.*, 2004; Jimenez-Macedo *et al.*, 2005-2007).

In vitro matured oocytes and ICSI outcomes: *In vitro* matured oocytes are more vulnerable to damage by *in vitro* manipulation than *in vivo* matured oocytes (Leibfried-Rutledge *et al.*, 1987; Pivko *et al.*, 2003). If the IVM conditions are not optimal, it can lead to decrease in inositol IP₃ and glutathione stored via Cumulus Cells (CCs) which may give rise to the possibility that this may adversely affect oocyte activation and MPN formation (Chian *et al.*, 1994; Geshi *et al.*, 1999). The amount of stored glutathione in the ooplasm of bovine oocytes was low that underwent IVM in unsuitable culture conditions (Geshi *et al.*, 1999), that is depletion of glutathione in the oocytes block decondensation of the MPN during fertilization (Sutovsky *et al.*, 1997; Sirard *et al.*, 2006). Most of the oocytes from caprine ovarian follicles with diameter of 2 to 6 mm progress to MII stage after IVM (De Smedt *et al.*, 1994; Crozet *et al.*, 2000). However, these oocytes might not be effectively activated by artificial activation because IVM causes disturbances in nuclear, ooplasmic and CC maturation which would normally occur simultaneously *in vivo* (Sato, 1991; Kikuchi *et al.*, 2000). It is found that bovine oocytes after 28 h of IVM can be easily activated, although the developmental competence of the embryos obtained was low (Long *et al.*, 1994). Hence a period of 26 to 27 h after IVM is probably the optimum activation timing of non-aged oocytes as this time duration provided the highest number of viable embryos after ICSI (Wang *et al.*, 2003; Rahman *et al.*, 2008a). In bovine it is found that extended time after IVM adversely affect PN formation and subsequent embryo developmental competence of IVF or ICSI-derived embryos (Long *et al.*, 1994; Balakier *et al.*, 2004).

Oocyte morphology: Oocyte quality is an important prognostic factor as the nuclear and ooplasmic maturity of the oocyte may be directly related to the success rate of ICSI (Kahraman *et al.*, 2000). A search of available literature could not generate any work that relates oocyte quality or morphology with the outcome of ICSI in farm animals especially in goat. However, there are a number of studies reported in human in this regard. Oocyte denudation prior to an ICSI procedure has revealed that a significant proportion of oocytes exhibit different morphological abnormalities, such as excessive granularity, vacuolarization, clustering of smooth endoplasmic reticulum, refractile bodies, large PVS and fragmented PB. Although, morphological appearance of goat oocyte is quite different from that of human, it is likely that a similarity in oocyte dysmorphism may exist. The extent to which these abnormalities can interfere with the normal process of fertilization following ICSI is controversial. Some authors did not find any correlation

between oocyte morphology, fertilization rates and embryo quality (De Sutter *et al.*, 1996; Balaban *et al.*, 1998), others have reported a significant influence of oocyte morphology on fertilization rates and embryo quality after ICSI (Sehral *et al.*, 1997; Xia, 1997; Rahman, 2008). In their study Lourtradis *et al.* (1999) found human oocytes with dark ooplasm; ooplasmic vacuoles and fragments led to poor-quality embryos. Although normal fertilization and embryo development were achieved in oocytes with abnormal ooplasm, however, the resulting embryos failed to demonstrate the same implantation potential as those derived from normal oocytes (Sehral *et al.*, 1997). In contrast, no significant differences in fertilization, embryo quality, implantation and pregnancy rates were found after ICSI with normal and abnormal oocytes (Balaban *et al.*, 1998) or oocytes with abnormal morphology or Centrally Located Granular Cytoplasm (CLGC) (Kahraman *et al.*, 2000), although chromosomal abnormality was found with CLGC oocytes in preimplantation genetic diagnosis (Kahraman *et al.*, 2000). Although, not with ICSI, a few reports on oocyte quality and embryo developmental competence after IVF in cattle have been published. Except one (Nagano *et al.*, 1999), all were in the opinion that poor quality oocytes had lower developmental competence than those of good quality oocytes (Nagano *et al.*, 2006, 2007). As till now, no reports published on ICSI outcome in relation to oocyte quality in farm animals.

Sperm parameters: ICSI results are not correlated with the total sperm count as high fertilization and pregnancy rates can be obtained in cases of very low sperm count (extreme oligozoospermia) (Nagy *et al.*, 1995). Sperm morphology is also not much related with ICSI outcome. In their study Lee *et al.* (1996) reported that although some morphological abnormalities, namely amorphous, round and elongated heads are associated with chromosome defects; surprisingly, no increase in chromosome aberrations were found in sperm with large or small heads compared with normal ones. Burrueal *et al.* (1996) injected sperm with grossly misshapen heads into mouse oocyte and obtained high fertilization and embryo development rates (>80%); some of the embryos developed to normal fertile adults. This indicated that a proportion of abnormal sperm carry all the genome and organelles necessary for embryonic development and growth to fertile maturity. Therefore, morphological abnormality of sperm might not necessarily reflect a genetic abnormality of the male gamete rather its inability to cross physiological barriers (Ben-Yosef and Shalgi, 2001). In contrast to other sperm parameters stated above, sperm motility and livability are correlated with ICSI

outcomes as motility reflects sperm vitality. Normally sperm are considered dead when their plasma membranes are severely damaged or lost. Such sperm are no longer motile and will not fertilize oocytes; and obviously these are dead as cells (Goto, 1997). However, normal offspring were obtained from killed or dead sperm from a number of species by using frozen-thawed sperm without cryoprotectant (Goto *et al.*, 1990; Hatakeyama *et al.*, 1994; Hoshi *et al.*, 1994; Wakayama *et al.*, 1998). These reports indicated that sperm injected into the oocytes were dead as cells but the nuclei were alive in terms of reproduction and genetics (Goto, 1997). It was found that initially immotile sperm show some motility after 2 to 3 h of incubation (Nijs *et al.*, 1996). Lower quality embryos tended to be produced from totally immotile (dead) sperm compared with initially immotile sperm. This is probably due to the fact that some of the totally immotile (dead) sperm DNA have already started degeneration process, although pregnancies were reported after ICSI in both cases (Goto, 1997). It was reported that even after freezing-thawing, a significant amount of SF is still present in the sperm because after this treatment sperm are still able to activate mouse oocyte, whereas immotile (dead) sperm failed to do so (Dozortsev *et al.*, 1995b). However, in clinical situation, it is impossible to know which sperm has a normal DNA as some of the immotile sperm may have degenerating or degenerated DNA. Therefore, motile sperm should be selected for ICSI.

CONCLUSIONS

The present state of ICSI in human and animals shows a great deal of promise. Although, nowadays ICSI become a very widely applied means of overcoming infertility in humans, clinical use of this technique has not been started in animals yet. However, in addition to its clinical usefulness, ICSI can be applied for the production of transgenic animals. Besides, ICSI can be a valuable research tool for studying fundamental aspects of how the two gametes interact during fertilization and also the mechanism of fertilization in normal versus abnormal and *in vivo* versus *in vitro* condition, both in humans and animals. Although this technique has been used to produce farm animals (ruminants) offspring, such as cattle, sheep and goats, the efficiency is still far from satisfactory. Although ICSI technique in human has spread rapidly due to its relative simplicity, short-term safety and high rate of success in achieving apparently normal fertilization in cases of previously intractable male infertility, its long-term safety is still unknown. The effects of sperm morphology on pregnancy outcome and congenital malformation are also unknown. The efficacy

and safety of sperm immobilization process and use of PVP has been questioned as PVP is currently accused of having a potential harmful, mutagenic or carcinogenic effect. As ICSI is still a novel technique, there is ongoing concern about the possible risks to the resulting offspring, particularly with regard to the chromosomal abnormalities. Unlike in human, clinical use of ICSI in goat may not be economically feasible at this moment due to fewer investigations to solve the basic developmental constrains both *in vitro* and *in vivo*.

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