PRONUCLEI FORMATION SUBSEQUENT TO INTRACYTOPLASMIC SPERM INJECTION IN BOVINE

G.GODJA, Ana-Maria RAŢIU, Georgiana UNGUREANU, T. KELLER, Simona ZARCULA, Andreea CIOBOTA, G. OTAVĂ, Camelia TULCAN, I. HUŢU, C. MIRCU
University of Agricultural Sciences and Veterinary Medicine King Michael I of Romania from Timișoara, Faculty of Veterinary Medicine
calinmircu@usab-tm.ro

Abstract
ICSI represents the top of the range of the assembly protocols of assisted reproduction which presumes the existence of sophisticated equipments, prior acquired routine in preparing the oocytes and sperm and a very good organization and synchronization of the working time. The aim of the present paper is represented by the assembly ICSI technique in the assisted reproduction laboratory within CLC-HC Timisoara . 70% of the oocytes were considered able for ICSI and out of this number 12 (17%) were destroyed meanwhile micromanipulation; the remaining were fertilized using sperm treated with PVP (G1), TritonX (G2) or untreated sperm (control). Taking into account the outcome results the superiority of the method that prepares the sperm with Triton X, even though the fertilization percentage (35%) is clearly inferior toward the ones reported (60-80% by Sekhavati et al., 2012). Using PVP to prepare the sperm has generated a lower percentage of success (30%) besides a overwhelming proportion of oocytes with one pronucleus (1PN-80%) or unfertilized (NE-20%). Previous reports emphasize the fact that the volume of PVP which gets in the oocyte cytoplasm consecutively injecting the sperm can have a harmful effect on the zygote. The efficiency of Triton X to remove the acrosome, in this way dislodging a consistent enzymatic volume and allowing to decondense the male pronucleus it is demonstrated by the 7 fertilized oocytes (35%) by injecting the sperm treated previous with this solution. Untreated sperm it has generated the oocytes fertilization only in proportion of 11% whereas the male pronucleus does not cover in an useful time and requested proportion the transformations needed to support the fecundation. The culture media used for gamete and zygote preparation had generated lower results toward the reports in the literature. The modest outcomes can be explained both through the culture media composition and through the long execution of ICSI. The presence of both pronuclei in some of the oocytes subdued to ICSI (between 30 and 35% depending on the substance used to treat the sperm), proves our capacity to assemble ICSI technique in the assisted reproduction laboratory within CLC-HC.
Key words: *ICSI, bovine, PVP, TritonX*

Assisted reproduction has massive benefited of the appearance and using the intracytoplasmic sperm injection (ICSI), followed by the birth of many products. Despite the success, there is still pondering the likelihood that ICSI be responsible for the increased infertility in males, hetero- and autosomal chromosome aberrations and mental, physical or reproductive anomalies registered in the offspring that have resulted from the use of this technique. Currently, due to the progress recorded in the use of ICSI, using immature sperm or those with abnormal morphology it is likely to have increased the risks associated with the use of this technique.

Cattle have represented a promise due to the technique well established, but the results were not as expected, with a limited success or even getting a single descendant (Goto et al., 1990), although the published results of Wei and Fukuy (2000) proofed that the oocytes of cattle, consecutive ICSI, are activated and undergo normal fertilization, comparable with IVF, but the division and the blastocyst formation are modest. Unlike in human, in cattle is necessary to apply an activating stimulus of electrical or chemical nature subsequent ICSI, in order to ensure the resumption of meiosis (ionophore calcium A23187 or ethanol).

Keskintepe and Brackett (2000) have enabled oocyte at 30 minutes after injecting the spermatozoon by incubating in a medium containing 50 µM of the ionophore of calcium (A23187) for 5 minutes; the bull sperm was capacitated before injection by incubating it for 1 hour in medium containing heparin. Thus were obtained the rates of division of the blastocyst of 52.4% and 24.4% with reference to the development of the blastocyst.

Chung et al. (2000) have analyzed a method of activation that mimic the oscillations of calcium observed during natural fertilization of cow: the oocytes were activated by three consecutive incubations of 5 minutes each with ionomycin, the periods of incubation being separated by breaks of 30 minutes. Their results have confirmed the observations of those who claimed that only the technique of ICSI itself is not sufficient for the activation of oocytes. They called the partial activation that they have identified as metaphase III (a abnormal stage in which the chromosomes remain condensed after telophase II due to insufficient activation of the ooplasm). They also assumed that such a partial activation could sporadically ensure sufficient cytoplasm factors to initiate the formation of female pronucleus but not enough to process the very stable spermatozoon of bull (they have a very tightly packed core because it contains protamins of type I that are the most cross-linked of all protamins). They assumed in equal measure that the partial activation could be occurring due to the membrane of the spermatozoon that it remains intact. Subsequent investigations have shown that the use of immobilized sperm has led to the improvement of the activation degree of the oocyte as well as the percentage of fertilization.

The successful use of ICSI in cattle is mainly limited due to the reduced capacity of bull sperm to decondense after ICSI. In the last stages of sperm maturation, the nucleus powerful condenses by replacing the histones with protamins and stabilizes by forming numerous disulfide links between protamine. Reduced glutathione (GSH), the endogenous reducer of disulfide plays a key role in the development of the germinal vesicle in the stage of metaphase II as well as in ensuring the protection of the cell against oxidative stress. GSH plays an important role in spermatozoon decondensation and formation of the male pronucleus by reducing the S-S ties to S-H groups in the chromatin structure of the spermatozoon during fertilization and the degree of decondensation of the SPE nucleus at the time of ICSI affect its success (Sekhavati et al., 2012). ICSI avoids the competition that occurs between the sperms in the female reproductive tract as well as the interaction of sperm-oocyte at the levels of zona pellucida and oolema and through these features proves its effectiveness when the number and the quality of the SPE are inadequate for the completion of IVF. In cattle, the success rate of ICSI is restricted by the low proportion of head decondensation of the sperm consecutive ICSI. Activating the oocytes with additional stimuli during ICSI increases the free fraction of cytosolic calcium that will determine the destruction of the cytostatic factor as well as the degradation of the maturation promoting factor (MPF) essential for the resumption of the second meiosis as well as for the formation of the two pronuclei. The activation of the oocyte can be induced by different physical or chemical factors, including electric throb, ionomycin, calcium ionophore, 6-dimetylaminopurine and ethanol, obtaining different results depending on the species.

Consecutive ICSI, the intact acrosome as well as the attendant perinuclear sheath must be removed by the cytoplasm of the oocyte. These events generate extension of the remodeling process of the spermatozoon head as well as the creation of an asymmetry in the chromatin decondensation. Due to the persistence of the perinuclear sheath at the base of the acrosome, the access to the apical part of the spermatozoon chromatin as well as its remodeling are affected creating a situation in which the rear portion of the spermatozoon chromatin is decondensated when the portion of the apical remains unaffected. This postponement of the partial decondensation of chromatin generates a delay in the process of nuclear remodeling including the recruitment of the constituents of nuclear pore as well as delaying the replication of DNA and the first cell cycle after fertilization. From these considerations, the removal of the acrosome or at least its deterioration would lead to superior results of ICSI (Varghese et al., 2005).

The use of Percoll to select useful sperm presents two major advantages: first, the mobile sperms are effectively isolated and secondly, the mobile sperm will be easily selected from the seminal plasma, somatic cells, and dead sperms or immobile. In spite of the indisputable efficiency in the sperm selection, the Percoll method can lead to the calcium coupling and in this way the fertilization interferes.

The failure of ICSI in cattle seems to be due to the incomplete activation of the oocyte generated by the late disintegration of the plasmatic membrane of the spermatozoon inside the oocyte as well as the incorporation of the acrosome into the oocyte which contains a wide spectrum of hydrolysing enzymes. Between the attempts to improve these shortcomings it is stated the individual spermatozoon dismemberment shortly before the injection with the help of lysolecithin- a hydrolysis product generated from membrane phospholipids.

The radical difference between fertilization and ICSI lies in the fact that in the latter, both the plasma membrane and the acrosome are introduced into the oocyte, the contents of the acrosome may exert a harmful effect on the oocyte (Morozumi et al., 2006). Another notable difference between fertilization and ICSI is represented by the fact that the repeated transient elevations of intracellular Ca^{2+} from the oocyte (Ca^{2+} oscillations) - the key signal of the oocyte activation - begins much later in the oocytes subjected to ICSI. The oocyte activation, as evidenced by the completion of meiosis, appeared much earlier in the cases in which the spermatozoon was released from the plasma membrane prior to ICSI.

The quick disintegration of the spermatozoon membrane is important because the activation of the oocyte depends on the oocyte activation factor induced by the spermatozoon (SOAF – sperm-borne oocyte activating factor). In mammals, the SOAF is represented by phospholipase C, localized both in the perinuclear sheath of the acrosomial region (TPRA) and under the plasma membrane of the equatorial segment of the acrosome (MPSEA). Injecting a whole SPE allows the exposure of the SOAF to the oocyte cytoplasm just after the plasma membrane of the two regions disintegrates. Degradation may occur more quickly in some eggs than in others. If the plasmatic membrane of the spermatozoon is removed before ICSI, the SOAF will leak out into the environment and it will be lost. The SOAF coupled to the perinuclear sheath (TPRA) will remain and will be exposed to the ooplasma only after the injection that triggers the Ca^{2+} oscillations. The phenomenon is almost similar with natural fertilization, when SOAF comes into contact with the ooplasma, consecutive to the membranes merger of the spermatozoon and the oocyte.

Another reason for enhancing the embryo development consecutively using chemically treated sperms could be also the fact that the acrosome could remain on the outside. TritonX removes both the membrane of the spermatozoon and the acrosome which contains many enzymes (hydrolases) with a potential negative effect on zygote.

The curtailment of the interval elapsed between the destruction of the membrane of the spermatozoon and the time of ICSI is essential. As this interval is longer, the greater is the likelihood of damaging the spermatozoon nucleus. Endogenous nucleases, which are activated by Ca^{2+}, can cleave the sperm DNA when the core of the spermatozoon is directly exposed to the artificial environment.

The fusion of the sperm and oocyte increase the intracellular concentration of the free Ca^{2+} and stop the histonH1-kinase activity. This determines the production of IP_3 (inositol 1, 4, 5-tri phosphate) at the receptor level via protein G as well as the production of tyrosine-kinase. Through a specific receiver, IP_3 stimulates the type 1 receptor IP_3. This generates an increase of free Ca^{2+} through its exit from the endoplasmic reticulum. The Ca^{2+} ionophore is one of the most used artificial factors for the oocyte activation and balancing the maturation supporting factor. Ethanol stimulates the activation of metaphase II of oocytes and leads to the

IP_3 stimulation from the plasma membrane in the late embryo stages. The combined treatment of oocyte activation consists in phosphorylation or inhibitors of protein synthesis (Korkmaz et al., 2013).

The intracytoplasmic sperm injection technique (ICSI) represents the top of the range of the assembly protocols of assisted reproduction and some of the stages that form it represent the initial steps for cloning, generating and using of stem cells or induced pluripotent cells.

The execution of this technique presumes besides the existence of sophisticated equipment and a prior acquired routine in preparing the oocytes and sperms, and a very good organization and synchronization of the working time. Considering this aspects, by going through all the necessary steps, the aim of the present paper work was represented by the assembly of ICSI technique in the assisted reproduction laboratory within CLC-HC Timisoara and also by the evaluation of the obtained results reflected by attesting the fecundation, phenomenon mirrored by the presence of both pronucleus (female and male).

Materials and methods

Oocytes were obtained from slaughtered cows ovaries. The ovaries were transported in 0.9% NaCl solution within two hours to the CLCHC laboratory of assisted reproduction. Oocytes were harvested by suction (Simona Marc-Zarcula et al, 2015) and only first quality oocytes were cultivated, according to the protocol described by Simona Marc-Zarcula et al, 2014. Since the cultivation time for cattle may be between 22 and 24 hours, after 22 hours from the time of placing in the incubator, the oocytes were subjected to examination for the presence of the first polar body. Oocytes were denuded by vortexing for 30 seconds in TCM199 medium containing 0.1% hyaluronidase (Sigma) and washed in two steps of PBS. Oocytes suitable for the next stages of the ICSI technique were distributed to group 1 (G1) and injected with sperm treated with PVP, Group 2 (G2) - injected with sperm treated with TritonX and the control group (M), in which the injected sperm was not subjected to any treatment affecting their movements or cell membrane.

After thawing, the sperm was evaluated from the point of view of motility on a scale from 0 to 5 and was used only if it was at least 4. The defrosted sperm was selected with mini-Percoll gradients (90% and 45% - Mircu et al, 2015) and resuspended in Sperm-TALP medium. Afterwards they were treated with PVP, TritonX or used directly. PVP is a polymer used in ICSI procedure to increase the viscosity of the sperm solution, facilitating sperm handling and immobilization. For ICSI, the sperm is first suspended in a medium containing PVP and a single sperm is selected and injected into the oocyte, along with a small amount of agent. PVP (10%) (40,000 IU) was prepared by dissolving PVP in culture medium (MSOF active solution). The solution was aliquot and store in refrigerator for up to 2 weeks. Triton X - 50 µl of the sperm suspension was mixed with an equal volume of TCM. The mixture was vortexed 0^0C for 1 minute. The mixture was vortexed at 0^0C for 1 minute. Sperm is washed in TCM by centrifugation (2000x G for 3 minutes) before ICSI. According to literature, the time between preparation of sperm and injection into the oocyte should not exceed 30-45 minutes (Nakai et al., 2011). All steps were performed at the NARISHIGE micromanipulator Axiovert 40 CFL, equiped with a NIKON inverted phase contrast microscope. After removal of cumulus cells, oocytes were put in drops containing 5 µl of IVF-TALP (in vitro fertilization-Tyrode's albumin lactate pyruvate). The sperm was transferred into 10µl culture medium (Sp-TALP) containing 10µl / ml heparin. Before the injection of SPE, 4% PVP was used to slow them down. Each SPE for injection was selected from those who placed into 10µl drop of PVP acceded there head to the bottom of Petri plate. The aspiration needle was used to pierce the oolemma, forming a access to the clear area of the ooplasm, and then was sucked into a small volume

pipette ooplasm, , and then was sucked a small volume of ooplasm into the pipette. SPE and ooplasm sucked were then injected into oocyte with a minimum volume of PVP. ICSI was performed under a microscope, magnification of x200, in drops of 30µl of medium TCM199 + BSA 3 mg / ml covered by the silicone oil, the culture plate 60x15 mm (Falcon, Fisher Scientific) and maintained at 37 ° C in the plate heating microscope. Two drops of TCM199 medium + BSA were positioned on the fertilization micro-plate in witch were introduced five oocytes and were covered with a drop of mineral oil. On the same micro-plate were positioned two drops SOFplus PVP medium or TritonX from which was extracted one sperm. Each SPE for injection was selected from those who placed into 10µl drop of PVP acceded there head to the bottom of Petri plate. The aspiration needle was used to pierce the oolemma, forming access to the clear area of the ooplasm, and then was sucked into a small volume pipette ooplasm, and then was sucked a small volume of ooplasm into the pipette. SPE and ooplasm sucked were then injected into oocyte with a minimum volume of PVP. The oocyte was fixed in micromanipulator with polar body at 6 o'clock and sperm was injected perpendicular (about 3 o'clock) in the perivitelin space after the oocyte membrane cell was dotted and a small amount of cytoplasm drained. After ICSI, the oocytes were transferred to the culture medium represented by TCM199 + BSA 3 mg / ml covered by the silicone oil, the culture plate 60x15 mm (Falcon, Fisher Scientific) and placed in an incubator at 37 ° C, humidity 100% and 5% CO2. 18 hours after sperm injection, oocytes were examined for the presence of pronucleus (Sekhavati et al., 2012). The existence of both pronuclei attests the full completion of fertilization process. Depending on the aspects observed under a microscope, the oocytes were classified into the following categories: 2N (presence of both pronuclei and absence of sperm), 1N (there presence of female pronucleus and the sperm head) and NE (oocytes inactivated / unfertilized inside which no pronucleus was observed). Garcia-Mengual et al. (2015) attests activation of the oocyte through its ability to resume meiosis without the presence of a visible metaphasic plate and having at least one pronucleus.

Results and discussions

The results achieved by the ICSI technique are presented in Table 1 and Chart 1.

Table 1.

The results obtained by the treatment of sperm

Group	Oocytes x ICSI	2PN		1PN		NE	
		n	%	n	%	n	%
G1	20	6	30	10	50	4	20
G2	20	7	35	8	40	5	25
G3	18	2	11,11	3	16,6	13	72,44

During the passage through the epididymis the mammals SPE is highly resistant to chemical and physical disintegration due to conversion of protamines sulfhydryl group in disulfide groups. SPE cattle nucleus possesses particularly strong disulfide bonds, which could explain the presence of stable chromatin, which could prevent decondensing of the nucleus. In ICSI procedure, puncture of the oolemma or aspiration of cytoplasm with micropipette can contribute to oocyte activation. Centrifuging clarifies ooplasm by polarizing lipids, which are recognized in the mask of cellular organelles in most ungulates. Tatham et al. (1996) states that the centrifugation of oocytes (15800xg for 10 minutes) prior to the ICSI procedure contributes

to better visualization of the injected SPE, while Wall and Hawk (1988) believe that the centrifuging does not influence the further development as the polarization of the ooplasm is only temporary. Rho et al. (1998) observed redistribution of lipids in the ooplasm 10 hours after centrifugation. After removal of the cumulus cells, about 70% of the in vitro matured oocytes had GP visible and a dense and uniform ooplasm making them thereby suitable for ICSI (Rho and col., 1998). Of the 761 oocytes injected, 57 (8%) were destroyed in the process of micromanipulation and degenerated within 3-24 hours. Other 32 (or 4%) expelled the SPE into perivitelin space.

The amplitude and the increased concentrations of Ca2 + was believed to play an important role in the events resulting in activation of the oocyte, including regulation of maternal mRNA stored in the early zygote, which could affect gene expression in the late stages of embryonic development and the cellular composition of the blastocyst (Nakai et al., 2011). In pigs, formation rate of pronucleus was lower when using sperm treated with Triton X compared to those resulting consecutively using sperm intact (Nakai et al., 2011).

Concentration and activity of phospholipase C in SPE may be slower due to the treatment of SPE by processes commonly used prior to ICSI in rent animal species. It seems that the procedures used to decondensing the head of SPE before ICSI in pig also causes the loss of phospholipase C, resulting in a weaker signal oocyte activation. Korkmaz et al's study (2013) demonstrated that transferring oocytes after ICSI in culture medium containing 3 mg / ml BSA and CR1aa and then (on the 5th day) in freshly prepared culture medium and supplemented with 5% calf serum affected positively embryonic development. The same study revealed that the best ICSI results were achieved consecutively ICSI using an ionophore calcium in combination with 6-DMAP and high quality oocyte.

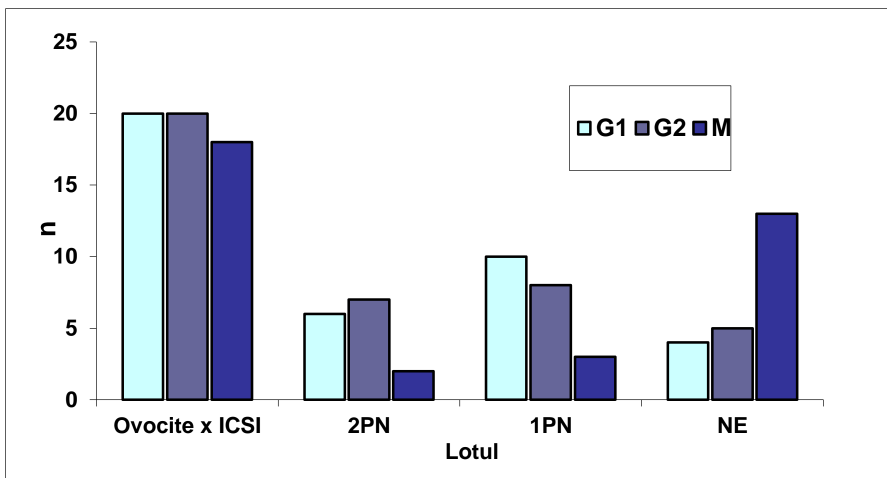

Chart 1. Number of pronuclei variation related to spermatozoa treatment

Out of the 70 oocytes considered fit for ICSI 12 (17%) were destroyed during micromanipulation, a proportion which can vary from case to case depending on both the biological material used and skills of the performer.From the data presented in Table 2 result the superiority of the method which appeals to preparation of sperm with Triton X, even if the percentage of fertilization (35%) is significantly lower than those reported in the literature (60-80% Sekhavati et al., 2012). Chart 1 captures the dynamics of unfertilized oocyte whose percentage rate clearly increases in the situation in which the injected sperm was not subject to any treatment.

Using PVP to prepare sperm generated besides a lower success rate (30%) and an overwhelming proportion of essential active oocytes (1 PN – 80%) or completely inactive (NE – 20%). Specialized studies show that PVP volume reaching oocyte cytoplasm consecutively sperm injection can be harmful for the zygote. The 7 fertilized oocytes (35%) by injecting sperm prepared with Triton X can demonstrate its efficacy knowing that Triton X has the ability to remove the acrosome, deploys in this way a consistent volume of enzyme and allowing decondensing the male pronucleus. Untreated sperm generated only 11% oocyte fertilization – a value highlighted by the literature that the male pronucleus does not make the necessary transformations in useful time to produce and sustain fertilization. ICSI has exceeded the time required to achieve the allowed 30 minutes for contact with sperm with PVP and Triton X in witch in both cases the values close to 60 minutes (59 PVP and Triton X-56). Unlike data reported consecutively human studies, bovine oocytes are not sufficiently activated only by puncture the oolema or aspirating the cytoplasm (Rho et al., 1998). Data presented by Morozumi and Yanagimachi (2005) reveal that when invariably a certain amount of PVP or Triton X reaches the oocyte, injection did not affect the oocyte whether ICSI was removed before the acrosome.

Conclusions

Culture medium and preparation of the zygote generated inferior results those presented in the literature. The modest results could be explained both by composite of the culture medium and long service and execution of ICSI. Both pronuclei in some of oocytes subjected to ICSI (between 30 and 35% depending to the substance used to treat sperm), demonstrates our ability to assemble ICSI assisted reproductive in the CLC-HC laboratory.

Aknowlegdements

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