Generating bovine embryos through ICSI

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Abstract

Through ICSI, competition between sperms and also sperm-oocyte interaction are avoided thus ICSI proving reliable when sperm is not suitable for IVF. In bovine, the limiting step is represented by low rate of sperm head decondensation subsequent ICSI. Intracytoplasmatic sperm injection allows avoiding many critical moments that may occur during normal or in vitro fertilization. Oocytes were obtained from ovaries from slaughtered cows. These were transported in 0.9% NaCl solution in isothermal bags at a temperature of 25-30 ° C. The ovaries were brought from the slaughterhouse within 2 hours. Harvesting of the oocytes was made through the aspiration method. After maturation, oocytes were fertilized using sperm that was prepared using Percoll method and then treated with TritonX. The volume of the TritonX solution that accompanies the sperma and which remains in the oocyte is extremely important given that by its action, TritonX removes the acrosome, thus releasing a rich enzyme content and facilitating the dehydration of the male pronucleus. Even though the number of 2 nucleus, 2 cells or 4 cells oocytes is inferior to the data found in the literature, compared to the results achieved last year in the assisted reproduction laboratory within CLC-HC Timisoara, it marks significant progress. At the 2 cells stage, there were several oocytes from group 1 (24.39% vs. 12.5%), while at the 4 cells stage there were 14.63% oocytes from group 1 and 25% group 2. The use of TritonX solution for sperm treatment as well as shortening the duration of ICSI execution allowed us to get encouraging results. The results obtained are inferior to those presented in the literature but are far superior to those we obtained last year when the ICSI technique was assembled. Achieving the two- and four-cell embryonic stages justifies us thinking that we are mastering the ICSI technique.

Keywords: *bovine, embryos, ICSI, TritonX*

Introduction

Intracytoplasmatic sperm injection allows avoiding many critical moments that may occur during fertilization. Applying this technique involves sophisticated equipment as well as detailed specialist knowledge. It is essential to have a good knowledge of working steps equal to the use of certain specific reagents of the highest quality (Godja et al., 2016).

The ICSI technique in cattle was assembled in CLCHC Assisted Reproduction Laboratory last year, and the results obtained determined us to continue its application. In the present paper, we attempted to demonstrate the implications of using TritonX for sperm treatment as well as reducing working times in order to shorten both the interval in which the sperm is in contact with the slowing solution of their movements and the period of oocytes outside the incubator.

In cattle, intracytoplasmic sperm injection (ICSI) has low efficacy (Canel et al., 2014; Zambrano et al., 2016). The content of acrosome may be considered responsible for this effect due to the large number of hydrolytic enzymes released in oocytes.

In order to eliminate the acrosome and destabilize sperm membranes, researchers at the University of Frontera (Zambrano et al., 2016) chose the use of Lizolecitin and Triton-X 100. The rate of oocyte development was assessed along with pronuclear formation and embryo quality. The use of Lizolecitin and Triton-X 100 (0.01%, 0.02%, 0.03%, and 0.04%) decreased the sperm viability based on the dose used. At the same time, an improvement in the acrosome reaction for all Lizolecitin and Triton-X 100 concentrations was observed, reaching already a concentration of 100% from 0.05% of both treatments.

A higher rate of cell division was observed in the intracytoplasmic injection of sperm treated with Triton-X 100 (66%) and Lizolecitin (65%) compared to the untreated sperm group (51%). At the same time, a significant increase in blastocyst formation was observed in the Lizolecitin treated group (29%) compared to the control group (21%). There was no difference in the formation of pronucleus and the number of embryos developed.

The researchers concluded that sperm treatment with Lizolecitin and Triton-X 100 improves the rate of embryo development without affecting the quality of embryos produced by using this technique.

Galli et al. (2003) studied the effect of various substances used for sperm pretreatment. In the study, the sperm was activated with heparin, D-penicillamine, hypotaurine and epinephrine before ICSI.

The effect of the use of dithiothreitol for pretreatment of spermatozoa or oocytes to be injected was then studied. Activation of sperm with heparin in combination with epinephrine did not improve the development of embryos following ICSI. Instead, the use of dithiothreitol for pre-treatment of oocytes improved cell division and blastocyst formation in the case of inactivated embryos and an acceleration of blastocyst development when embryos were activated. At the same time, sperm pretreatment with dithiothreitol followed by ICSI showed a significant increase in embryonic development rates over the first 7 days.

The study by Canel et al. (2014) aimed primarily at identifying how different treatments applied to semen, such as sperm pretreatment with heparin and L-glutathione or sexing of semen, can influence the development of embryos following intracytoplasmic sperm injection in cattle.

Cell division and blastocysts were assessed at 2 and 7 days after ICSI. The results of the study show a significant increase in the rate of cell division and blastocysts in the pretreated batch with heparin and L-glutathione group compared to the untreated group. The use of sperm semen has also improved the cell division rate, with no major differences in blastocyst counts.

Research has also been carried out on the quality of oocytes and semen (Ohlweiler et al., 2013). During the study, experiments were performed using better or worse quality oocytes and sperm.

The blastocyst formation rate was significantly higher in the case of good quality oocytes (23.3% versus 11.1% in the case of inferior quality oocytes), regardless of the characteristics of the semen. At the same time, there was no major difference in the rate of blastocyst formation using poor quality semen (regardless of oocyte quality). However, the use of good quality semen has been shown to have a major influence on the blastocyst formation rate (25.7% versus 9.2% in the case of poor quality oocytes).

The influence of oocytes and sperm quality on ICSI was subsequently evaluated. No major differences were observed in the blastocyst formation rate irrespective of the quality of oocytes or semen.

The conclusion of the researchers was that the use of intracytoplasmic sperm injection is an effective way to achieve the in vitro generation of bovine embryos irrespective of the quality of oocytes or sperm.

Materials and methods

Oocytes were obtained from ovaries from slaughtered cows. These were transported in 0.9% NaCl solution in isothermal bags at a temperature of 25-30 ° C (Mayes, 2002). The ovaries were brought from the Nojag slaughterhouse, which is 167 km (2h) from the Assisted Reproduction Laboratory of the CLCHC and from Macea, Arad County, which is 80.5 km (1h 9min) from the laboratory. For the harvesting of the oocytes, the aspiration method (Chung et al., 2000) was chosen

and cultivated for those of category I. After harvesting, COC, together with the follicular fluid, were placed in a 50 ml conical tube for sedimentation. After 5 minutes of rest, they were sucked from the bottom of the tube with the help of a pipette. This operation was repeated 4-5 times to make sure all oocytes were sucked together with the cumulus cells.

The next step was to wash the COC by passing successively into 2 PBS plates. Thus, they are prepared for maturing in 4-well plates. After being held in 400 µl of TCM 199 enriched with 10% ESC and coated with mineral oil to prevent oxygen action, for 22h for maturation in the incubator, they were removed from the PBS-washed incubator and then denuded in 0.4 ml of 0.1% hyaluronidase (Sigma) and washed in two steps of PBS.

The sperm strains stored in liquid nitrogen were thawed at 37 degrees for one minute (Rahman, 2010). Sperm was prepared using Percoll method. Through the density gradient (Percoll), dead, abnormal (low density) sperm and detritus (cell contamination) of ejaculate accumulate in the corresponding densities, while the density of the sperm (mobile, normal) crosses the gradient and is found on the bottom of the tube. To eliminate all traces of gradient used, sperm isolated by this method is washed in a culture medium. In order to centrifuge in the concentration gradient, 90% and 45% Percoll solutions were prepared by dilution with Earl 1x solution. The Percoll concentration gradient solution was kept on a water bath for 4 hours at 37 degrees before use. 200 µl of semen from each bull was used. After bringing the samples to 37 ° C, they were slowly added dropwise onto the walls of the centrifuge tube over the Percoll solution in a concentration gradient (90%-45%) pre-heated for 4 hours at 37 ° C. Then the mixture was centrifuged (Hettich 350R) for 20 minutes. After which the supernatant was removed and 2 ml of Earl 1X solution was added. The mixture was homogenized and analyzed for the determination of seminal material parameters. To facilitate manipulation of the sperm and to immobilize it, the use of Triton-X 100 (Sigma) was chosen. It is necessary to mix 50 µl of sperm suspension with an equal volume of TCM 199 and 0.1% Triton-X 100. The mixture is centrifuged at 2000xG for 3 minutes.

After removing the cumulus cells, the oocytes were placed in a drop containing 5 µl of IVF-TALP (in vitro fertilization-Tyrode's albumin pyruvate albumin). The sperm was transferred to 10 µl of the culture medium (Sp-TALP) containing 10 µl / ml heparin. ICSI was performed at a magnification x200 microscope in drops of 30µl of TCM199 + BSA 3mg / ml medium coated with silicone oil and kept at 37 ° C on the microscope warm plate. On the fertilization microplate were placed two drops of TCM199 + BSA medium into which the oocytes were introduced and the drop was covered with mineral oil. A drop containing spermatozoa treated with TritonX was placed on the same microplate, from which a single sperma was extracted (Figure 1 A). The oocytes were fixed in the micromanipulator pipette polar body at 6 o'clock, and the sperm was injected perpendicularly (approximately at 3 o'clock) (Figure 1 B).

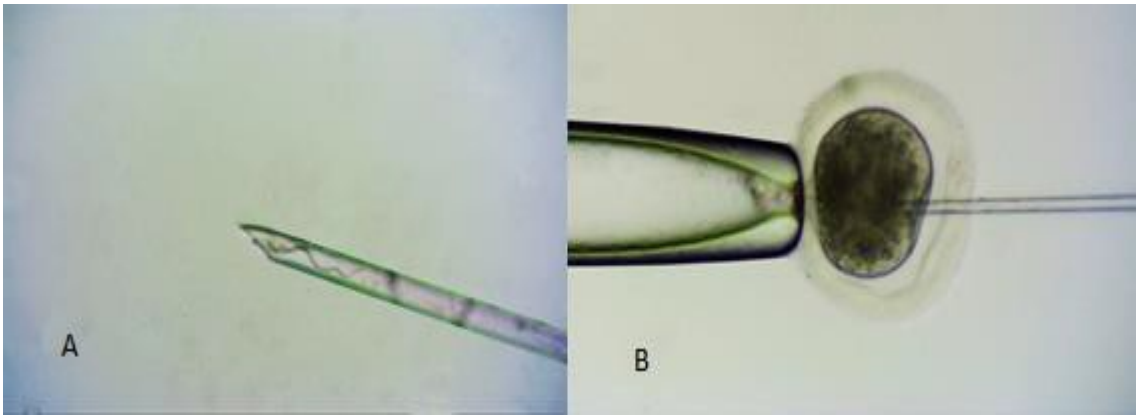

Fig.1. Sperm in micropipette (A) and injecting of sperm into oocyte (B)

After ICSI, the oocytes were transferred to the culture medium of TCM199 + 20% ESC coated with silicone oil in 60x15 mm culture plates (Falcon, Fischer Scientific) and placed in the incubator at 37 ° C, 100% humidity and 5% CO2. 18 hours after sperm injection, oocytes were examined for the presence of the pronucleus. After 72h of ICSI, some oocytes were found to have 2 cells (Figure 2 A) and at 96 hours ICSI had 4 cells (Figure 2 B).

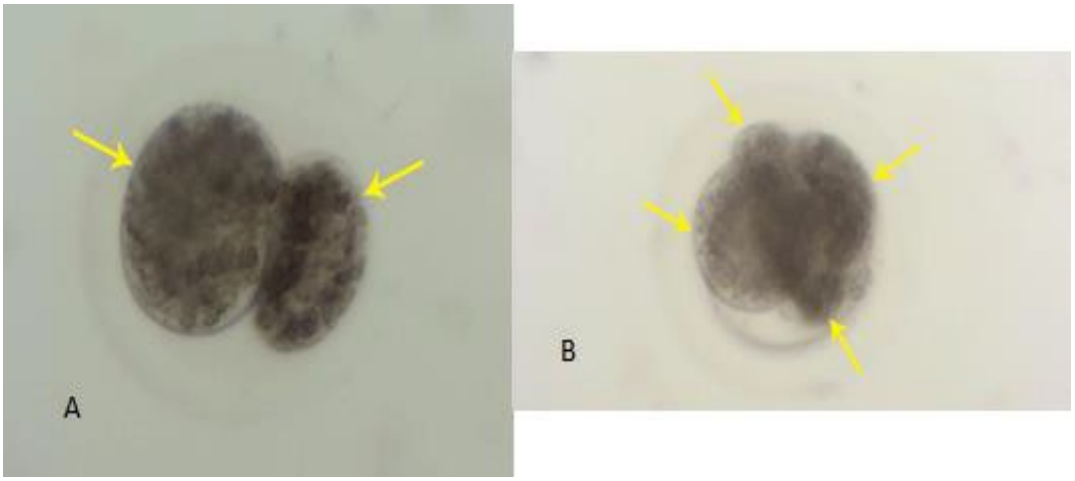

Fig.21. Oocytes with 2 cells (A) and 4 cells (B)

To highlight the division, oocyte staining with Hoechst 33342 (Thermofischer Scientific) was chosen. After they were removed from the incubator they were washed in a PBS step after which they were placed in Hoechst for 15 minutes in the incubator. After this period, they were examined in an immunofluorescence microscope to highlight the cell division (Figure 3).

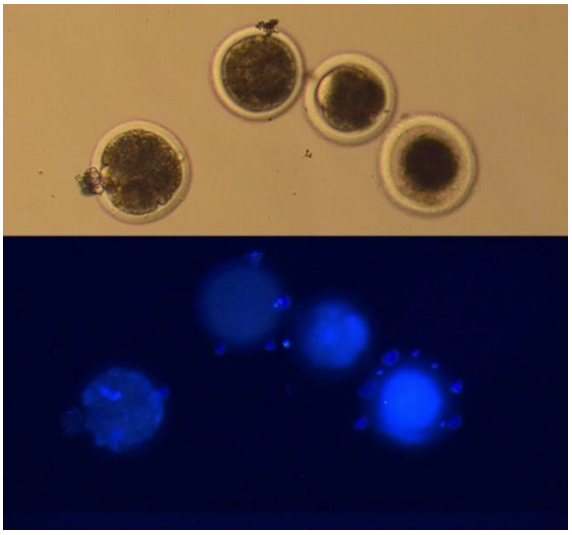

Fig.3. Before and after **using the Hoechst staining**, the presence of 4 cells is evidenced

Results and discussions

Depending on the length of ICSI's own workload (from sperm contacting to injection into oocytes), we divided the 73 oocytes injected into two groups (Table 1):

- Group 1, in which we included the 41 oocytes injected in less than 7 minutes;
- Group 2, containing 32 oocytes injected in more than 7 minutes.

We chose the 7 minute threshold taking into account both the recommendations in the literature and our values (the shortest time was 4 minutes and 3 seconds and the longest 17 minutes and 22 seconds), Values to which we calculated average and standard deviation. ICSI was performed in TCM supplemented with 20% ESC, the oocytes remaining in this medium for the first 24 hours. Subsequently, they were moved for another 24 hours in the maturation medium containing cumulus cells and 48 hours after ICSI time they were again moved to the TCM + ESC20% medium where they remained during the observation period. The 2N stage was also highlighted by the 24-hour examination from ICSI, 2 cells (2C) at 72 hours from ICSI and 4-cell (4C) at 96 hours from ICSI. The embryos remained in those stages for more than 24 hours, at which time we found degenerative processes and lack of further development.

Table 1. Results obtained from ICSI on cattle oocytes

Group	Number of OV x ICSI	Nonfertilized oocytes	Oocytes with one pronucleus	Oocytes with two pronucleus	Fertilized oocytes that have reached the stage of 2 cells	Fertilized oocytes reaching the 4-cell stage
Group 1	41	1	6	18	10	6
Group 2	32	4	4	12	4	8

Group 1, including oocytes subjected to ICSI in less than 7 minutes, had only unfertilized oocytes (2.45%). There were 6 oocytes with one pronucleus (14.63%) and 18 oocytes (43.90%) with two pronucleus. Of these 18, 10 fertilized oocytes (24.39%) reached the stage of 2 cells and 6 oocytes (14.63%) - the stage of 4 cells (Figure 4).

The percentages of fertilized oocytes (as evidenced by the presence of the two pronucleus) as well as those representing the 2 or 4 embryonic stages obtained at G1 are inferior to those presented in the literature but clearly superior to those we personally obtained (Godja et al., 2016).
Gali (2003) reported only poor results subsequent ICSI as cell division and blastocist development, despite bovine oocytes were activated and supposed to develop fertilization similarly with IVF.
Keskintepe and Brackett (2000) activated the oocyte at 30 minutes consequent sperm injection by incubation with A23187 ionophore for 5 minutes. Bull sperm was previously capacitated by incubation in a heparin-containing media. Authors reported 52,4% blastocyst division and 24,4% for blastocyst development.

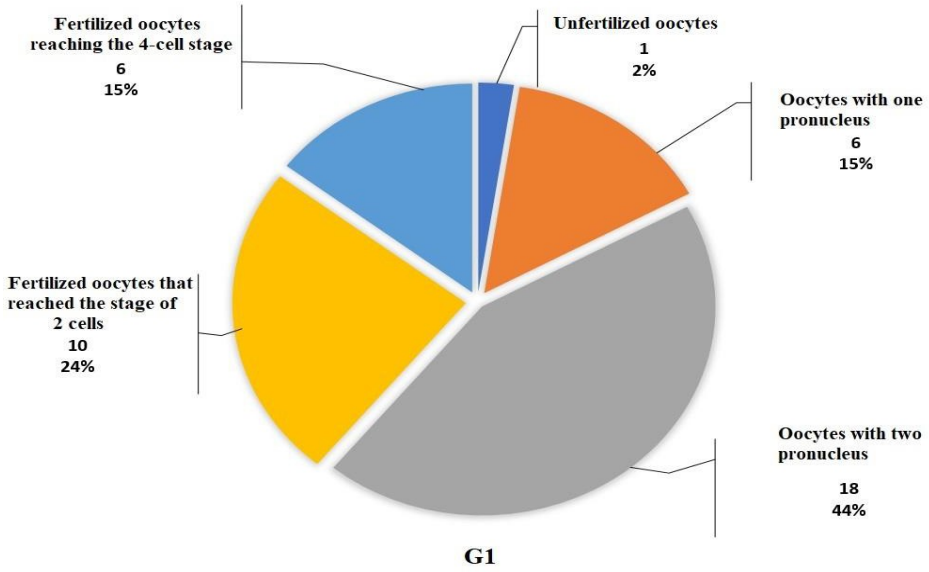

Fig. 2. Results from ICSI on cattle - Group 1

For group 1- containing oocytes at which ICSI occurred over a period of time longer than 7 minutes, there were 4 nonactive oocytes (12.5%) and 4 (12.5%) with a single pronucleus. Of the 12 oocytes (37.5%) who presented the stage of 2 pronucleus, 4 oocytes (12.5%) reached the stage of 2 cells and 8 oocytes (25%) - the embryonic stage of 4 cells (Figure 5).

Figure 1 shows eloquently the differences between the two groups. Even though the number of 2N, 2C or 4C oocytes is inferior to the data reported in the literature, compared to the results we achieved last year (11) marks significant progress. It should be noted that at the 2C stage there were several oocytes from G1 (24.39% vs. 12.5%), while at the 4C stage there were 14.63% oocytes from G1 and 25% G2 (Figure 6).

We consider that the volume of the TritonX solution that accompanies the sperm and which remains in the oocyte is extremely important given that by its action, TritonX removes the acrosome, thus releasing a rich enzyme content and facilitating the dehydration of the male pronucleus.

The results of the ICSI were also influenced by the running time of this work, and it is desirable that it be as short as possible. The experience gained by the ICSI person makes it possible to shorten this interval permanently, ensuring for oocytes spending as short a time as possible outside the incubator.

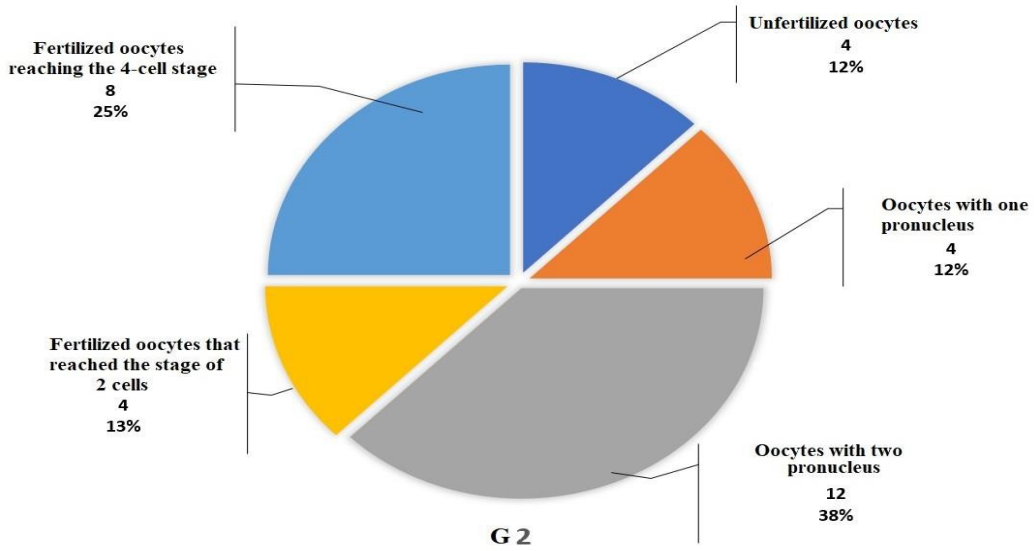

Fig. 3. Results from ICSI on cattle - Group 2

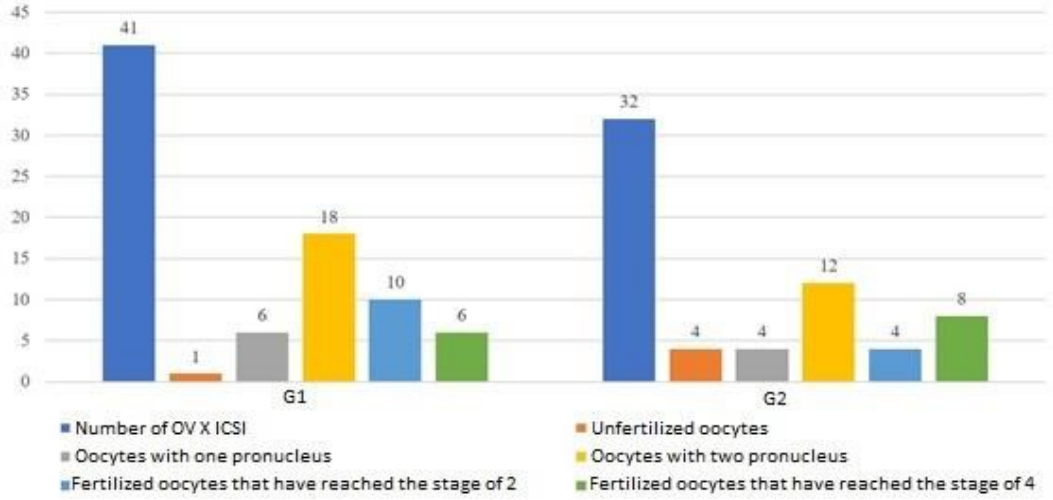

Fig. 4. Results obtained from ICSI on cattle oocytes

We consider important to keep oocytes subjected to ICSI in the culture medium containing cumulus cells, because this significantly improves oocyte metabolism by facilitating the exchange of nutrients.

To grasp any statistical differences, we used the chi test, and a statistically significant difference occurred at $p \leq 0.01$ between G1 and G2, which is of particular interest when we look at the results obtained with two or four cell embryos.

Stages C2 and C4 occurred 24 hours later than the accepted physiological time in bovine embryogenesis, which discusses both the moment of potential oocyte activation and subsequent survival.

The divergent opinions in the literature on whether or not the oocyte is activated following the simple ICSI can support the underlying assumptions regarding the installation of the delayed cell division and its closure after a short time as well as in the case of the 4C embryos the higher success rate registered at G2 compared to G1. In other words, according to our results, the longer duration of ICSI execution has enhanced cell division.

Conclusions

The use of TritonX solution for sperm treatment as well as shortening the duration of ICSI execution allowed us to get encouraging results.

The results obtained are inferior to those presented in the literature but are far superior to those we obtained last year when we assembled the ICSI technique.

Achieving the two- and four-cell embryonic stages justifies us thinking that we are mastering the ICSI technique.

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