Embryo development after ICSI, using spermatozoa from bovine testicular tissue treated with three membrane-destabilizing agents

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ABSTRACT

Objective. To determine the differences in the embryo development of bovine oocytes fertilized with frozen/thawing (F/T) spermatozoa or with the intracytoplasmic sperm injection (ICSI) of F/T, spermatozoa from fresh testicular tissue (FTT), and cryopreserved testicular tissue (CTT), using three spermatozoa membrane-destabilizing agents.

Methodology. Four treatments were used. Treatment (TRT-1): *In vitro* fertilization (IVF) with F/T. TRT-2: ICSI with F/T. TRT-3: ICSI with FTT. TRT-4: ICSI with CTT. The spermatozoa membranes were destabilized with Triton X-100 (TX), Lysolecithin (LL), and Heparin-Glutathione (Hep-GSH). Embryonic division was recorded at 48 h and grade 1 and 2 blastocysts (BL) were recorded 8 days (D8) after the fertilization. The means were compared using Fisher's least significant difference method.

Results. At D8, the blastocysts formation between ICSI treatments (F/T 13±3, FTT 6±3, and CTT 6±3, $p>0.05$) were lower than control (IVF 23±5). There was a lower cleavage at 48 h using Hep-GSH than when LL and TX were used (35±5 *vs.* 50±5 and 56±5, $p<0.05$). Embryo division at 48 h obtained better results with the ICSI+F/T and LL treatment, while the highest blastocyst percentage at D8 was obtained using TX.

Conclusions. Blastocysts can be produced through ICSI, using spermatozoa from fresh or cryopreserved testicular tissue. The spermatozoa treated with TX and LL produced a higher percentage of BL than the spermatozoa treated with Hep-GSH. Further experiments should be carried out using spermatozoa obtained from different sources, in order to improve embryo development after the ICSI.

Keywords: ICSI, vitrification, testicular tissue, oocytes, bovine, fertilization.

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INTRODUCTION

In vitro embryo production (IVP) has benefited genetic improvement and it has also been a research model for the embryo development of several species (Diaza *et al.*, 2013). IVP includes *in vitro* maturation (IVM), in vitro fertilization (IVF), and *in vitro* embryo culture (IVC). Every step is equally important for the production of embryos with a high implantation potential (Consensus Group, 2020). Devroey and Steirteghem (2004) pointed out that the intracytoplasmic sperm injection (ICSI) enables fertilization, even when there are few spermatozoa or they lack motility. This is the case when the spermatozoa come from bad quality testicular spermatozoa or semen.

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The *in vitro* fertilized oocytes percentage in cattle is usually high; however, the fertilization rate using ICSI is lower than in other species (Canel *et al.*, 2017; Canel *et al.*, 2018; Ressaissi *et al.*, 2021). Some of the events that can cause a drop in fertilization include defective sperm head decondensation, inadequate sperm capacitation, and the integrity of the spermatozoa membrane (Rho *et al.*, 1998, Sekhavati *et al.*, 2012, Arias *et al.*, 2015, Águila *et al.*, 2017). One of the main problems with defective sperm head decondensation is the inconsistent reduction of the disulfide double bond of the protamine in the spermatozoa nucleus that takes place after the fertilization (Malcuit *et al.*, 2006).

Sutovsky *et al.* (1997) and De Matos and Furnus (2000) reported that the glutathione (GSH) is a reducing agent of the disulfide double bond and plays a fundamental role in the defective sperm head decondensation of the spermatozoon and the formation of the pronucleus during fertilization. Meanwhile, heparin (Hep) accepts and releases protamine into the DNA, consequently, helping the decondensation of chromatin (Delgado *et al.*, 1999 and Romanato *et al.*, 2003).

Another factor that can damage the cattle embryo development produced with ICSI is the following: when whole spermatozoa are injected in the oocyte, the enzymes in the acrosome are released into the ooplasm and can damage the organelle. Various strategies have been evaluated in order to remove the acrosomal membrane and consequently to facilitate the DNA decondensation and the formation of the male pronucleus. Zambrano *et al.* (2016) reported that the destabilization of the spermatozoa plasm membrane and the release of the acrosomal content through the use of Lysolecithin (LL) and Triton X (TX-100) improved development, without impacting the quality of cattle embryos produced with ICSI.

The above mentioned experiments used ejaculated spermatozoa. However, there is scarce information about the use of these destabilizing agents for the treatment of fresh or cryopreserved sperm cells from testicular tissue. Therefore, the objective of this study was to determine the fertilization rate and the development of cattle oocytes fertilized with ICSI, using testicular spermatozoa treated with Hep-GSH, LL, and TX.

MATERIALS AND METHODS

Sampling collection

The testicles were obtained from a slaughterhouse. They were dissected in their middle area, obtaining approximately 1.0 g of tissue. This sample was kept in a HTF medium, supplemented with 10% human serum albumin until it was used or cryopreserved. The cryopreserved semen of high-fertility bulls was donated by Genética Bovina de México S.A. de C.V.

In vitro maturation of oocytes

The 3-6 cm wide follicles were aspirated (Seita and Kashiwazaki, 2009; Gupta *et al.*, 2016) and placed in a BO-HEPES medium, supplemented with 2 μl/mL of heparin (HP, PiSA, Guadalajara, México). Cumulus oocyte complexes (COC) with dark and homogeneous cytoplasm and at least 2 cellular layers of the cumulus were selected for IVM (Hansen, 2013). The selected COC were washed with BO-HEPES and were transferred

to a BO-IVM medium (IVF Bioscience®, Monterrey, NL, Mexico). They were incubated for 23 h, at 38.5 °C, in an atmosphere with 6% CO_2 and 100% humidity. Maturation was evaluated using the cumulus cell expansion, cytoplasm appearance, zona pellucida integrity, and the presence of a polar body (Gupta *et al.*, 2016).

Preparation of the spermatozoa and in vitro fertilization (IVF)

After the IVM, the COCs were placed in BO-INF droplets (IVF Bioscience®, Monterrey, NL, Mexico) and were cultivated at 38.5 °C, in an atmosphere of 6% CO_2 and 100% humidity. Regarding sperm capacitation, the fertilization straws were defrosted for 45 s in water at 37 °C. The semen was centrifuged at 400 G for 5 minutes (Hansen, 2013). The sediment was reconstituted in 200 μl of BO-IVC (IVF Bioscience®, Monterrey, NL, Mexico) and the concentration was adjusted to 6×10^6 spermatozoa/mL. Ten μL of this semen dilution were added to each BO-IVF droplet (5 oocytes per droplet). Afterwards, they were cultivated during 22 h, at 38.5 °C, in an atmosphere of 6% CO_2, 5% O_2 (balanced with nitrogen), and 100% humidity (Gupta *et al.*, 2016).

Testicular tissue (TT) cryopreservation

The TT was diluted with Tryladil® (Minitube, Querétaro, Mexico). They were aspirated with 0.25-ml French straws, sealing the straws with polyvinyl chloride dust (PVC). The straws were kept at a 5-8 °C temperature for 4 h. Subsequently, they were put in nitrogen vapor for 20 min, before being finally immersed in liquid nitrogen.

Testicular tissue (TT) defrosting

The straws were defrosted for 1 minute using water at 37 °C. The TT portions were washed in HTF-Hepes (In Vitro Care®, Frederick, MD, EUA) at 37 °C. Subsequently, they were placed in a HTF medium supplemented with 10% synthetic serum substitute (SSS) (Irvine Scientific®, García, NL, Mexico). Afterwards, the TT portions were cut using 22" gauge needles and their content were extracted pressing the seminiferous tubules (ST) against the bottom of a Petri dish. The ST content (20 μl) was placed in a Petri dish; it was covered with mineral oil and placed in a Diaphot 300 inverted microscope (Nikon®, New York, NY, USA), set at 200X, in order to locate the spermatozoa.

Spermatozoa treatment

Heparin (Hep) -Glutathione (GSH)

The frozen-defrosted spermatozoa and the spermatozoa from fresh or cryopreserved TT were placed in a BO-SEMEN PREP medium (IVF Bioscience®, Monterrey, NL, Mexico), supplemented with 80 μM Hep and 15 mM GSH, in 20-μL droplets covered for 20 h with mineral oil, at 38.5 °C, in 6% CO_2, before the ICSI process (Canel *et al.*, 2018).

Lysolecithin (LL) and Triton X-100 (TX-100)

The spermatozoa were incubated in BO-SEMEN PREP, supplemented with 0.05% LL or TX (Sigma-Aldrich, Toluca, Mexico). The spermatozoa were stirred for 1 min in a vortex (Zambrano *et al.*, 2016). Once all the treatments had ended, the spermatozoa were

centrifuged at 400 g for 5 min and the sediment was reconstituted with 200 μL of BO-SEMEN PREP.

Intracytoplasmic sperm injection (ICSI)

The ICSI was carried out using an inverted microscope equipped with a micromanipulator and ICSI needles (Zambrano *et al.*, 2016). The injected oocytes were activated placing them in an HTF-SSS medium, supplemented with 7% ethanol for 5 min. Subsequently, the oocytes were washed 10 times in a BO-IVC medium and were cultivated in the same medium at 38.5 °C, in 6% CO_2, 5% O_2 (balanced with N_2), and 100% humidity.

Embryo culture

After a 20 h period of fertilization, the presumed zygotes of the control treatment (conventional IVF) were washed with BO-IVC and were cultivated in 30 μL droplets at 38.5 °C, in 6% CO_2, 5% O_2 (balanced with N_2), and 100% humidity. The injected oocytes were placed in cultivation right after the ICSI and the activation were carried out (Zambrano *et al.*, 2016).

Embryo development evaluation

Forty-eight hours after the fertilization (early cellular division), the divided embryos were transferred to fresh 30 μL BO-IVC drops and were cultivated for 144 h in groups of 5. Embryo development was evaluated at 120 and 144 h after the first evaluation. The final scores were recorded according to the AETA classification (Barfield, 2015).

Statistical analysis

The data were analyzed using a general lineal model, assuming a binomial distribution for the dependent variables (embryo development in D2 or D7-8). The model included a Probit function for the main effects (destabilizers) and their interactions.

RESULTS AND DISCUSSION

Regarding TRT-1 (IVF conventional treatment), the percentages recorded for embryo division and blastocyst development were 61% and 23%, respectively. These results are similar to those obtained by Rizos *et al.* (2001), who recorded 77% (embryo division) and 44% (blastocyst development) results.

The statistical analysis showed significative differences in the embryo development at D2; however, this was not the case in D7-8 for the embryos produced by ICSI. Therefore, the spermatozoa source did not have a significative impact on embryo development until the blastocyst stage (after the ICSI). This would seem to be the first experiment aimed to compare fresh or cryopreserved testicular tissue (TT) spermatozoa in cattle. ICSI —carried out using TT spermatozoa— is a treatment frequently used to deal with human infertility. Additionally, embryos produced by ICSI, using testicular spermatozoa from men that suffer cryptozoospermia or necrozoospermia, had a better implantation and pregnancy

Table 1. Means of least square and standard error (SE) for the cellular division in D2 and embryo development in D7-8, indicating the right standardization of the IVF technique to produce *in vitro* embryos.

Source of spermatozoid	Ovocyte (n)	% Cellular division 48 h	% Development D8
FIV (control)	64	61 ± 6^{a}	23 ± 5^{a}
ICSI F/T	113	57 ± 5^{a}	13 ± 3^{b}
ICSI FTT	96	43 ± 5^{b}	6 ± 3^{b}
ICSI CTT	92	41 ± 5^{b}	6 ± 3^{b}

^{a,b} means with different superscripts are different ($P<0.05$). F/T=Frozen-defrosted; FTT=Fresh testicular tissue; CTT=cryopreserved testicular tissue.

rates than the embryos produced by ICSI using ejaculated spermatozoa (Negri, 2014; Kang *et al.*, 2018). Some authors have proposed that spermatic DNA does not completely condense when entering the epididymis and, therefore, is more sensitive to oxidative stress. If, for whatever reason, oxygen reactive species are found in the epididymis, the DNA of the sperm will be damaged, causing a low fertilization rate. In this case, the immature spermatozoa of the seminiferous tubules would have greater chances to fertilize an oocyte and produce a viable embryo, since they have not been exposed to oxidative stress (Kang *et al.*, 2018).

In this experiment, when TX was used as membrane-destabilizing agent, 56% of the oocytes recorded cellular division 48 h after the ICSI (Table 2). These results are lower than the control group (61%), higher than the Hep-GSH group (35%), and similar to the LL group (50%).

Zambrano *et al.* (2016) reported a 66% division rate, 72 h after the ICSI. The discrepancy between the results of this experiment and the findings of those authors could be the result of the moment in which the embryo development was evaluated (48 *vs.* 72 h) and the influence of the oocyte activators (ethanol vs cycloheximide-ionomycin). In order to carry out the ICSI, the acrosomal membrane enters the injected oocyte, which can cause damage and a low development rate. LL has had positive effects, removing the acrosomal membrane of the spermatozoa of different species (Kang *et al.*, 2018).

There were no significative statistical differences in the interaction of the destabilizing agent with the sperm source (Table 3). Previously, Seita and Kashiwazaki (2009) reported a 10% blastocyst development in rats, when their spermatozoa were treated with LL. These

Table 2. Means of the least square and standard error (SE) for embryo division on D2 and embryo development on D7-8 (destabilizing agent).

Agent destabilizing	Cellular division D2	Development D 7-8
FIV	61 ± 6^{a}	23 ± 5^{a}
ICSI-Hep-GSH	35 ± 5^{c}	5 ± 2^{b}
ICSI-LL	50 ± 5^{b}	6 ± 3^{b}
ICSI-TX	56 ± 5^{b}	14 ± 3^{b}

^{a,b} means with different superscripts are different ($P<0.05$). Hep-GSH=heparin-glutathione; LL=lysolecithin; TX=Triton X-100.

Table 3. Means of the least square and standard error (SE) for the treatment × embryo development* interaction, after the ICSI.

Source of spermatozoid	Destabilizing	Ovocyte (n)	División celular of 48 h	Development to D 7 y 8
F/T	Hep-GSH	34	47±9 ^{a,b,c}	12±6 ^{a,b}
F/T	LL	38	63±8 ^{a}	11±5 ^{a}
F/T	TX	41	61±8 ^{a}	17±6 ^{a,b}
FTT	Hep-GSH	30	27±8 ^{b}	3±3 ^{a}
FTT	LL	32	56±9 ^{a,c}	6±4 ^{a}
FTT	TX	30	47±9 ^{a,b,c}	10±5 ^{a,b}
CTT	Hep-GSH	31	32±8 ^{b,c}	3±3 ^{a}
CTT	LL	31	32±8 ^{b,c}	3±3 ^{a}
CTT	TX	34	59±8 ^{a}	15±6 ^{a,b}
FIV		64	61±6^{a}	23±3^{b}

* There were no significative differences (P>0.05). F/T=Frozen-Defrosted; FTT=Fresh testicular tissue; CTT=Cryopreserved testicular tissue; Hep-GSH=heparin-glutathione; LL=lysolecithin; TX=Triton X-100.

results match the findings of this study for the ICSI using F/T spermatozoa treated with LL (10% blastocyst development).

LL not only had a positive effect as membrane-destabilizing agent on the F/T spermatozoa, but also on the spermatozoa from the FTT or CTT: (56, 6, 32 y 3% for early cleavage and blastocyst formation for FTT and CCT respectively). This is a potential opportunity to produce viable cattle embryos from testicular spermatozoa that have not reached their final maturation and have not developed motility. Additionally, this tissue can be cryopreserved and thawed, before the spermatozoa are extracted. Subsequently, they can be treated with membrane destabilizing agents and the oocytes can be fertilized with ICSI, in order to produce cattle blastocysts. The capacity of these blastocysts to achieve a complete term gestation when transferred to the uterus of a recipient cow remains to be elucidated.

Under the conditions of this experiment, Hep-GSH seems to be a less efficient spermatozoa membrane-destabilizing agent than TX or LL. When the ICSI was carried out using F/T spermatozoa treated with Hep-GSH, the percentage of embryos that recorded early cellular division in D2 (47.0%) was lower than the results obtained by Sekhavati *et al.* (2012; 74-83%). However, our result was similar to that of Canel *et al.* (2017; 60.8%). This situation can be the result of the time during which the spermatozoa were incubated with Hep-GSH in this study (20 h), compared with the incubation time with GSH (7 h) reported by Zambrano *et al.* (2016). Perhaps 20 h was an excessive period, resulting in an increase of the spermatic DNA fragmentation, which affected embryo division.

Finally, the percentage of embryos that reached blastocyst stage from those that cleaved at 48 h was similar to that published in other studies (Canel *et al.*, 2018; Sekhavati *et al.*, 2012). This situation suggests that the prolonged exposition of the spermatozoa to Hep-GSH could impact the embryo division, but not the blastocyst development.

Further studies are required to improve the efficiency of these techniques for two main reasons. On the one hand, this technique could be used to obtain testicular tissue from genetically valuable bulls that have to be slaughtered for one reason or another and whose sperm could not have been previously frozen. On the other hand, results like the ones obtained in this study open the possibility of applying this technique to preserve endangered species.

CONCLUSIONS

Cattle spermatozoa from fresh or cryopreserved testicular tissue can be used to fertilize an oocyte by ICSI and can promote development until the blastocyst stage. Under the conditions of this experiment, TX and LL were better membrane-destabilizing agents than Hep-GSH. Further experiments with spermatozoa from different sources are required, in order to improve embryo development after the ICSI.

REFERENCES

Águila L, Felmer R, Arias ME, Navarrete F, Martin-Hidalgo D, Lee HC, et al. Defective sperm head decondensation undermines the success of ICSI in the bovine. *Reproduction* 2017;154:207-218. https://doi.org/10.1530/REP-17-0270

Arias ME, Risopatrón J, Sánchez R, Felmer R. Intracytoplasmic sperm injection affects embryo developmental potential and gene expression in cattle. *Reprod Biol* 2015;15:34-41. https://doi.org/10.1016/j.repbio.2014.11.001

Barfield J. Evaluation of in vitro-produced bovine embryos 2015 CETA/ACTE & AETA JOINT CONVENTION – NIAGARA FALLS, ONTARIO SESSION: IVP vs IVD EMBRYO EVALUATION. https://www.aeta.org/docs/Evaluation_of_in_vitro_produced_bovine_embryos.pdf (consultado 02/febrero/2023).

Cairo Consensus Group. There is only one thing that is truly important in an IVF laboratory "everything". Cairo consensus guidelines on IVF culture conditions. *RBMO* 2020; 40 (1):33-60. https://doi.org/10.1016/j.rbmo.2019.10.003

Canel NG, Bevacqua RJ, Hiriart MI, Rabelo NC, de Almeida Camargo LS, Romanato M, et al. Sperm pretreatment with heparin and l-glutathione, sex-sorting, and double cryopreservation to improve intracytoplasmic sperm injection in bovine. *Theriogenology* 2017; 93:62-70. https://doi.org/10.1016/j.theriogenology.2016.12.018

Canel NG, Suvá M, Bevacqua RJ, Arias ME, Felmer R, Salamone DF. Improved embryo development using high cysteamine concentration during IVM and sperm co-culture with COCs previous to ICSI in bovine. *Theriogenology* 2018;117:26-33. https://doi.org/10.1016/j.theriogenology.2018.05.017

Delgado NM, Sánchez-Vázquez, ML, Reyes R, Merchant-Larios H. Nucleons I: A model for studying the mechanism of sperm nucleus swelling *in vitro*. *Arch Androl J* 1999;43:85–95. https://doi.org/10.1080/014850199262779

De Matos DG, Furnus CC. The importance of having high glutathione (GSH) level after bovine in vitro maturation on embryo development: effect of βmercaptoethanol, cysteine and cystine. *Theriogenology* 2000;53:761–71. https://doi.org/10.1016/S0093-691X(99)00278-2

Devroey P, Van Steirteghem A. A review of ten years experience of ICSI. *Hum Reprod* Update 2004;10:19–28. https://doi.org/10.1093/humupd/dmh004

Diaza AMG, Bustos JEA, Ulloa SMB, Jaramillo LC. Generalidades de la producción de embriones bovinos *in vitro*. *RIAA* 2013; 4(1):65-80. DOI: https://doi.org/10.22490/21456453.1967

Gupta A, Singh J, Anzar M. Effect of cryopreservation technique and season on the survival of *in vitro* produced cattle embryos. *Anim Reprod Sci* 2016;164:162-168. https://doi.org/10.1016/j.anireprosci.2015.11.026

Hansen P.J. In Vitro Production of Bovine Embryos. Laboratory Dept. of Animal Sciences, University of Florida Version 10.16. 2013. https://animal.ifas.ufl.edu/hansen/ivf_docs/University%20of%20Florida%20Bovine%20IVP%20Manual%20ver%2010.16.2013.pdf (Consultado en 10-01-2023)

Kang, YN., Hsiao, YW., Chen, CY. et al. Testicular sperm is superior to ejaculated sperm for ICSI in cryptozoospermia: An update systematic review and meta-analysis. *Sci Rep* 8, 7874 (2018). https://doi.org/10.1038/s41598-018-26280-0

Malcuit C, Maserati M, Takahashi Y, Page R, Fissore RA. Intracytoplasmic sperm injection in the bovine induces abnormal [Ca^{2+}] I responses and oocyte activation. *Reprod Fert Dev* 2006;18:39-51. DOI:10.1071/RD05131

Negri L., Patrizio P., Albani E., Morenghi E., Benaglia R., DesgroM. y Levi Setti P.E. (2014) ICSI outcome is significantly better with testicular spermatozoa in patients with necrozoospermia: a retrospective study, *Gynecological Endocrinology*, 30:1, 48-52. https://doi.org/10.3109/09513590.2013.848427

Ressaissi, Y., Anzalone, D. A., Palazzese, L., Czernik, M., & Loi, P. The impaired development of sheep ICSI derived embryos is not related to centriole dysfunction. *Theriogenology* 2021;159:7-12. https://doi.org/10.1016/j.theriogenology.2020.10.008

Rho GJ, Kawarsky S, Johnson WH, Kochhar K, Betteridge KJ. Sperm and oocyte treatments to improve the formation of male and female pronuclei and subsequent development following intracytoplasmic sperm injection into bovine oocytes. *Biol Reprod* 1998;59:918-24. https://doi.org/10.1095/biolreprod59.4.918

Rizos D, Ward F, Boland MP, Lonergan P. Effect of culture system on the yield and quality of bovine blastocysts as assessed by survival after vitrification. *Theriogenology* 2001;56:1-16. https://doi.org/10.1016/S0093-691X(01)00538-6

Romanato M, Cameo MS, Bertolesi G, Baldini C, Calvo JC, Calvo L. Heparan sulphate: A putative decondensing agent for human sperm cells *in vivo*. *Hum Reprod* 2003;18:1868–73. https://doi.org/10.1093/humrep/deg354

Seita Y, Ito J, Kashiwazaki S. Removal of acrosome membrane from sperm head improves development of rat zygotes derived from intracytoplasmic sperm injection. 2009;55 (5): *J. Reprod. Dev*. 475-479. https://doi.org/10.1262/jrd.20216

Sekhavati MH, Shadanloo F, Hosseini MS, Tahmoorespur M, Nasiri MR, Hajian M, Nasr-Esfahani MH. Improved bovine ICSI outcomes by sperm selected after combined heparin-glutathione treatment. *Cell Reprogram* 2012;14:295-304. https://doi.org/10.1089/cell.2012.0014

Sutovsky P, Schatten G. Depletion of glutathione during bovine oocyte maturation reversibly blocks the decondensation of the male pronucleus and pronuclear apposition during fertilization. *Biol Reprod* 1997;56:1503–12. https://doi.org/10.1095/biolreprod56.6.1503

Zambrano F, Águila L, Arias M.E., Sánchez R. Felmer R. Improved preimplantation development of bovine ICSI embryos generated with sperm cells pretreated with membrane-destabilizing agents lysolecithin and Triton X-100. *Theriogenology* 2016;86;1489-1497. https://doi.org/10.1016/j.theriogenology.2016.05.007