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Sperm pretreatment with heparin and L-glutathione, sex-sorting, and double cryopreservation to improve intracytoplasmic sperm injection in bovine

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ABSTRACT

In bovine, intracytoplasmic sperm injection (ICSI) remains inefficient partially due to low levels of sperm decondensation. The aim of this study was to determine whether the injection of normal size sperm pretreated with heparin (Hep) and L-glutathione (GSH), the use of sex-sorted sperm, the double round of sperm freezing/thawing (re frozen), or the combination of these approaches can improve sperm decondensation and embryo development after ICSI in cattle. Cleavage and blastocyst rates were evaluated on days 2 and 7 post ICSI. Quality of ICSI blastocysts was analyzed by the relative expression of four genes by qPCR and the DNA fragmentation index by TUNEL assay. For all assays, semen samples were co-incubated with pCX-*EGFP* 50 ng/μl before ICSI. GFP expression, which can be detected by fluorescence microscopy, was used as a tool to estimate the success of sperm decondensation after ICSI. The use of normal size sperm pretreated with 80 μM Hep-15 mM GSH for 20 h (Hep-GSH) increased cleavage, blastocyst and *EGFP* + blastocysts rates (60.8, 19.4 and 61.9%) compared to control ICSI (35, 4.9 and 20%) ($p < 0.05$). Moreover, *HMGN1, GLUT5, AQP3* and *POU5F1* transcription levels did not differ between ICSI Hep-GSH and IVF embryos. The use of sex-sorted sperm (X, Y) improved cleavage rates and *EGFP* expression at day 4 (83 and 30.2% for ICSI Y and 83.2 and 31.7% for ICSI X) compared to non-sorted group (50.9 and 15.1%), not showing differences at the blastocyst stage. Finally, sex sorting (X) was combined with Hep-GSH and/or re frozen treatments. The use of Hep-GSH diminished cleavage rates from ICSI X re frozen group (80.4% vs. 94.2%) and blastocyst development from ICSI X group (3.3% vs. 10%), compared with their controls ($p < 0.05$). While Hep-GSH pretreatment induced lower transgene expression at day 4, no differences were found at the blastocyst stage between ICSI groups (from 58.3 to 80%). TUNEL assay showed higher DNA fragmentation indexes for all ICSI treatments ($p < 0.05$), except for ICSI X Hep-GSH, which did not differ from IVF X control. In conclusion, the use of normal size sperm pretreated with Hep-GSH, combined or not with sex-sorting by flow cytometry could improve ICSI outcomes in cattle.

1. Introduction

Intracytoplasmic sperm injection (ICSI) is the technique most widely used to solve male factor infertility in humans [1]. However, ICSI efficiency has remained low in domestic species, especially in bovine [2–4]. The failure of sperm decondensation, the capacitation status of injected spermatozoa, and the integrity of the sperm

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membrane have been identified as the sperm factors that can influence ICSI outcome [5–7].

ICSI in cattle is characterized by a low frequency of sperm decondensation [7–9]. This could be partially due to inconsistent levels of protamine disulfide bond reduction in the sperm nucleus [10], which normally occurs after fertilization, and allows male pronucleus formation [11,12]. Reduced glutathione (GSH) acts as an endogenous disulfide bond reducer and plays a critical role in sperm decondensation and male pronucleus formation during fertilization [13,14]. Heparin, among other functions, acts as a protamine acceptor, inducing the release of protamines from DNA and subsequent chromatin decondensation [15,16]. The combined treatment of sperm with heparin and glutathione (Hep-GSH) was first reported by Delgado et al. [17] to induce bovine sperm decondensation *in vitro*; and Sekhavati et al. [7] applied this pretreatment for 7 h to inject fully decondensed spermatozoa by ICSI. Even though Sekhavati et al. [7] observed increased blastocyst production in the cow, the injection of completely decondensed sperm renders ICSI very complex and time-consuming, and enhances the risk of losing nuclear material. In this context, we determined the maximum incubation time in Hep-GSH to induce *in vitro* decondensation of sperm from six different bulls. On the basis of this analysis we used Hep-GSH treatment for a longer period of time (20 h) than Sekhavati et al. [7] and performed ICSI with treated spermatozoa showing heads of normal size (non-decondensed). Since the proposed treatment would extremely simplify ICSI procedure, we evaluated its effects over cattle embryo development.

Currently, sperm cryopreservation and sex-sorting by flow cytometry are well-established techniques in the livestock industry. Both technologies induce changes on the sperm membrane [18–20] and increase the percentage of acrosome reacted sperm, indicating that these methodologies could induce premature capacitation [21–23]. Nevertheless, further evidences have demonstrated that sperm chromatin integrity is conserved after freezing or sorting [24–26]. Therefore, even though sperm motility could be diminished by both cryopreservation and sex-sorting technologies in a bull dependent fashion [27], they have not impacted negatively on the overall ICSI efficiency [28]. All these observations, combined with the necessity to break the sperm membrane before injection to produce blastocysts by ICSI, led to hypothesize that the changes to the sperm membrane induced by cryopreservation and flow cytometry could result in improvement of sperm decondensation and embryo development in cattle.

In contrast to rodent or human embryos, it is not possible to visualize the pronuclei in bovine zygotes after fertilization, unless centrifugation or DNA staining are performed. As well, ICSI in cattle must be followed by chemical activation to assure subsequent embryo development, which makes difficult to discern between parthenogenetic embryos (produced merely by artificial activation) and ICSI embryos (which are products of proper sperm decondensation). For this reason, we performed the joint injection of plasmid pCX-*EGFP* with the spermatozoon, and the subsequent evaluation of GFP expression was used as an indicator of efficient sperm decondensation, and its contribution to the resulting embryo genome. Previous reports from our group [29–31] showed a strong association between the expression of pCX-*EGFP* plasmid and sperm head decondensation after ICSI. Bevacqua et al. [29] evaluated the presence of a condensed sperm head in ICSI embryos at day 4 of *in vitro* culture, which were injected with sperm previously incubated with pCX-*EGFP*. The authors did not observe condensed sperm heads inside any of the embryos showing expression of the transgene, indicating that all GFP expressing embryos had successfully undergone pronuclei formation. By contrast, more than 50% of embryos without GFP expression showed a condensed sperm head inside them. On the basis of these results, all the experiments assessed in this paper included the co-incubation of sperm with pCX-*EGFP* before ICSI. GFP expression, which can be detected by fluorescence microscopy, was used as a tool to identify ICSI embryos.

In the present study we assayed the use of normal size sperm pretreated with Hep-GSH, the use of sex-sorted sperm, the double round of sperm freezing/thawing (re frozen), or the combination of these approaches to improve sperm decondensation and embryo development after ICSI. *EGFP* expression was used as an indicator of successful fertilization, and developmental rates and blastocyst quality were analyzed to determine the most adequate sperm pretreatment to assist ICSI in bovine.

2. Materials and methods

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.1. Experimental design

The co-incubation of sperm with pCX-*EGFP* before injection was employed to indirectly asses sperm decondensation. Experiment 1: normal size sperm pretreated with Hep-GSH for 20 h were used for ICSI, and blastocysts quality was determined by measurement of relative expression of *HMGN1, GLUT5, AQP3* and *POU5F1* genes. Experiment 2: the effect of sex-sorting of sperm by flow cytometry on ICSI performance was evaluated and compared to IVF, using semen from the same bull. Experiment 3: the effect of Hep-GSH treatment of sex-sorted sperm from different bulls, in combination or not with a second event of cryopreservation (re frozen) on ICSI efficiency and DNA fragmentation index of blastocysts was tested.

2.2. Cumulus–oocyte complexes (COCs) collection and in vitro maturation (IVM)

Both procedures were performed as previously described by Canel et al. [32]. After 20 h of IVM, oocytes with an extruded first polar body were selected for ICSI or chemical activation. In the case of IVF groups, COCs were matured *in vitro* for 21 h, washed in Hepes-TALP, and immediately co-incubated with sperm.

2.3. Sperm pretreatment with Hep-GSH and typing

Sperm pretreatment was performed as previously described by Romanato et al. [16] with the following modifications. Cryopreserved semen straws (CIALE, Buenos Aires, Argentina) from six different bulls were thawed individually, and washed twice by centrifugation at 490 × g for 5 min with Brackett's defined medium (see section 2.9, IVF procedure). The remaining pellet was diluted in 100 μL of Brackett's fertilization medium and introduced into a 1.5 mL tube with the same medium containing 80 μM Hep and 15 mM GSH, under mineral oil. Incubation was performed at 39 °C in a humidified atmosphere of 6% CO_2 in air for 1, 3, 7 or 20 h. For all cases, a control group incubated in Brackett's fertilization medium alone was also included. After incubation, treated sperm were fixed in 2.5% glutaraldehyde, and the percentage of decondensed spermatozoa was determined by phase contrast in a Zeiss 47-30-11-9901 microscope at X 400 magnification. Spermatozoa were classified as normal size (non-decondensed, with defined membranes) or decondensed (enlarged heads with non-defined membranes). A minimum of 200 sperm cells from each sample were evaluated.

2.4. Sex-sorting of sperm by flow cytometry/cell sorting

Sperm samples were sex-sorted by Semex (Guelph, Ontario, Canada), according to company's routine procedures.

2.5. Sperm re-freezing

After sperm pellet resuspension in NaCl solution for ICSI, the gametes that are usually discarded were reconstituted in 200 μL of the same medium per straw, and re frozen using Andromed® cryoprotectant (13503/0200; Minitub, Germany), according to the manufacturer's instructions. The straws were preserved in storage tanks until their use.

2.6. DNA construction

The plasmid used was pCX-*EGFP* (kindly provided by Dr. Masaru Okabe, Osaka University, Osaka, Japan). This plasmid contains the enhanced green fluorescent protein gene (*EGFP*) under the chimeric cytomegalovirus-IE-chicken β-actin enhancer-promoter control [33]. The plasmid was linearized by *Hin*d III digestion.

2.7. Intracytoplasmic sperm injection

After 21 h of IVM, ICSI was performed as previously described by Bevacqua et al. [29]. For each assay, a quarter of a straw was thawed at 37 °C for 30 s and sperm were washed twice by centrifugation at 490×g for 5 min in Hepes-TALP. For Hep-GSH experimental groups, sperm samples were subjected to a 20 h incubation with Hep-GSH and washed twice by centrifugation at 310×g for 5 min, first in Hepes-TALP and then in NaCl solution (NaCl 2.8% w/v and EDTA 100 μM). For all cases, the resulting pellet was finally diluted in NaCl solution. Before ICSI, sperm were co-incubated with 50 ng/μL of pCX-*EGFP* for 5 min on ice, as previously described by Perry et al. [34]. Sham controls were injected with the medium used for spermatozoa, but with no sperm, using a volume equivalent to that used for ICSI. Diploid (Diplo PA) and haploid (Haplo PA) parthenogenetic groups were also included as controls of *in vitro* development. After injection, all groups were subjected to chemical activation as described below.

2.8. Chemical activation

Metaphase II oocytes (Haplo PA and Sham controls) and sperm injected oocytes (ICSI groups) were treated with 5 μM ionomycin (I24222; Invitrogen, Carlsbad, CA, USA) in Hepes-TALP for 4 min, followed by incubation for 3 h in TCM-199 medium (to allow second polar body extrusion), and finally treated with 1.9 mM 6-DMAP (D2629) diluted in TCM-199 medium for 3 h. In the case of the Diplo PA control group, oocytes were treated with 6-DMAP immediately after ionomycin incubation to inhibit second polar body extrusion. Afterwards, oocytes were thoroughly washed in Hepes-TALP and cultured as described below.

2.9. In vitro fertilization (IVF) with sex-sorted and non-sorted sperm

The IVF procedure was previously described by Brackett and Oliphant [35]. Briefly, frozen semen was thawed in a 37 °C water bath for 30 s. Sperm were washed twice by centrifugation at 490×g for 5 min with Brackett's defined medium. Sperm concentration was adjusted to 15×10^6/mL in Brackett's fertilization medium and co-incubated for 5 h with groups of 20–25 COCs. Afterwards, presumptive zygotes were vortexed for 30–60 s and washed several times in Hepes-TALP and cultured *in vitro* as described below.

2.10. In vitro culture (IVC), evaluation of pCX-EGFP expression and assessment of pronuclear formation

IVC and evaluation of pCX-*EGFP* expression were performed as previously described by Bevacqua et al. [36]. For pronuclear formation assessment, some ICSI embryos were permeabilized for 15 min in 0.2% (v/v) Triton X-100 (T-9284) in PBS, 16 h post ionomycin. Immediately, oocytes were stained with 5 μg/ml propidium iodide (P4170) for 15 min in the dark. Presumptive zygotes were observed under an epifluorescence microscope using an excitation wavelength of 544 nm to evaluate the presence of pronuclei or of non-decondensed spermatozoa.

2.11. RNA extraction and Real-Time PCR

Three pools of ten blastocysts from each group were kept in RNA later® (AM 7020, Ambion, CA, USA) at −20 °C until RNA extraction. Total mRNA was extracted using the RNeasy® Micro Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Reverse transcription and cDNA synthesis were performed with the SuperScript III® First-Stand Synthesis Supermix Kit (Invitrogen, Carlsbad, CA, USA), using primer oligo dT_{20}, following the manufacturer's recommendations. The quantification of complementary DNA was performed by NanoDrop 1000 Spectrophotometer (Thermo Scientific). Real-Time PCR reactions were performed with Quanti Tect SYBR® Green PCR kit (QIAGEN®) with 200 ng of cDNA for all genes, with the exception of HMGN1, for which 400 ng were used. Amplification reactions were made in triplicate for each sample, with ABI PRISM® 7300 Sequence Detection Systems (Applied Biosystems). A negative template control was prepared by replacing cDNA with water and run in parallel. The gene expression pattern of the ICSI group was compared with the IVF control. Template cDNA was denatured at 94 °C (3 min), followed by 35 cycles at 94 °C (45 s), 50 °C–59 °C (30 s), 72 °C (1:30 min), and a final extension at 72 °C (10 min). After normalization of real time PCR, the dissociation temperature and the reaction efficiency were determined for each analyzed gene. The efficiency of the primers was calculated by LinReg PCR program [37]. For the analysis of relative quantification *of HMGN1, GLUT5, AQP3* and *POU5F1* messengers, *β-ACTIN* and *YWHAZ* genes were used as the endogenous references, using the comparative CT method (cycle threshold). The primers used to assess the relative abundance of transcripts were designed from sequences available in the GenBank database using the program Prime3 [38]. For details regarding primer design see Supplementary data, Table 1. All primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA), except for YWHAZ, which was synthesized by Invitrogen (Life Technologies Sao Paulo, Brazil).

2.12. TUNEL assay and confocal microscopy

DNA fragmentation was detected by TUNEL assay as was previously described by Moro et al. [39]. Complete Z series of 10–12 optical sections at 4–5 μm intervals were acquired from each embryo and three-dimensional images were constructed using the software EZ-C1 2.20. Total cell numbers and TUNEL positive cells (TUNEL+) were counted. To determine the DNA fragmentation index, the mean TUNEL + cell number of each group was divided by the mean blastocyst cell number.

2.13. Statistical analysis

Each experiment was repeated at least three times. Differences between treatments of pronuclei formation, developmental and *EGFP* expression rates were determined by Fisher's exact test using

Table 1
ICSI using bull sperm pretreated with Hep-GSH.

Treatment	N	Cleavage (%)	ä embryos at Day 4 (%)	Blastocysts (%)	ä blastocysts (%)
ICSI Hep-GSH	217	132 (60.8)[a]	64 (29.5)[a]	42 (19.4)[a]	26 (61.9)[a]
ICSI	206	72 (35)[b]	52 (25.2)[a]	10 (4.9)[b]	2 (20)[b]
Sham	105	84 (80)[c]	1 (1)[b]	6 (5.7)[b]	0[b]
Haplo PA	108	82 (75.9)[c]	–	20 (18.5)[a]	–
Diplo PA	140	104 (74.3)[c]	–	59 (42.1)[c]	–

[a,b,c:] Values with different superscripts within columns differ (Fisher's test, $p < 0.05$).

Graph Pad PRISM software 5.01 version. Differences between total cell numbers were analyzed by the Kruskal-Wallis non-parametric test, with Dunn's multiple comparisons correction. Percentages of decondensed spermatozoa were analyzed for significance using Two-way Repeated Measures ANOVA and Tukey's Multiple Comparisons Test. To compare differences between each bull and their own controls, Fisher's exact test was employed. The proportion of fragmented nuclei over total cell number was analyzed with the proportion difference test, using the Statistics 8.0 software. Calculations of relative mRNA quantification were performed with the Relative Expression Software Tool (REST®) [40] by the Pairwise Fixed Reallocation Randomization TEST® (version 384-Beta, 2005). This model was developed on the basis of the permutation tests, which are nonparametric. For all analyses a difference of $p < 0.05$ was considered to be significant.

3. Results

3.1. Experiment 1: ICSI with Hep-GSH pretreated sperm

3.1.1. Sperm decondensation kinetics

Prior to performing ICSI assays, the timing of Hep-GSH pretreatment (1, 3, 7 and 20 h of incubation) and its effect on sperm morphology from 6 bulls was analyzed. Results of sperm decondensation kinetics are detailed in Supplementary data, Figs. 1–3. Briefly, sperm decondensation population values (average for the 6 bulls used in this study) were almost null for untreated spermatozoa at each time point tested, but gradually increased following incubation in Hep-GSH ($p < 0.0001$). Increases were significant between 1 h and all other periods of incubation assayed ($p = 0.004$, $p < 0.0001$ and $p < 0.0001$ for 3, 7 and 20 h, respectively), and between 3 and 20 h of incubation ($p = 0.0017$). On the other hand, decondensation did not increase significantly either from 3 to 7 h of incubation, or from 7 to 20 h. Sperm decondensation increased significantly after 3 or more hours of incubation with heparin compared to control ($p = 0.0003$, $p < 0.0001$ and $p < 0.0001$ for 3, 7 and 20 h, respectively).

3.1.2. ICSI using normal size sperm pretreated with Hep-GSH

On the basis of decondensation kinetics results, sperm were pretreated with Hep-GSH for 20 h and those showing normal size heads were used for ICSI. Results from ICSI assay are shown in Table 1. The Hep-GSH ICSI group showed higher cleavage, blastocyst and *EGFP* expressing blastocysts rates than the ICSI control group ($p < 0.05$). Additionally, some ICSI embryos were fixed at the pronuclear stage and sperm decondensation was evaluated by propidium iodide staining. Hep-GSH pretreatment induced higher rates of zygotes with 2 pronuclei (77.4%, $n = 31$) than the control group (41.4%, $n = 29$) ($p < 0.05$).

ICSI Hep-GSH: ICSI using sperm pretreated with 80 μM heparin and 15 mM glutathione for 20 h. ICSI: ICSI using sperm with no pretreatment. Sham: parthenogenetic embryos injected with sperm incubation medium alone and activated with Ionomycin+3 h in TCM199+6-DMAP. Haplo PA: parthenogenetic embryos activated with Ionomycin+3 h in TCM199+6-DMAP. Diplo PA: parthenogenetic embryos activated with Ionomycin+6-DMAP. ä embryos: *EGFP* expressing embryos. ä blastocysts were calculated as a percentage of total blastocysts.

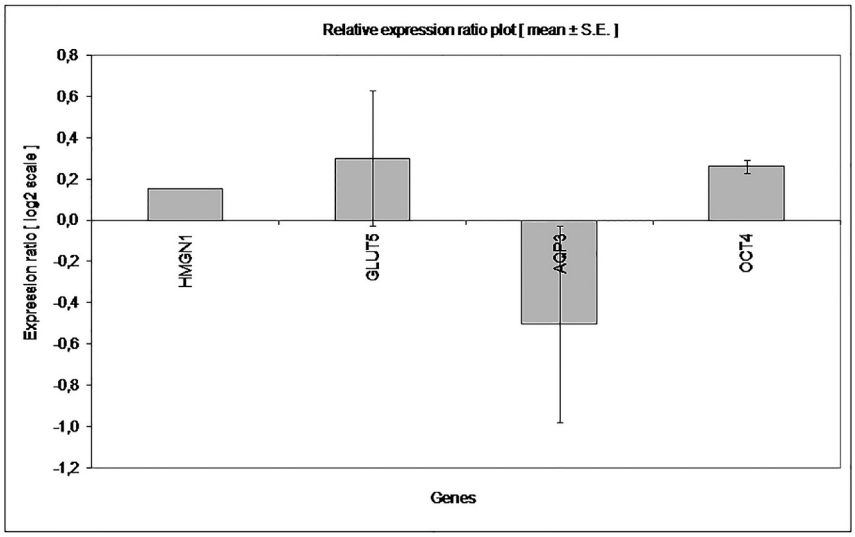

Fig. 1. Relative quantification of mRNA by qPCR from bovine blastocysts produced by ICSI using normal size sperm pretreated with 80 μM heparin and 15 mM glutathione for 20 h. Values from IVF group were converted to 0. No significant difference was observed for *HMGN1*, *GLUT5*, *AQP3* and *POU5F1* expression between groups (REST test, $p > 0.05$).

Table 2
In vitro development and EGFP expression of ICSI and IVF bovine embryos fertilized with sex-sorted (X and Y) and non-sorted (NS) sperm.

Treatment	N	Cleavage (%)	♂ embryos at Day 4 (%)	Blastocysts (%)	♂ blastocysts (%)
ICSI Y	106	88 (83)[a]	32 (30.2)[a]	14 (13.2)[ac]	4 (28.6)[a]
ICSI X	101	84 (83.2)[a]	32 (31.7)[a]	11 (10.9)[ac]	4 (36.4)[a]
ICSI NS	106	54 (50.9)[b]	16 (15.1)[b]	5 (4.7)[ab]	2 (40)[a]
IVF Y	139	19 (13.7)[c]	–	0[b]	–
IVF X	114	18 (15.7)[c]	–	0[b]	–
IVF NS	135	91 (67.4)[d]	–	23 (17)[c]	–
Sham	116	99 (85.3)[a]	1 (0.9)[c]	9 (7.8)[a]	0[b]
Haplo PA	88	67 (76.1)[b]	–	0[b]	–
Diplo PA	165	157 (95.2)[e]	–	72 (43.6)[d]	–

[a,b,c,d:] Values with different superscripts within columns differ (Fisher's test, $p < 0.05$).

3.1.3. Relative quantification of mRNA by qPCR from ICSI Hep-GSH vs. IVF blastocysts

In order to analyze the quality of ICSI embryos, we compared the differential quantitative expression of *HMGN1, GLUT5, AQP3* and *POU5F1* genes between ICSI Hep-GSH blastocysts and IVF controls, fertilized with sperm from the same bull with no pretreatment (Fig. 1). No differences were found between groups for the relative expression of the analyzed genes.

3.2. Experiment 2: ICSI with sex-sorted and non-sorted sperm

To assess the effect of sex-sorting of sperm by flow cytometry on ICSI efficiency, we performed ICSI and IVF with sex-sorted (X and Y) and non-sorted (NS) semen from the same bull. Embryo development and *EGFP* expression are shown in Table 2. Although cleavage and transgene expression rates at day 4 of ICSI Y and ICSI X groups were higher than those of ICSI NS group ($p < 0.05$), no differences were found among ICSI Y, ICSI X and ICSI NS groups in terms of blastocyst and transgene expression rates. All ICSI groups showed higher rates of transgene expression than the Sham controls, both at day 4 and 7 of *in vitro* development ($p < 0.05$). IVF sex-sorted groups produced the lowest cleavage rates and did not produce blastocysts ($p < 0.05$). ICSI embryos expressing *EGFP* are shown in Fig. 2.

Sham: parthenogenetic embryos injected with sperm incubation medium alone and activated with Ionomycin+3 h in TCM199+6-DMAP. Haplo PA: parthenogenetic embryos activated with Ionomycin+3 h in TCM199+6-DMAP. Diplo PA: parthenogenetic embryos activated with Ionomycin+6-DMAP. ♂ embryos: *EGFP* expressing embryos. ♂ blastocysts were calculated as a percentage of total blastocysts.

3.3. Experiment 3: ICSI with sex-sorted sperm pretreated with Hep-GSH and/or re frozen

On the basis of results obtained, we employed Hep-GSH pretreatment to improve ICSI embryo development using sex-sorted sperm. Additionally, we compared *in vitro* development of ICSI embryos fertilized with sex-sorted sperm frozen/thawed once (ICSI X) or twice (ICSI X re frozen), and previously treated (+) or not (−) with Hep-GSH. Results are shown in Table 3. Cleavage rates did not differ between ICSI groups, except for ICSI X re frozen Hep-GSH, which was lower than those of ICSI controls with or without re-frozen sperm ($p < 0.05$). Hep-GSH diminished blastocyst development compared with the control ($p < 0.05$). No effect on blastocyst production was observed when re frozen sperm were used ($p > 0.05$). While Hep-GSH pretreatment induced lower transgene expression levels at day 4, no differences were found at the blastocyst stage between ICSI groups.

Hep-GSH +: sperm pretreated with 80 μM heparin and 15 mM glutathione for 20 h. ICSI X: ICSI performed with frozen/thawed sex sorted sperm. ICSI X re frozen: ICSI performed with frozen/thawed/frozen/thawed sex sorted sperm. Sham: parthenogenetic embryos injected with sperm incubation medium alone and activated with Ionomycin+3 h in TCM199+6-DMAP. Haplo PA: parthenogenetic embryos activated with Ionomycin+3 h in TCM199+6-DMAP. Diplo PA: parthenogenetic embryos activated with Ionomycin+6-DMAP. ♂ embryos: *EGFP* expressing embryos. ♂ blastocysts were calculated as a percentage of total blastocysts.

3.4. DNA fragmentation in ICSI blastocysts obtained using sex-sorted sperm pretreated with Hep-GSH and frozen/thawed once (ICSI X) or twice (ICSI X re frozen)

Blastocysts produced by ICSI using sex-sorted sperm pretreated with Hep-GSH and/or re frozen were evaluated to determine the presence of fragmented DNA by TUNEL assay (Table 4, Fig. 3). IVF X blastocysts were used as a control. The mean of total cell number and TUNEL + cells from blastocysts did not differ between ICSI and IVF groups. However, the rate of DNA fragmentation was only comparable to those of IVF-X for ICSI-X Hep-GSH + group ($p < 0.05$).

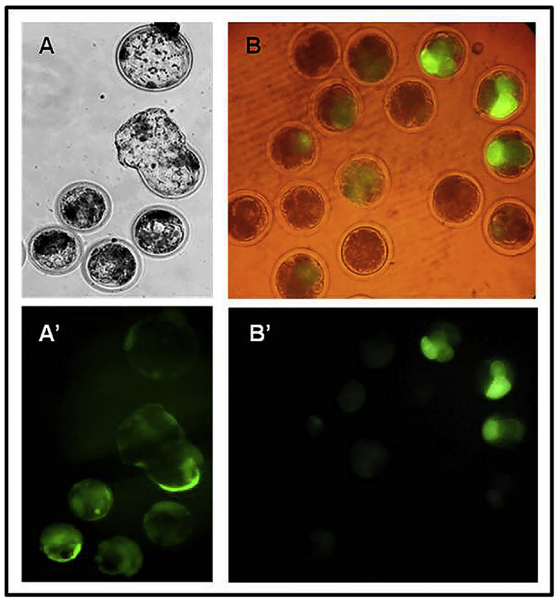

Fig. 2. Embryos produced by ICSI with sex-sorted semen. A) Blastocysts under bright field. B) Embryos at Day 4 of *in vitro* development observed under bright field and fluorescent light. A′, B′) The same embryos observed by fluorescence microscopy. 40× magnification.

Table 3
ICSI with sex-sorted sperm (ICSI X) pretreated with Hep-GSH and/or re frozen.

Treatment	Hep-GSH	N	Cleavage (%)	≥ embryos at Day 4 (%)	Blastocysts (%)	≥ blastocysts (%)
ICSI X	+	121	111 (91.7)^{ac}	46 (38)^{a}	4 (3.3)^{a}	3 (75)
	–	200	187 (93.5)^{a}	120 (60)^{b}	20 (10)^{bc}	16 (80)
ICSI X re frozen	+	92	74 (80.4)^{bc}	42 (45.7)^{ac}	8 (8.7)^{ac}	6 (75)
	–	120	113 (94.2)^{a}	72 (60)^{bc}	12 (10)^{bc}	7 (58.3)
Haplo PA	*NA*	159	138 (86.8)^{c}	–	17 (10.7)^{bc}	–
Diplo PA	*NA*	167	158 (94.6)^{a}	–	88 (52.7)^{d}	–

$^{a,b,c,d:}$ Values with different superscripts within columns differ (Fisher's test, $p < 0.05$).

4. Discussion

In the present study, we evaluated single or combined treatments to promote sperm decondensation following ICSI in cattle. Our results indicate that the use of normal size sperm after single treatment with Hep-GSH or sex-sorting improves the rates of sperm decondensation. In addition, Hep-GSH pretreatment induced higher blastocyst rates than the other single and combined pretreatments tested.

In Experiment 1, we evaluated Hep-GSH pretreatment to assist ICSI in cattle. Sekhavati et al. [7] observed that the injection of completely decondensed bull sperm after 7 h of treatment led to higher fertilization and blastocyst rates. However, the injection of fully decondensed sperm is more technically complex, since it requires the use of larger diameter pipettes, which inevitably increase the lysis rates of injected oocytes. Additionally, decondensed sperm become sticky, thus raising the risk of sperm remaining in the pipette after injection. This in turn, reduces the percentage of oocytes that are effectively injected with sperm, and thereby the overall efficiency. Finally, fully decondensed sperm lose their structural integrity, at least in the head region, where the genetic material resides, which led us to hypothesize that the use of these sperm could raise the risk of losing DNA during ICSI. In this work, we exposed sperm to a 20 h incubation with Hep-GSH and selected normal sized sperm for ICSI, which simplifies the procedure and also avoids injection of spermatozoa with morphological abnormalities. Following ICSI, the spermatozoon proceeds an initial decondensation step which is believed to be induced by the oocyte, and to be independent of oocyte activation [41]. As the sperm nuclear decondensing activity of the oocyte lasts for a short time after the onset of activation [42], the increased cleavage, blastocysts and *EGFP* + blastocyst rates obtained after Hep-GSH incubation of sperm prior to ICSI indicates that this treatment facilitates the first steps of sperm decondensation. Indeed, the evaluation of pronuclei formation supported these results, showing increased numbers of zygotes with 2 pronuclei for the group injected with sperm pretreated with Hep-GSH.

In order to evaluate embryo quality, we analyzed the transcript levels of *HMGN 1*, *GLUT 5*, *AQP 3* and *POU5F1* genes, which have been demonstrated to be indicators of blastocyst quality [43–47]. We observed no differences in relative expression of those genes between ICSI embryos generated with sperm treated with Hep-GSH, and IVF embryos produced with sperm from the same bull, without pretreatment. These results suggest that the injection of normal sized sperm pretreated with Hep-GSH does not affect embryo quality, at least in regard to the expression of the analyzed genes at the blastocyst stage.

In Experiment 2, we assessed whether sex-sorting of sperm by flow cytometry affects ICSI efficiency in cattle. Results showed that the use of sex-sorted semen significantly increases cleavage rates and *EGFP* expression at day 4 after ICSI, compared to non-sorted semen from the same bull. These results suggest that flow cytometry improves male pronucleus formation of ICSI cattle embryos. In contrast, we were unable to produce IVF blastocysts with sex-sorted semen from the same bull. The results obtained could be attributed to the reversible alteration of sperm membranes induced by the sorting process, which reduce the lifetime and fertilizing capacity of sperm *in vitro*, favouring sperm membrane capacitation patterns and the occurrence of acrosome reaction [48]. This could well decrease the production of IVF blastocysts with sex-sorted sperm, but would not affect, or could even improve the production of ICSI embryos. However, in terms of blastocyst production, we did not find differences neither between sex-sorted and non-sorted ICSI groups, nor with Sham controls. Nonetheless, *EGFP* expression rates indicate that at least 28.6% of ICSI Y; 36.4% of ICSI X and 40% of ICSI NS blastocysts are not the result of parthenogenetic activation, but have an input from both the maternal and the paternal genome. Given that the flow cytometry equipment currently used has significantly improved the quality of sex-sorted samples [25,26,49], our results suggest the use of sex-sorted sperm could be an alternative to improve ICSI outcomes in dairy cattle production.

In an attempt to modify the sperm membrane and facilitate male nucleus decondensation, in Experiment 3 we subjected the

Table 4
DNA fragmentation index of ICSI blastocysts using sex sorted sperm frozen/thawed once (ICSI X) or twice (ICSI X re frozen), and pretreated (+) or not (–) with Hep-GSH.

Treatment	Hep GSH	N	Total cell n° of blastocysts (Mean ± SD)	TUNEL + cell n° of blastocysts (Mean ± SD)	DNA fragmentation index*
ICSI-X	+	2	101 ± 52.3	14 ± 2.8	13.9^{a}
	–	9	60.11 ± 38.4	14.56 ± 5.9	24.2^{b}
ICSI-X Re Frozen	+	4	85.25 ± 12.5	31.5 ± 16	37^{c}
	–	6	67.33 ± 28	15.5 ± 8.4	23^{b}
IVF-X	–	10	104.8 ± 59	18.5 ± 9.6	17.7^{a}

$^{a,b,c:}$ Values with different superscripts within columns differ (difference of proportions test, $p < 0.05$).
ICSI X: ICSI performed with frozen/thawed sex sorted sperm. ICSI X re frozen: ICSI performed with frozen/thawed/frozen/thawed sex sorted sperm. Hep-GSH +: sperm pretreated with 80 μM heparin and 15 mM glutathione for 20 h. TUNEL + cells: cells that resulted positive for the analysis of DNA fragmentation. No differences were found for the Mean of total cell n° of blastocysts (±SD) and the Mean of TUNEL + cell n° of blastocysts (±SD), between the 5 groups analyzed (Kruskal-Wallis test, with Dunn's correction for multiple comparisons, $p < 0.05$). *: (Mean of total cell n° of blastocysts/Mean of TUNEL + cell n° of blastocysts) × 100.

sex-sorted sperm that are usually discarded after ICSI, to a second event of cryopreservation, and used them for a later ICSI procedure. Some samples were additionally treated with Hep-GSH. For most of the ICSI groups, cleavage rates did not differ from those of parthenogenetic embryos activated with Io-DMAP, which are usually high [50,51]. Since sperm from different bulls were used for Experiment 3, taken results from Experiments 2 and 3 together suggest that the sex-sorting process facilitates bull sperm decondensation after ICSI. According to Hep-GSH treatment, it showed a positive effect on cleavage, blastocyst and *EGFP* expression rates when using non-sorted sperm (Experiment 1), while no effect or even a negative effect was observed for those parameters when sex-sorted sperm was employed (Experiment 3). Interestingly though, embryos produced with sex-sorted sperm pretreated with

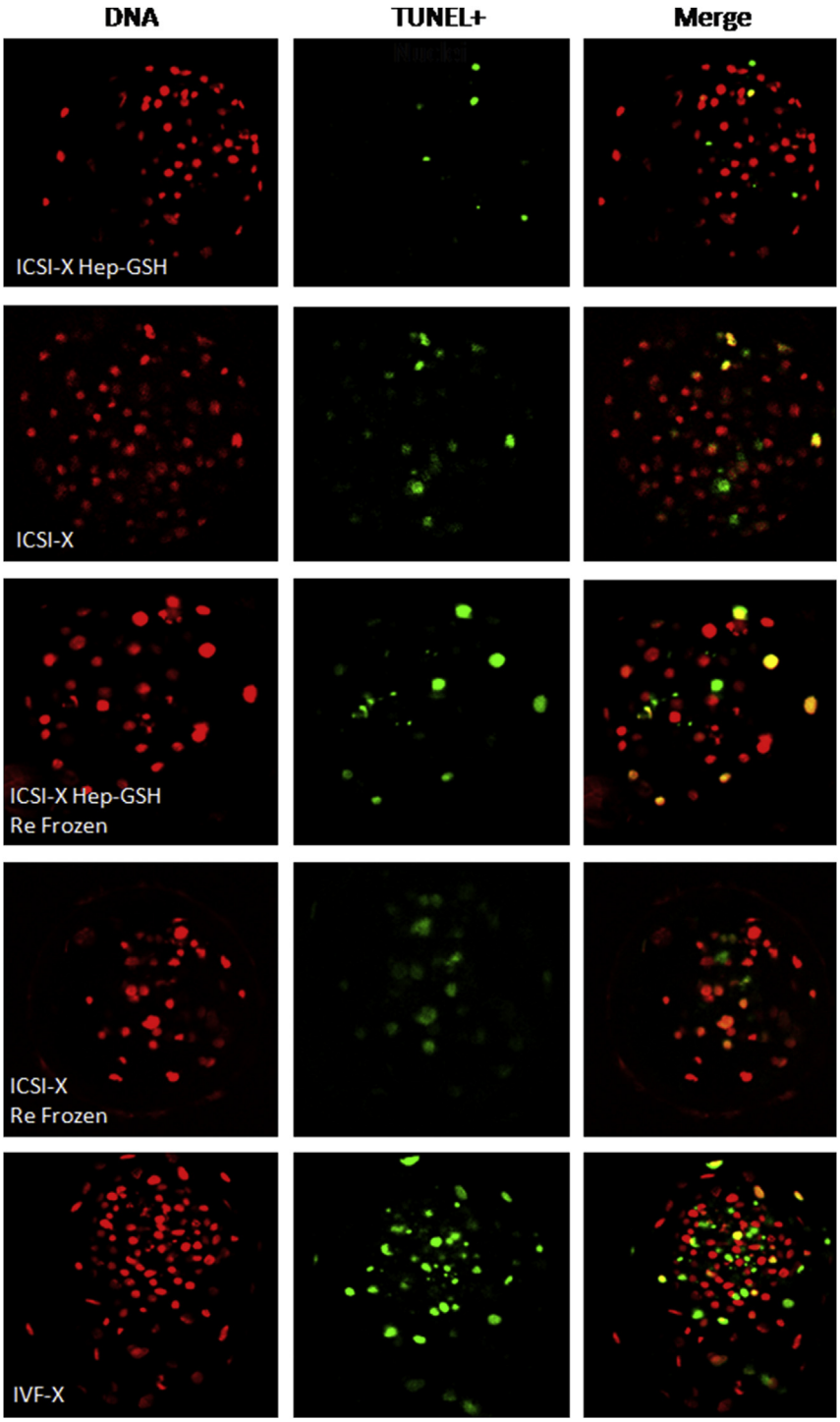

Fig. 3. DNA fragmentation analysis by TUNEL assay. Blastocysts produced using sex-sorted sperm subjected to different pretreatments: frozen/thawed sperm in combination (ICSI X Hep-GSH) or not (ICSI X) with Hep-GSH pretreatment for 20 h, and/or with a second event of cryopreservation (ICSI X Hep-GSH re frozen and ICSI X re frozen). IVF X: IVF using sex-sorted sperm with no pretreatment. TUNEL positive cells (TUNEL+) are labeled with fluorescein-12-dUTP (green), and nuclei are counterstained with propidium iodide (red). 400× magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Hep-GSH showed higher DNA integrity, except when re frozen sperm were used, suggesting that treatment of sex-sorted semen with Hep-GSH could reduce DNA damage in ICSI embryos. One possible explanation is that, by improving sperm decondensation, treatment with Hep-GSH could facilitate the access of the oocyte DNA repair machinery to the sperm nucleus prior to the first phase of DNA synthesis, thus repairing any damage caused by the sorting process. However, this would not be enough to restore the damage caused by double cryopreservation and thawing after sorting. Therefore, these results contraindicate the use of an additional event of sperm cryopreservation in ICSI programs.

4.1. Conclusions

The use of normal size sperm treated with Hep-GSH and sex-sorted sperm by flow cytometry is a methodology equally complex as conventional ICSI, that facilitates sperm decondensation and improves embryo development after ICSI in bovine. The results presented on this paper provide new insights into the potential use of combined treatments to induce the disruption of sperm membranes and the alteration of chromatin structure, in order to improve ICSI efficiency in cattle.

Authorship

N.C. performed oocyte collection and *in vitro* maturation, ICSI, TUNEL and sperm decondensation kinetics assays, the experimental design, data analysis, and manuscript writing. R.B. took part in ICSI protocols, study design, manuscript drafting, critical discussion and data analysis. M.I.H. was involved in oocyte collection and *in vitro* maturation, IVF and real time PCR. N.C.R. performed RNA extraction and real time PCR, and L.S.A.C. analyzed the data. M.R. and L.P.C. were involved in the design and performance of sperm decondensation kinetics assay. D.S. was in charge of research coordination, contributed to the experimental design, critical discussion and manuscript editing and submission. All the authors revised and accepted the draft version of the manuscript.

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Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.theriogenology.2016.12.018.

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