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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.