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Improved preimplantation development of bovine ICSI embryos generated with spermatozoa pretreated with membrane-destabilizing agents lysolecithin and Triton X-100

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ABSTRACT

In cattle, intracytoplasmic sperm injection (ICSI) has a low efficiency. The acrosome content may be responsible for this effect because of the large amount of hydrolytic enzymes that are released within the oocyte. With the aim of removing the acrosome and destabilize the membranes, cryopreserved bovine spermatozoa were treated with lysolecithin (LL) and Triton X-100 (TX) at different concentrations. We evaluated the membrane integrity, the acrosome integrity, DNA integrity, and the variation of phospholipase C zeta. The rates of development (cleavage and blastocysts) were also evaluated along with pronuclear formation and the embryo quality. Spermatozoa incubated with LL and TX (0.01%, 0.02%, 0.03%, and 0.04%) decreased ($P < 0.0001$) sperm viability in a dose-dependent manner. The acrosome reaction was also increased ($P < 0.0001$) in all tested concentrations of LL and TX achieving 100% at 0.05% concentration in both treatments. Terminal deoxynucleotidyl transferase dUTP nick-end labeling assay reported an increase ($P < 0.05$) in DNA fragmentation only with the highest concentration of LL (0.06%), whereas all concentrations assessed of TX reported an increased respect to the control. Phospholipase C zeta expression decreased ($P < 0.05$) in spermatozoa treated with LL and TX at all concentrations tested. A higher cleavage rate was observed in ICSI-TX (66%) and ICSI-LL (65%) groups compared with the untreated control group (51%) and the blastocyst formation rate significantly increased in the ICSI-LL group (29%) compared with the control (21%). No differences were observed in the pronuclear formation and quality of the embryos. In conclusion, the destabilization of the plasma membrane and the release of the acrosomal content with LL and TX before ICSI improve the rate of embryonic development, without affecting the quality of the embryos produced by this technique.

1. Introduction

The physiological ability to fertilize the oocytes *in vivo* and *in vitro* is hampered in situations where mammalian spermatozoa lose their motility, but its ability to contribute to the embryonic development remains intact because of the intracytoplasmic sperm injection (ICSI) technique [1]. In humans, the ICSI technique is currently the treatment of choice to overcome fertility problems related mainly to the male factor. In addition to its clinical applications, ICSI represents a valuable research tool for studying molecular aspects of the interaction between gametes during fertilization [2].

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In cattle, ICSI can also be used as a reproductive option to solve fertility problems related to the failure of artificial insemination and/or *in vitro* fertilization [3]. Furthermore, the technique allows the use of samples without or very low sperm motility as result of fertility problems related to disorders in the sperm production [4]. However, the low efficiency of ICSI in cattle determined by the low rates of *in vitro* embryo production [2,5,6] and the generation of live offspring has limited its widespread use [1]. Therefore, its commercial and clinical use has not been reported yet, except in the equine and human species [3,4].

The oocyte activation is naturally produced by the sperm–oocyte activating factor, described as a specific isoform of phospholipase C, called phospholipase C zeta (PLCζ) [7–11]. In the bovine species, the spermatozoon is not capable of inducing the oocyte activation in 90% of cases [12] because the release of PLCζ may be compromised after its injection. Therefore, external activation stimuli are necessary for meiosis resumption, extrusion of the cortical granules, formation of the male pronucleus, and the start of the embryonic development [2]. A number of exogenous activation protocols have been used to solve this limitation [2,13–15]. The first challenge is to induce intracellular calcium waves that are not generated spontaneously within the oocyte. This in turns contributes to a rapid disintegration of the membranes that makes PLCζ exposed within the oocyte cytoplasm, later triggering the earlier mentioned events [16]. The acrosomal content (composed of many acid hydrolases commonly found in the lysosome), injected by the spermatozoon during the ICSI procedure, has also a potential harmful effect within the oocyte, which is directly correlated with the size of the sperm head [17,18]. This potential damage caused by the acrosomal enzymes involves a disorganization of the cytoskeleton in fertilized oocytes undergoing polyspermy and a distribution of the microtubules to the cortex, which in the natural fertilization process are distributed throughout the oocyte cytoplasm [18]. Different strategies to remove the acrosome and plasma membrane have been evaluated to facilitate the sperm decondensation and pronuclear formation including sonication, freeze-thawing, freeze-drying, and treatments with chemicals, such as dithiothreitol, Triton X-100 (TX), among others [19–23]. However, some of these treatments generate damage in the sperm DNA, which may affect the further embryonic development [24]. Other protocols have used caffeine [20] and calcium ionophore [25] to increase the permeability of the membrane and to contribute to a more rapidly sperm head decondensation; however, the results so far have not been satisfactory in bovine ICSI.

Therefore, the objective of the present study was to establish sperm incubation conditions for the bovine species to destabilize the acrosome and plasma membranes using lysolecithin (LL) and TX. We evaluated the effects of these pretreatments on different sperm quality parameters, and the preimplantation development of bovine ICSI embryos was also examined. We hypothesized that removal of the plasma membrane and the acrosomal content before injection would improve the developmental rates and quality of embryos produced by ICSI in cattle.

2. Materials and methods

Unless stated otherwise, all chemicals were purchased from Sigma–Aldrich (St Louis, MO, USA).

2.1. Preparation of spermatozoa and treatments

Commercial cryopreserved bull semen with proved fertility was used for all the analyses (Alta Genetics Inc., Alberta, Canada). Frozen sperm was thawed in a water bath at 38.5 °C for 1 minute and selected by Percoll gradient [26]. The selected sperm were brought to a concentration of 6×10^6 sperm/mL in each treatment group in 400 μL of Tyrode's Lactate (TL)–sperm medium. Sperm were incubated for 1 minute in different concentrations of LL and TX (range 0.01–0.06%). Additionally, a control untreated group was also included. All experimental groups were vortexed during the incubation time according to the methodology proposed by Seita et al. [27]. Immediately after incubation, spermatozoa were washed by centrifugation at 400 × *g* for 5 minutes and then the supernatant was discarded.

2.2. Plasma membrane integrity

The integrity of the plasma membrane was evaluated using the LIVE/DEAD Sperm Viability Kit (Molecular Probes, Eugene, OR, USA). Spermatozoa treated with LL and TX (range 0.01–0.04%) were resuspended in 400 μL of TL–sperm medium and incubated with SYBR-14 at 1 nM concentration for 10 minutes at 38.5 °C in darkness. Propidium iodide (PI) was added at 18 μM and incubated for 10 minutes. Finally, the sperm were washed by centrifugation at 400 × *g* for 5 minutes and resuspended in 300 μL of PBS 1×. Samples were analyzed by flow cytometry with a BD FACS Canto II Flow Cytometer (Becton, Dickinson and Company; BD Biosciences, San Jose, CA, USA) and by confocal microscopy (FV 1000; Olympus, Miami, FL, USA) at 520 and 610 nm of emission of SYBR-14 and PI, respectively.

2.3. Acrosome membrane integrity

Acrosome membrane integrity was evaluated using the fluorescence probe peanut agglutinin (PNA) conjugated with fluorescein isothiocyanate (FITC) and combined with PI. Sperm groups treated with LL and TX (range 0.01–0.06%) were resuspended in 400 μL of TL–sperm medium and incubated with PNA/FITC (60 μg/mL) and PI (18 μg/mL) for 15 minutes at 38.5 °C in darkness. Then, samples were washed by centrifugation at 400 × *g* for 5 minutes. Finally, the pellet was resuspended in 300 μL of PBS for evaluation by flow cytometry and confocal microscopy at 550 and 610 nm emissions of PNA/FITC and PI, respectively.

2.4. DNA fragmentation

DNA fragmentation was evaluated by the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay, using the *In Situ* Cell Death Detection Kit with fluorescein (Roche Biochemical, Indianapolis, Indiana, USA), according to the manufacturer's instructions. Sperm treated with LL and TX (0.04%, 0.05%, and 0.06%) were washed with