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Effects of sperm pretreatment and embryo activation methods on the development of bovine embryos produced by intracytoplasmic sperm injection

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ABSTRACT

The aim of the study was to examine the effects of different embryo activation methods and sperm pretreatments on the activation and development of bovine embryos produced by intracytoplasmic sperm injection (ICSI). Four activation agents, *i.e.*, calcium ionophore (A23187), ionomycin (Ion), electric pulse (EP) and ethanol (Eth) were used in various combinations to activate bovine ICSI embryos. The normal fertilization rate was similar in bovine ICSI embryos activated by A23187 + Eth, Ion + Eth, Ion + EP + Eth, and 2-Ion (Ion administered two times) + Eth. Increasing the frequency of ionomycin stimulation from two (2-Ion + Eth) to three times (3-Ion + Eth) significantly ($p < 0.05$) increased the cell number per embryo at the blastocyst stage. In addition, spermatozoa were pretreated with dithiothreitol (DTT), glutathione (GSH) or GSH + lysolecithin (LL) and used for producing bovine ICSI embryos. The blastocyst rate of bovine ICSI embryos produced from sperm pretreated with GSH was significantly ($p < 0.05$) higher than those of embryos produced from sperm pretreated with DTT and GSH + LL. In conclusion, the embryo activation methods and sperm pretreatments examined in the present study did not affect the normal fertilization rate of bovine ICSI embryos. However, activation with 3-Ion + Eth and sperm pretreatment with

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GSH increased the cell number per embryo at blastocyst stage and the blastocyst rate, respectively, in bovine ICSI embryos.

1. Introduction

Intracytoplasmic sperm injection (ICSI) has been widely applied in studying fertilization of mammals [1], production of transgenic animals [2] and male infertility in humans [3] and endangered species [4]. ICSI technique involves a direct injection of a single spermatozoon into a metaphase II arrested oocyte. Such procedure circumvents many gamete interactions that occur during the course of physiological fertilization including sperm acrosome reaction, sperm–zona binding and penetration and sperm–oolemma binding and fusion [5]. As a consequence, the development of embryos produced by ICSI is relatively low [6,7]. Application of ICSI in mice [1], rabbits [8], horses [9] and humans [3] demonstrated that micromanipulation and spermatozoon alone are capable to activate embryos produced by ICSI and to propagate the subsequent embryonic development. In contrast, the activation rate of bovine ICSI embryos produced by micromanipulation is lower than 5% [6,10]. It appears that additional activation is required for producing bovine ICSI embryos [7,11–13].

The activation agents commonly used for bovine ICSI embryos are: electric pulse (EP) [14], ionomycin (Ion) [12,13], calcium ionophore (A23187) [15], ethanol (Eth) [7,11,13], 6-dimethylaminopurine (6-DMAP) [13] and cyclohexmide (CHX) [13]. It has been shown that a single treatment with only one activation agent is not enough to activate bovine ICSI embryos [7]. Moreover, it was demonstrated that combination of multiple activation agents or repeated treatment of a single activation agent are able to improve the activation efficiency and embryonic development [11–13,16]. The blastocyst rate of bovine ICSI embryos activated by the various combination of Ion, 6-DMAP, CHX, and ethanol ranged from 14.7% to 30.0% [13,16,17].

In physiological fertilization, the nucleus and cytoplasmic content of sperm are released into the oocyte after fusion, leaving the sperm acrosome and membrane outside of the oocyte [18]. In addition, protamines of DNA in the condensed sperm are replaced by histones in the oocyte cytoplasm. Meanwhile, the disulfide bonds of protamine are broken down to make the sperm nuclear materials decondensed and to form the male pronucleus [19,20]. During ICSI, an intact spermatozoon (with acrosome and membrane) is directly injected into an oocyte. The acrosome and membrane block the interaction between the sperm nucleus and the oocyte cytoplasm, thereby reducing the decondensation of sperm nuclear material, formation of male pronucleus, and subsequent development of ICSI embryos [21,22]. These undesired phenomena can be prevented, at least partially, when the acrosome and membrane are removed [21] or the disulfide bonds of protamine in sperm DNA are disrupted [13,21,23] before performing ICSI. Agents used in ICSI to remove the acrosome and membrane of mammalian spermatozoa include Triton X-100 [21], lysolecithin (LL) [24] and alkyltrimethylammonium bromide (ATAB) [25,26]. On the other hand, dithiothreitol (DTT) [21,23] and glutathione (GSH) [23] have been used to breakdown the disulfide bonds of protamine in sperm DNA. All the above-mentioned agents have been shown to increase pronuclear formation and development in porcine ICSI embryos [21,23]. In bovine ICSI embryos, sperm pretreatment with DTT, the most commonly used activation agent [12,13,27], had no effects on the blastocyst rate (21.9–24.3%) [27]. To the best of our knowledge, the effects of sperm pretreatment with LL and GSH in bovine ICSI embryos have not been investigated.

Taken together, the effects of different combinations of activation agents and sperm pretreatment schemes on the activation efficiency and subsequent development of bovine ICSI embryos were examined in the present study. Our results may provide fundamental information for improving the *in vitro* development of bovine ICSI embryos.

2. Materials and methods

Chemicals and reagents used in this study were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA) unless otherwise specified.

2.1. *Oocyte collection and* in vitro *maturation (IVM)*

Bovine ovaries were obtained from a local slaughterhouse. The cumulus–oocyte complexes (COCs) were aspirated from ovarian surface follicles (2–8 mm) by a syringe attached to an 18 gauge needle. Only COCs with homogenous ooplasm and at least four layers of tightly surrounded cumulus cells were selected. The COCs were *in vitro* matured in 80 μL drops (15–20 COCs/droplet) of M-199 (Gibco, Grand Island, NY, USA) supplemented with 5% (v/v) fetal bovine serum (FBS; Gibco) and 5 μg/mL gentamycin and covered with mineral oil in 3.5 cm culture dishes (Nunc, Roskilde, Denmark) at 38.5 °C, 2% CO_2 for 22 h. Thereafter, the cumulus cells were removed from the COCs by gentle pipetting in the hyaluronidase solution (1 mg/mL in Dulbecco's phosphate buffered saline, DPBS; Gibco) for 3 min. Only oocytes with the first polar body (PB I) were selected for ICSI [28].

2.2. *Preparation of sperm*

Commercially available Holstein semen was thawed in a water bath (37 °C, 25 sec) and washed twice with 10 mM theophylline in Brackett–Oliphant (theophylline-BO) medium [29]. Before ICSI, the sperm pellets were pretreated with one of the