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Improved fertilization and embryo development resulting in birth of live piglets after intracytoplasmic sperm injection and *in vitro* culture in a cysteine-supplemented medium

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Abstract

The effects of cysteine treatment on fertilization rate, intracellular concentration of glutathione, and embryo development *in vitro* and after embryo transfer were examined following intracytoplasmic sperm injection (ICSI) of *in vitro*-matured porcine oocytes using a piezo drive unit. Culture of presumed zygotes after ICSI with 1.71–3.71 mM cysteine for 3–12 h improved ($P < 0.05$) fertilization rates as compared to treatment with 0.57 mM cysteine or to controls (0 mM) (56 to 68%, 48%, 35%, respectively). Extension of treatment time with cysteine beyond 3 h did not further increase fertilization rates, suggesting that cysteine promoted early developmental events after ICSI (e.g. decondensation of sperm chromatin). There was no effect of cysteine supplementation on oocyte glutathione levels after ICSI. Pretreatment of spermatozoa for 3 h with 1.71 mM cysteine did not improve fertilization rates. The incidence of blastocysts formation when cultured in 1.71 mM cysteine for 3 h after ICSI was 31%, which was higher ($P < 0.05$) than controls (18%). Transfer of 20–38 embryos cultured with 1.71 mM cysteine for 3 h after ICSI to each of seven recipients yielded three deliveries with an average litter size of 4.0. We concluded that cysteine supplementation for the first 3 h after ICSI improved fertilization and embryo development rates, with no influence on glutathione levels in oocytes, and that the cysteine-treated ICSI embryos developed to full term. The study also showed that porcine oocytes matured in a chemically defined medium had the ability for full-term development after piezo-ICSI without additional treatments for oocyte activation.

Keywords: ICSI; Pronuclear formation; Glutathione; Embryo transfer; Pigs

1. Introduction

High incidence of polyspermy after *in vitro* fertilization (IVF) and the ensuing aneuploidy remains an unsolved problem in the *in vitro* production of pig blastocysts [1]. Intracytoplasmic sperm injection (ICSI) provides a possible remedy to produce high numbers of monospermic zygotes. However, the efficiency of ICSI in pigs is low, mainly due to a failure of oocyte activation [2,3] and low incidence of sperm head decondensation [4].

Low frequencies of male pronuclear formation observed in early IVF studies in pigs were dramatically improved by supplementations of oocyte maturation media with cysteine [5] and EGF [6]. However, male pronuclear formation after ICSI is not well supported in

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porcine oocytes matured in media with these supplements. The persistence of intact sperm acrosome, plasma membrane and perinuclear theca (PT) [7–9] which are removed during the process of sperm penetration in natural fertilization [10–12] is considered as one of the reasons for low male pronuclear formation after ICSI in the oocytes which are capable to support male pronuclear formation after IVF.

Pretreatments of spermatozoa to remove the plasma membrane have been applied to improve rates of fertilization and embryo development of ICSI in domestic animals [13–15]. In mice, the existence of the PT in sperm heads after the pretreatments is critical for oocyte activation after ICSI [16], artificial activation of oocyte is required for full term development when the PT is removed from spermatozoa by the pretreatments or freeze-dry spermatozoa are used for ICSI [17,18]. These findings were consistent with the later studies showing the localization of sperm borne oocyte-activating factors in postacrosomal PT [19–21]. As in mice, injection of spermatozoa which lost all exterior structures of sperm head except the PT and nuclear envelope improved fertilization rates without further artificial oocyte activation in pigs [15]. However, in domestic animals, birth rates after embryo transfer were low or zero even after fertilization and embryo development were improved by disruption of sperm plasma membrane before injection [14,22] or after the injection of freeze-dried spermatozoa [23,24], followed by artificial activation of ICSI-oocytes. This is in contrast to mice, where artificial activation of oocytes can substitute for sperm activating factors lost by pretreatments of spermatozoa [17,18]. In addition to sperm nuclei and oocyte activating factors, other sperm components participate in unpacking of sperm chromatin and paternal genome expression after fertilization [25]. Ward et al. [25] observed that the sperm perinuclear structures were easily disrupted by disulfide-bond-reducing agents, such as dithiothreitol. To develop normally, domestic animal zygotes may require multiple sperm components which are lost or damaged by destruction of sperm plasma membrane. In turn, artificial treatments for oocyte activation may be insufficient to support full term development when spermatozoa are chemically or physically demembranated and deprived of oocyte-activating factors before ICSI in domestic animals.

For the reasons mentioned above we prefer to inject non-chemically treated, intact spermatozoa to fertilize porcine oocytes by ICSI and to produce ICSI-piglets. However, we disrupt the plasma membrane of motile spermatozoa by applying piezo pulses with microinjection pipettes for sperm immobilization [9]. It is considered that most sperm components can be introduced as intact into oocytes if spermatozoa are injected immediately after immobilization [20,26]. Contrary to the accepted opinions in humans and mice, the membrane damage by sperm immobilization may not cause sufficient disassembly of sperm acrosome and PT to allow sperm nuclei to decondense after ICSI in cows [27] and pigs [9]. We considered that the fertilization after ICSI and embryo development to yield birth of piglets could be improved if disassembly of sperm plasma membrane and underlying perinuclear skeleton is promoted in ooplasm after injection of immobilized spermatozoa. Generally, cell surface is stabilized by disulfide-bond formation among cysteine residues of proteins [28]. The disassembly of sperm plasma membrane in ooplasm may be promoted by maintaining high levels of GSH in ooplasm after ICSI, that could be achieved by the supplementation of cysteine to the medium after ICSI as suggested by IVF studies in pigs [29,30] and cows [31]. In the present study, we examined the effect of cysteine treatment of presumed zygotes after ICSI on fertilization, intracellular concentration of GSH, embryo development *in vitro* and after embryo transfer. Also, the effect of pretreatment of spermatozoa with cysteine on fertilization was examined since the pretreatment of spermatozoa has improved fertilization rates in domestic animals [13–15,23,24,32].

2. Materials and methods

2.1. Collection of oocyte cumulus complexes (OCCs) and in vitro maturation

Oocytes were aspirated from antral follicles (3–6 mm in diameter) of ovaries collected from slaughtered prepubertal gilts. The oocytes surrounded by compact cumulus mass (oocyte-cumulus complexes: OCCs) were collected from the follicular fluid and washed twice. Groups of 50–100 OCCs were transferred into a well of a 4-well multidish containing 500 μL of TCM-199 (Gibco, Grand Island, NY, USA) including 0.1% (w/v) polyvinyl alcohol (PVA; Sigma Chemical Co., St. Louis, MO, USA), 10 ng/mL epidermal growth factor (Sigma), 10 IU/mL FSH (Sigma), 10 IU/mL LH (Sigma), 0.57 mM L-cysteine (Sigma; mTCM-199) at 39 °C in atmosphere of 5% CO_2 in humidified air. After 22–24 h, OCCs were transferred to mTCM-199 without FSH and LH, and then cultured an additional 20 h under the same conditions.