Theriogenology 80 (2013) 58–64

Contents lists available at SciVerse ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Development and quality of porcine parthenogenetically activated embryos after removal of zona pellucida

Rong Li*, Ying Liu, Hanne Skovsgaard Pedersen, Peter M. Kragh, Henrik Callesen

Department of Animal Science, Aarhus University, Aarhus, Denmark

ARTICLE INFO

Article history:
Received 30 December 2012
Received in revised form 16 March 2013
Accepted 18 March 2013

Keywords:
Time-lapse
Developmental kinetics
Apoptosis
Vitrification
Zona free

ABSTRACT

The need of the zona pellucida (ZP) for *in vitro* development is controversial because it might be an obstacle to hatching of the blastocyst. This study investigated the development and quality of porcine parthenogenetically activated (PA) embryos by observation of the developmental kinetics, the developmental percentages, the frequency of apoptosis, and robustness after removal of the ZP by pronase. Three experiments were made between zona-free PA embryos and zona-intact embryos: (1) determination of the timing of developmental stages using time-lapse observations for 6 days; (2) determination of developmental percentages and occurrence of apoptosis on Day 6 and Day 7 (Time of PA, Day 0); and (3) investigation of the robustness of embryos using vitrification on Day 4. The developmental kinetics showed that there was a general trend for zona-free PA embryos to develop faster than zona intact PA embryos at all developmental stages, but the difference was only significant at the five-cell stage. When compared with development of zona-intact embryos, ZP removal decreased the overall blastocyst percentage (83.9 ± 2.0 vs. 72.5 ± 2.9, respectively) and especially the percentage of good morphology (grades 1 and 2 combined) blastocysts (69.5 ± 2.0 vs. 55.7 ± 2.4, respectively). However, the process showed a significant decrease in apoptosis indicating an increased embryonic quality. Still, the survival percentage of porcine PA embryos after vitrification was dramatically decreased after ZP removal at all observation times ($P < 0.05$). In conclusion, removal of the zona pellucida might improve the embryonic quality by accelerating the speed of embryonic development and decreasing the number of apoptotic cells in blastocysts even though developmental percentages and embryonic robustness might be decreased.

1. Introduction

The zona pellucida (ZP) is the coat that covers mammalian oocytes and embryos. The main functions of the ZP are to connect with sperm and protect against polyspermy during fertilization; to protect integrity of the preimplantation embryo and sustain a stable microenvironment; and to protect the embryo against bacteria, fungi, immune cells and mechanical injury as the embryo travels through the oviduct [1,2]. For subsequent *in vivo* development the ZP is lost during hatching, a process that not only depends on blastocyst expansion, but presumably involves lytic factors from the uterus [3,4]. In this last phase, the ZP surface of *in vivo* embryos changes from a porous structure to one with a compact surface [5].

However, the ZP can also become an obstacle during hatching and subsequent uterine implantation, as is known from *in vivo* development [6,7], but even more from *in vitro*-produced embryos [8–11]. During *in vitro* procedures the ZP seems to harden as a result of different chemical agents [8], and this leads to differences in the ZP structure [5]. These differences between the ZP of embryos produced *in vivo* and *in vitro* are known from different species from morphological studies [5], studies of hatching

* Corresponding author. Tel.: +45 87154254; fax: +45 87156000.
E-mail address: lirong14@gmail.com (R. Li).

0093-691X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2013.03.009

times [4] and from the resistance times against enzymatic digestion [12,13].

Because hatching can become a problem for implantation and pregnancy, assisted hatching has been introduced as a method to improve implantation and pregnancy. This is known already for *in vivo* and *in vitro* embryos from human [8–10] and different animal species [6,7,11,14]. To perform assisted hatching, four different methods seem to yield similar implantation and pregnancy rates: mechanical partial dissection, laser, acid Tyrode, and enzyme (pronase) [9,10,15]. In the first two methods, a hole is made in the ZP, and the acid and the enzyme treatment remove the ZP more or less completely. Among these methods, the most preferred seems to be partial dissection using pipette [6,10,16] or laser [17]. However, complete removal of the ZP using an enzyme is easy and inexpensive, and the subsequent handling of zona-free embryos is safe and easy using different micro-well systems such as the Well of the Well (WOW) [18]. The use of zona-free embryos did not result in differences in efficiency of embryonic development [9,15,18], in quality of embryos based on total cell number in bovine [18] and pig [19], and in ultrastructure of human embryos [20]. In fact, zona removal improved efficiency of embryonic development and total cell number during parthenogenetic activation (PA) of porcine metaphase II oocytes [21].

The importance of synchronization between the embryos and the endometrium is a contributing factor for establishment of pregnancy after IVF [22,23]. In mouse, Talansky and Gordon reported that opening of the ZP by drilling accelerated the pronuclear appearance and increased subsequent development rates [24]. The consequences of zona removal are well described, however, there is no literature to observe the developmental kinetics of embryos after removal of the ZP.

In the present study, therefore, we investigated the effect of ZP removal on *in vitro* development and robustness using porcine parthenogenetically activated embryos in two ways: (1) by comparing the developmental kinetics of zona-free versus zona-intact embryos; and (2) by evaluating the effect of removal of the ZP on embryonic development, quality, and robustness.

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA) except when otherwise indicated.

2.1. Porcine parthenogenetically activated embryo production

Cumulus–oocyte complexes were aspirated from 2- to 6-mm follicles in slaughterhouse-derived sow ovaries and matured as described earlier [25,26]. Briefly, Cumulus–oocyte complexes with compact and at least two layers of cumulus cells were selected and cultured for 42 to 44 hours in four-well dishes (Nunc, Skovlunde, Denmark) in bicarbonate-buffered TCM-199 supplemented with 10% (vol/vol) cattle serum (CS; Danish Veterinary Institute, Frederiksberg, Denmark), 10% (vol/vol) sow follicular fluid, 10 IU/mL pregnant mare serum gonadotrophin and 5 IU/mL human chorionic gonadotrophin (Intervet) at 38.5 °C in 5% CO_2 with maximum humidity.

After maturation, oocytes were used for PA as described earlier [25]. Briefly, oocytes were equilibrated for 10 to 15 seconds in drops of activation medium (0.3 M mannitol, 0.1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.01% polyvinyl alcohol). Using a 0.12 kV/cm alternating current, oocytes were aligned to the wire of a fusion chamber (Microslide 0.5-mm fusion chamber, model 450; BTX, San Diego, CA, USA). Meanwhile, a single direct current pulse (1.26 kV/cm, 80 μs) was applied for activation. After washing twice in drops of TCM-199 with 10% fetal bovine serum, oocytes were incubated for 4 hours in culture medium (PZM-3 medium supplemented with 4 mg/mL bovine serum albumin, 5 mg/mL cytochalasin B, and 10 mg/mL cycloheximide) at 38.5 °C in 5% CO_2, 5% O_2, and 90% N_2 with maximum humidity. Putative PA embryos were either made zona-free (PAZF) or directly cultured (PAZI) in microwells (WOW, [18,27]). Zona pellucida removal was performed by placing the embryos in 0.3% (wt/vol) pronase for 30 seconds followed by immediate washing two to three times in culture medium, and then the remaining ZP was removed mechanically using a glass pipette (diameter: 200–300 μm). The time of electrical oocyte activation was defined as Day 0, and the PAZF and PAZI embryos were then randomly divided for the three experiments.

2.2. Experimental design

A schematic of the experimental design is shown in Figure 1.

2.2.1. Experiment 1

To determine the developmental kinetics, PAZI and PAZF embryos were cultured in a time-lapse incubator system with frequent morphological observations.

2.2.2. Experiment 2

To determine the developmental percentages and quality of PAZI and PAZF embryos, they were cultured in an incubator until Day 6 and evaluated morphologically. In four replicates, the Day 6 embryos were examined for total cell number and number of apoptotic cells. In three other replicates, the embryos were further cultured until Day 7,

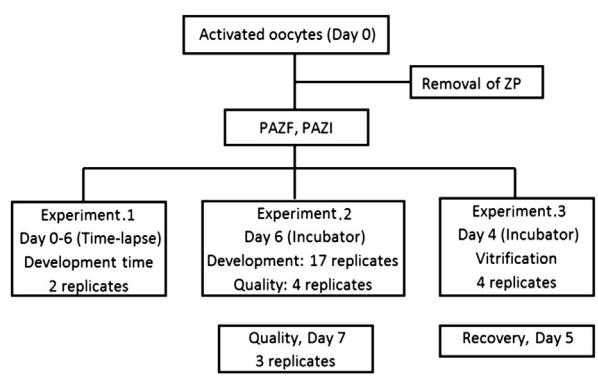

Fig. 1. Experimental design. PA, parthenogenetically activated; PAZF, zona-free PA embryo; PAZI, zona-intact PA embryo; ZP, zona pellucida.