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Distribution and content of lipid droplets and mitochondria in pig parthenogenetically activated embryos after delipation

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ABSTRACT

The present study examines the effect of delipation on developmental competence and the distribution pattern of lipid droplets (LDs) and mitochondria in parthenogenetically activated (PA) pig embryos. Mature oocytes were delipated by centrifugation after partial digestion of the zonae pellucidae, subjected to parthenogenetic activation after total removal of zonae pellucidae by pronase, and then cultured *in vitro* up to the blastocyst stage. The contents and distributions of LDs and mitochondria in the oocytes and/or embryos were observed by staining with Oil Red O and MitoTracker Red CMXRos, respectively. The LD and mitochondrial contents were significantly reduced by the delipation process, and only smaller LDs remained in the delipated oocytes and/or embryos. Their content remained constant from the metaphase II oocyte to the blastocyst stage, but they became gradually smaller as the oocytes and/or embryos developed. The distribution pattern of the LDs in the delipated embryos changed over time and in a manner different to that seen in the controls. In the early developmental stages (1- to 4-cell stages), they were distributed peripherally and formed a ring around the nucleus. However, by the blastocyst stage, a homogeneous distribution of LDs was observed in both the inner cell mass and trophectoderm. The distribution pattern of mitochondria also changed with the development of the delipated PA embryos and again, in ways different to those seen in the controls. In the early 1- to 4-cell stages, a peripheral distribution of mitochondrial foci was observed in each blastomere. However, in blastocysts, the mitochondria were homogeneously distributed throughout the inner cell mass and trophectoderm. Although the cleavage rate at the 2- and 4-cell stages of the PA embryos was not affected by delipation (95.83 ± 2.25% vs. 97.44 ± 0.67%; 79.17 ± 4.47% vs. 84.62 ± 1.19%), it was reduced significantly in the blastocyst compared with the controls (21.67 ± 3.78% vs. 49.36 ± 1.77%). The distribution pattern of the LDs in oocytes and/or embryos at different developmental stages, and that of the mitochondria in metaphase II oocytes, was affected by delipation. The developmental competence of porcine PA embryos would appear to be affected by delipation.

1. Introduction

In the past, lipid droplets (LDs) were only regarded as organelles for the storage of neutral lipids, triacylglycerols, and steryl esters [1–3]. Recently, however, it has been noticed that different types of lipids participate in a range of

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cellular processes [4–7] and interact with various organelles [8,9]. Indeed, LDs are now recognized as common subcellular structures [9–12], the number and distribution of which can change under different conditions. They are present in almost all cell types but especially in oocytes, in which their numbers can be very high [13]. The LD content of pig oocytes through perihatching blastocysts is particularly large [13–16], and the physical damage caused by them during cryopreservation leads to very low embryo survival rates [14,17–22]. Several authors have reported that the delipation of pig embryos improves the survival rate [17–25] and can even result in the birth of piglets [17,18,24,25]. However, most of these reports focused on the improvement of cryosurvival; few set out to observe the effect of delipation on the distribution of LDs and mitochondria.

Mitochondria are crucial to embryo viability. Their numbers in oocytes/embryonic cells are indicative of the energy and ion requirements associated with oocyte maturation, fertilization, and early embryonic development. Dysfunctions and/or abnormalities of the mitochondria in oocytes can induce apoptosis in embryos, compromising their developmental ability [26–29]. Interestingly, mitochondria may colocalize with LDs and together function as metabolic units in the oocytes of domestic species [30–33].

Lipid droplets can be removed from the mature oocytes by delipation [17–23]. The aims of the present work were to determine what effect this might have on LD and mitochondrial content and distribution in pig oocytes and parthenogenetically activated (PA) embryos and on the latters' developmental competence. The results obtained are important for understanding basic organelle and oocyte function, whereas knowledge of the distribution and content of LDs and mitochondria in PA embryos might provide an indicator of embryo viability.

2. Materials and methods

2.1. Chemical reagents

All chemicals were purchased from Sigma–Aldrich Co., Inc. (St. Louis, MO, USA) unless otherwise indicated. All manipulations were performed on a heated stage adjusted to 38.5 °C, unless otherwise indicated.

2.2. Oocyte collection and in vitro maturation

Ovaries were obtained from prepubertal gilts at a local abattoir and transported within 3 hours to the laboratory in 0.9% (w/v) NaCl at 37 °C. *In vitro* oocyte maturation was performed as previously described [34]. Briefly, cumulus–oocyte complexes (COCs) were aspirated from 2- to 6-mm follicles using an 18-ga needle connected to a 20-mL disposable syringe. Cumulus–oocyte complexes were selected according to their morphologic characteristics, i.e., showing at least three layers of compact cumulus cells and evenly granulated ooplasm. After washing three times in HEPES-buffered tissue culture medium 199 (TCM-199) plus 0.8-mM L-glutamine and then in 2% (v/v) cattle serum (CS), the COCs were cultured in groups of 50 to 60 in 4-well dishes (Nunc, Roskilde, Denmark) containing TCM-199 supplemented with 10% (v/v) CS, 10% (v/v) porcine follicular fluid, 0.8-mM L-glutamine, 75 μg/mL potassium penicillin G, 50 μg/mL streptomycin sulfate, 15 IU/mL serum gonadotrophin, and 15 IU/mL hCG, at 38.5 °C for 42 to 44 hours in a 5% CO_2/20% O_2 atmosphere at maximum humidity.

2.3. Removal of cytoplasmic LDs from mature oocytes (delipation)

Oocytes were delipated as previously described [19,22]. Briefly, after removal of the cumulus cells by repeated pipetting in 1 mg/mL hyaluronidase, the zonae pellucidae (ZP) (Fig. 1A) of oocytes showing the first polar body were partially digested by 1 mg/mL pronase for 2 to 3 minutes. Oocytes with a swollen ZP (Fig. 1B) were washed three times for 1 minute in T20 (T for HEPES-buffered TCM-199 and the number 20 for the percentage [v/v] of CS supplement). Some 50 to 60 oocytes were then centrifuged for 20 minutes (12,000 rpm; ×7576*g*) at room temperature (RT) in T2 supplemented with 7.5 μg/mL cytochalasin B. After centrifugation (Fig. 1C), the now delipated oocytes were placed in IVM medium for a 1-hour recovery period before further manipulation.

2.4. Production of PA embryos

The recovered delipated oocytes (Fig. 1C) were then further treated with 3.3 mg/mL pronase for 1 minute to totally remove the ZP. These, along with control IVM oocytes with the intact ZP and showing the first polar body (Fig. 1 A), were washed twice in an activation solution consisting of 0.3-M mannitol, 0.1-mM $MgSO_4$, 0.05-mM $CaCl_2$, and 0.01% polyvinyl alcohol. They were then electrically activated in a BTX microslide 0.5-mm fusion chamber (model 450; BTX, San Diego, CA, USA) using a single direct current pulse of 0.86 kV/cm for 80 μs, followed by chemical activation with 10 μg/mL cycloheximide and 5 μg/mL cytochalasin B in PZM-3 medium [35] for 4 hours. The delipated oocytes were then cultured in a well of wells [36] and the control PA oocytes were cultured in PZM-3 medium in a 4-well plate, both at 38.5 °C in a 5% CO_2/20% O_2 atmosphere at maximum humidity.

2.5. Viability of delipated embryos

The *in vitro* developmental competence of the delipated oocytes was assessed by determining the percentage of embryos reaching the 2-cell (24 hours after PA), 4-cell (36 hours after PA), and blastocyst (144 hours after PA) stages. To determine the total cell number, Day 6 blastocysts (performed in triplicate, 6–10 per replicate) were randomly collected and stained with 10 μg/mL Hoechst 33342 in PBS for 20 minutes (Fig. 2). Their morphology was observed by epifluorescent microscopy using a UV-2A filter (Leica DMIRB, Cambridge, UK) and the total number of cells per blastocyst counted.

2.6. Staining of LDs

After washing in PBS, all processed metaphase II (MII) oocytes and PA embryos (1-cell, 2-cell, 4-cell, and blastocyst