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Peroxidized mineral oil increases the oxidant status of culture media and inhibits *in vitro* porcine embryo development

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ABSTRACT

The use of oils with undetected alterations is a long-recognized problem for *in vitro* embryo production systems. Since peroxides in oils have been associated with reduced embryo production outcomes, our goals were (1) to evaluate the effects of a batch of mineral oil (MO) that was suspected to be altered on the *in vitro* production of pig embryos and (2) to determine oil peroxide values throughout culture and the transfer of oxidant agents from oil to culture media. Sunflower oil, which has a completely different chemical composition than MO but a higher oxidative status, and unaltered MO were used as controls.

Oocyte maturation, fertilization and embryo development were affected differently depending on the oil overlay used. While the suspected MO was not able to sustain *in vitro* maturation and fertilization, the oocytes incubated in the presence of sunflower oil were matured and fertilized similarly to those of the unaltered MO group. Moreover, the cleavage rate of presumed zygotes cultured under the suspected MO was severely reduced compared with those cultured under the other oils, and none of the cleaved embryos developed to the blastocyst stage. Although the cleavage rates in the sunflower oil and unaltered MO groups were similar, embryos cultured under sunflower oil also failed to develop to the blastocyst stage. Our results revealed that the suspected MO and sunflower oil had similar levels of peroxides and that these levels were much higher than those of the unaltered MO. The total oxidant status was higher in media incubated under peroxidized oils than in fresh media or media incubated without an oil overlay or under unaltered MO, indicating that oxidant agents were transferred to the incubation media. However, unlike the sunflower oil group, the culture media incubated under the suspected MO had high levels of total oxidant status and low levels of hydrogen peroxide and reactive oxygen species, suggesting the presence of other unknown oxidant agents in that oil. These results indicate that a peroxidized MO overlay dramatically decreases embryo production outcomes. This decrease could be associated with the higher peroxide values of the oil but cannot be explained by the levels of hydrogen peroxide and reactive oxygen species transferred from the oil to the culture media. It is likely that different oxidant agent(s) and/or other toxic compounds present in the peroxidized MO are responsible for its damaging effects on oocytes and embryos.

1. Introduction

Mineral oil is widely used in embryo *in vitro* production (IVP) systems to cover drops of culture media because among other advantages, it helps to maintain a stable and proper pH and osmolality in the culture medium [1–5]. This fact is critical since variations in the pH of the medium might affect embryo metabolism [6,7], whereas osmotic stress may cause DNA and protein alterations that affect cell function [8]. However, the use of an oil overlay may also have adverse effects due to the possibility that toxic contaminants that accumulate in the oil during production, transport or storage may be introduced into the culture medium [9,10] and may negatively affect gametes and embryos. The use of

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oils with undetected contamination has long been recognized as a problem for embryo cultures. Oil degradation produces alkenals and aldehydes [11], which have been shown to be toxic to embryos when present in culture media [12]. Triton X-100, a nonionic surfactant that is used in research to permeabilize membranes or solubilize proteins, has also been found in oils used for embryo culture where the embryo development was severely inhibited [11]. In another study, zinc was identified as a possible toxic contaminant of silicone oil in mouse embryo cultures [13]. However, peroxides, which produce reactive oxygen species, are possibly the most dangerous contaminants found in altered oils. They can be formed throughout the shelf life of the oil and have been associated with decreased fertilization, cleavage, and blastocyst formation rates in rodents [11,14,15]. Although these findings suggest that peroxidized oil overlays have embryo-toxic effects, the transfer of oxidant compounds from the oil to the culture medium has not been clearly established.

The aims of this study were (1) to evaluate the effects of a batch of mineral oil, which was suspected to be altered (SMO), on the IVP of pig embryos and (2) to determine oil peroxide values (POVs) throughout culture and the transfer of oxidant agents from oil to culture media.

2. Materials and methods

All experiments were performed following the ethical guidelines of the 2010/63/EU EEC Directive for animal experiments and were approved by the Ethical Committee for Experimentation with Animals of the University of Murcia, Spain (research code: 183/2015).

2.1. Collection of cumulus-oocyte complexes (COCs)

Pre-pubertal gilts of 5.5–6 months of age and weighing 90–100 kg were used as COC donors. The pre-pubertal gilts were sacrificed in a local slaughterhouse, and their ovaries were transported to the laboratory at the University of Murcia at 35 °C within 1 h post-collection in 0.9% NaCl containing 70 μg/mL kanamycin. The COCs were collected with a surgical blade from medium-sized healthy follicles (3–6 mm diameter) in Tyrode's lactate medium supplemented with 10 mM HEPES and 0.1% polyvinyl alcohol (PVA) [16,17]. Oocytes surrounded by two or more compact cumulus mass complexes and with a dark and granulated cytoplasm were selected for maturation.

2.2. In vitro maturation (IVM) and assessment of maturation

The COCs were washed three times in IVM medium. This medium consisted of TCM-199 (Gibco Life Technologies S.A. Barcelona, Spain) supplemented with 0.57 mM cysteine, 0.1% PVA and 10 ng/mL epidermal growth factor. Groups of 40 COCs were matured in 500 μL of IVM medium supplemented with 10 IU/mL equine chorionic gonadotrophin (Folligon, Intervet International B.V., Boxxmeer, The Netherlands) and 10 IU/mL human chorionic gonadotrophin (Veterin Corion, Divasa Farmavic, S.A., Barcelona, Spain) for 22 h. The COCs were then incubated for an additional 22 h in the same medium but without hormones. All cultures were incubated under an oil overlay at 38.5 °C in a humidified atmosphere of 5% CO_2 in air.

To assess the maturation status, the oocytes were fixed in acetic acid:ethanol (1:3) for 72 h at room temperature and stained with 1% lacmoid in 45% acetic acid. The oocytes were considered immature when their chromatin was enclosed in a nuclear membrane (i.e., germinal vesicle stage) or when it was condensed in metaphase I. The oocytes were considered mature when their chromosomes were organized in metaphase and a clear first polar body was visible (MII). Oocytes with a broken cytoplasmic membrane or a cytoplasm with an abnormal appearance were considered to be degenerated. The degenerated, immature and mature oocyte rates were expressed as the ratio of the number of degenerated, immature and MII stage oocytes, respectively, relative to the total number of oocytes evaluated.

2.3. In vitro fertilization (IVF) and assessment of fertilization status

At the end of the maturation period, the COCs were denuded by vortexing at 1660 rounds/min for 2 min in IVM medium containing 0.1% hyaluronidase and washed three times in IVM medium. The oocytes were then washed three times in pre-equilibrated IVF medium [18] supplemented with 2.0 mM caffeine and 0.2% bovine serum albumin (BSA) and inseminated as described previously [19]. Briefly, the oocytes were placed in groups of 40 in 50-μL drops of IVF medium covered with oil and kept in the incubator at 38.5 °C and 5% CO_2 in humidified air for 30 min until sperm were added. Semen cryopreserved in 0.5-mL straws, as described by Carvajal et al. [20], was thawed (two straws for each replicate) at 37 °C for 20 s and washed three times at 1900 × g for 3 min in Dulbecco's PBS (Gibco, Grand Island, NY) supplemented with 0.1% BSA. The sperm pellet was then re-suspended in IVF medium, and after being appropriately diluted, 50 μL of the sperm suspension was added to the drop of IVF medium that contained the oocytes. The spermatozoa:oocyte ratio was 1000:1. The oocytes and spermatozoa were co-incubated at 38.5 °C in a humidified atmosphere of 5% CO_2 in air for 5 h under oil.

To evaluate fertilization parameters, presumptive zygotes were fixed and stained as described for the maturation assessment. Presumptive zygotes were classified as penetrated when at least a sperm head and/or male pronucleus, with the corresponding sperm tail, and two polar bodies were visible in their cytoplasm. Oocytes with an abnormal cytoplasm appearance were classified as degenerated. The penetration rate was defined as the ratio of the number of penetrated oocytes relative to the total number of mature oocytes. The monospermic rate was the ratio of the number of oocytes with one female pronucleus, one male pronucleus and two polar bodies relative to the total number of penetrated oocytes. The fertilization efficiency was defined as the ratio of the number of monospermic oocytes relative to the total number of oocytes inseminated. The degeneration rate was the ratio of the number of degenerated presumptive zygotes relative to the total number of oocytes evaluated.

2.4. In vitro culture (IVC) and assessment of embryonic development parameters

Following sperm-oocyte co-incubation, groups of 30 presumptive zygotes were washed three times and cultured in 500 μL of IVC medium, which consisted of glucose-free pre-equilibrated North Carolina State University culture medium (NCSU-23; [21]) supplemented with 0.4% BSA, 0.3 mM pyruvate and 4.5 mM lactate, for 2 days. Then, the cleaved embryos were cultured in fresh IVC medium supplemented with 0.4% BSA and 5.5 mM glucose for an additional 5 days. All embryo cultures were incubated at 38.5 °C in an atmosphere of 5% CO_2 in air under oil.

Embryonic development was evaluated morphologically under a stereomicroscope. Embryos with a morphologically abnormal appearance and those that were poorly developed were classified as degenerated embryos. The cleavage rate was defined as the ratio of the number of embryos developed to the 2- to 4-cell stage at day 2 of culture relative to the total number of oocytes inseminated. The blastocyst formation rate was the ratio of the number of 2- to 4-cell