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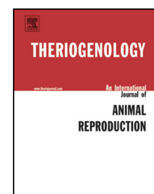

Protective effects of antioxidants on linoleic acid–treated bovine oocytes during maturation and subsequent embryo development

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ABSTRACT

Linoleic acid (LA; *n-6*, 18:2) is the most abundant polyunsaturated fatty acid in the ovarian follicular fluid and is known to inhibit oocyte maturation and its subsequent development. In the present study, we investigated how its effects on cumulus cell expansion, oocyte nuclear maturation, and blastocyst development are altered by supplementation of the media with vitamin E (VE; 100 μM) and glutathione peroxidase (GPx; 1 μM) either alone or in combination, and whether it has any effect on the mRNA expression of *GPx1*, *GPx4*, or superoxide dismutase (*SOD2*) in the bovine cumulus oocyte complexes (COCs). LA supplementation of the culture media significantly ($P \leq 0.05$) reduced the percentage of COCs exhibiting full cumulus cell expansion and the percentage of oocytes reaching metaphase II stage, and lowered the blastocyst rate compared with controls. And these inhibitory effects were associated with a reduction in the relative mRNA expression of *GPx1* and *SOD2* but not of *GPx4* compared with controls. However, VE and GPx, both alone and in combination, completely abrogated the inhibitory effects of LA on nuclear maturation of oocytes and blastocyst rate but failed to do so for cumulus cell expansion. In conclusion, these data suggest that the detrimental effects of LA on oocyte developmental competence are mediated, at least in part, by a reduction in *GPx1* and *SOD2* mRNA expression. Moreover, VE and GPx may provide protection to most of the inhibitory effects of LA.

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1. Introduction

Supplementation of diet with polyunsaturated fatty acids (PUFAs) can influence the reproductive performance of cattle [1–4]. Moreover, dietary PUFA type and content is reflected in the fatty acid profile of plasma and ovarian follicular fluid [5]. The biochemical characters of the follicular fluid determine the microenvironment in which oocyte growth and maturation takes place. Therefore, it is likely that the effects of PUFAs on animal fertility may be mediated by altering the characteristics of the follicular fluid with the ultimate effect on the oocyte maturation, which is critical for its subsequent development after fertilization [6]. It has been previously reported that treatment of bovine cumulus oocyte complexes (COCs) with physiological concentrations of LA affects the molecular mechanisms that control oocyte nuclear maturation, leading to a decreased proportion of oocytes reaching MII stage at 24 hours of culture, and inhibits subsequent early embryo development [7]. Further studies revealed that LA induces alterations in mitochondrial distribution and activity associated with increased reactive oxygen species (ROS) levels [8]. The resulting oxidative stress was suggested to mediate, at

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least in part, the inhibitory effects of LA on oocytes' developmental competence.

Production of ROS (which include superoxide anion [O_2^-] and hydrogen peroxide [H_2O_2]) is a physiological process and occurs in the cell mainly during the mitochondrial energy metabolism. The primary ROS generated by the mitochondria, O_2^-, is transformed by superoxide dismutase (SOD2) enzymes into a more stable ROS, H_2O_2, which then diffuses into the cytoplasm [9]. H_2O_2 plays important roles in cell's function through activation of cell signaling cascades, such as those involving mitogen-activated protein kinases [10]. ROS have also been shown to act as second messengers and regulate transcription factors [11]. In oocytes, short-term exposure to H_2O_2 for 1 hour at the beginning of maturation enhanced the development to blastocyst stage [12]. In addition, a transient increase in ROS in oocytes was found to signal resumption of meiosis by activation of phosphodiesterase 3A (PDE3A) that hydrolyzes cAMP in oocytes resulting in activation of maturation promoting factor [13]. When H_2O_2 concentrations in the cytoplasm reach above the physiological threshold, it can be removed by cytosolic antioxidant systems of the cell. These antioxidant defense mechanisms may include both enzymatic such as catalase, glutathione peroxidase (GPx) [14], and SOD2 [15], and non-enzymatic such as vitamin E (VE) (α-tocopherol), cysteamine, vitamin C, vitamin A, pyruvate, taurine, and hypotaurine [15] antioxidants.

Oxidative stress reflects an imbalance between production of ROS and cellular antioxidant defense mechanisms [16]. ROS are highly reactive with complex cellular molecules such as proteins, lipids, and DNA and may alter their functions, which may have serious consequences, for instance, enzymatic inactivation, DNA fragmentation, and irreversible damage of mitochondrial DNA, membrane lipids, and proteins, resulting in mitochondrial dysfunction and ultimately cell death [17,18].

Oxidative stress is one of the major obstacles to embryo development [15], and several exogenous factors can increase the production of ROS by embryos. These factors include oxygen concentration, metallic cations, visible light, and spermatozoa [15]. Many fatty acids have also been shown to cause oxidative stress by stimulating the production of ROS in different cell types [19], including COCs [8,20]. As oxidative stress has previously been suggested to mediate the inhibitory effects of LA on oocytes' developmental competence [8], the aims of the present study were to confirm whether LA inhibits oocyte maturation and/or further development, and if so, then to investigate the mechanism by which these effects are mediated. This was done by determining the mRNA expression of GPx and SOD2 in COCs treated with LA both in the presence and absence of VE and/or GPx. The study also investigated whether the inhibitory effects of LA could be alleviated by treatment with antioxidants like VE and/or GPx.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma Chemical Co. (Poole, UK).

2.2. Experimental design

In all experiments, grade 1 bovine COCs having homogenous ooplasm and more than four compact layers of cumulus cells were used. They were randomly allocated to one of the five treatment groups for *in vitro* maturation for a period of 24 hours. The treatment groups were as follows: (1) control maturation media free from LA or antioxidants, or media supplemented with (2) LA (water soluble LA, Sigma L5900, stock solution 100 mM in M199; final concentration 100 μM), (3) LA in the presence of VE (Sigma T3251, stock solution 100 mM in ethanol, final concentration 100 μM), (4) LA in the presence of GPx (Sigma 49753, stock solution 1 mM in M199, 1 μM), and (5) LA in the presence of both VE and GPx. Because ethanol was used to dissolve VE, it was also added to control and other treatment groups (0.1% vol/vol).

In experiment 1, cumulus cell expansion was recorded and oocytes were denuded, fixed, and stained to assess the stage of nuclear maturation. A total of 757 COCs were used in four independent replicates, allocating about 25 COCs per treatment per replicate.

In experiment 2, RNA was extracted from the treated COCs and reverse transcribed, and the mRNA expression of *GPx1*, *GPx4*, and *SOD2* was determined. A total of 375 COCs were used in three independent replicates, allocating 25 COCs per treatment per replicate.

In experiment 3, treated COCs were *in vitro* fertilized and cultured for 8 days. Cleavage and blastocyst rates were recorded on Days 3 and 7, respectively. A total of 477 COCs were used in three independent replicates, allocating about 30 COCs per treatment per replicate.

2.3. Collection of oocytes

Bovine ovaries were collected from a local abattoir, transported to the laboratory in PBS at 37 °C within 2 hours after slaughter, and washed with fresh PBS immediately after arrival. The COCs were retrieved from antral follicles 2 to 8 mm in diameter with a 19-gauge needle mounted on a 10-mL syringe. The grade 1 COCs were selected under a stereomicroscope and washed two times in TCM-199 supplemented with 20 mM HEPES and 0.4% (wt/vol) BSA.

2.4. In vitro maturation

Selected COCs were washed twice and cultured in four-well dishes (NUNC; Thermo Fisher Scientific, Loughborough, UK) using 500 μL of maturation medium per well. The maturation medium comprised medium TCM-199 supplemented with 0.6% (wt/vol) fatty acid–free BSA, 10 μg/mL follicle-stimulating hormone (Follitropin; Bioniche Animal Health, Belleville, ON, Canada), 10 μg/mL luteinizing hormone (Leutropin; Bioniche Animal Health), 1 μg/mL estradiol, and 50 μg/mL gentamycin. The COCs were incubated for 24 hours at 38.5 °C under 5% CO_2 in humidified air [21].

2.5. Assessment of cumulus cell expansion

The degree of cumulus expansion was assessed under a stereomicroscope after 24 hours of maturation subjectively

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