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Physiological responses of cultured bovine granulosa cells to elevated temperatures under low and high oxygen in the presence of different concentrations of melatonin

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ABSTRACT

Our understanding of the effects of temperature on granulosa cell (GC) physiology is primarily limited to *in vitro* studies conducted under atmospheric (~20% O_2) conditions. In the current series of factorial experiments we identify important effects of O_2 level (i.e. 5% vs 20% O_2) on GC viability and steroidogenesis, and go onto report effects of standard (37.5 °C) vs high (40.0 °C) temperatures under more physiologically representative (i.e. 5%) O_2 levels in the presence of different levels of melatonin (0, 20, 200 and 2000 pg/ml); a potent free-radical scavenger and abundant molecule within the ovarian follicle. Cells aspirated from antral (4–6 mm) follicles were cultured in fibronectin-coated wells using serum-free M199 for up to 144 h. At 37.5 °C viable cell number was enhanced and luteinization reduced under 5 vs 20% O_2. Oxygen level interacted ($P < 0.001$) with time in culture to affect aromatase activity and cell estradiol (E_2) production (pg/mL/10^5 cells). These decreased between 48 and 96 h for both O_2 levels but increased again by 144 h for cells cultured under 5% but not 20% O_2. Progesterone (P_4) concentration (ng/mL/10^5 cells) was greater ($P < 0.001$) under 20 vs 5% O_2 at 96 and 144 h. Cell number increased ($P < 0.01$) with time in culture under 5% O_2 irrespective of temperature. However, higher doses of melatonin increased viable cell number at 40.0 °C but reduced viable cell number at 37.5 °C ($P = 0.004$). Melatonin also reduced ($P < 0.001$) ROS generation at both O_2 levels across all concentrations. E_2 increased with time in culture at both temperatures under 5% O_2, however P_4 declined between 96 and 144 h at 40.0 but not 37.5 °C. Furthermore, melatonin interacted ($P < 0.001$) with temperature in a dose dependent manner to increase P_4 at 37.5 °C but to reduce P_4 at 40.0 °C. Transcript expression for *HSD3B1* paralleled temporal changes in P_4 production, and those for *HBA* were greater at 5% than 20% O_2, suggesting that hemoglobin synthesis is responsive to changes in O_2 level. In conclusion, 5% O_2 enhances GC proliferation and reduces luteinization. Elevated temperatures under 5% O_2 reduce GC proliferation and P_4 production. Melatonin reduces ROS generation irrespective of O_2 level and temperature, but interacts with temperature in a dose dependent manner to influence GC proliferation and luteinization.

1. Introduction

Thermal stress can have a detrimental effect on ovarian function and endometrial receptivity in the cow leading to reduced expression of estrus, impaired post-fertilisation development of oocytes and implantation failure [1–3]. It is particularly problematic in the metabolically challenged high-yielding dairy cow that struggles to dissipate heat under moderate to high (typically ≥25 °C) ambient temperatures [4,5]. Reduced blood flow to the ovary in such animals can contribute to observed delays in emergence of dominant/pre-ovulatory follicles [6] which in turn can indirectly compromise oocyte quality. However, the effects of heat stress are also believed to directly and negatively affect both pre- and early-antral stages of follicle development, and the pool of germinal vesicle-stage oocytes contained therein [7].

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In order to gain a better understanding of the mechanisms underlying the effects of thermal stress on follicular development several studies have undertaken short-term *in vitro* culture of granulosa and/or thecal cells simulating normal or high core-body temperatures. They confirmed reductions in cell viability associated with upregulation of apoptotic pathways and reduced steroidogenic capacity [1,8,9]. However, whilst providing valuable insights into underlying mechanisms, these and other related studies [10], invariably cultured cells under atmospheric (~20%) O_2 levels often in the presence of serum. To the best of our knowledge there are no *in vitro* culture studies that have assessed the effects of thermal stress on somatic cells of the ovary under more physiological O_2 levels (~5% O_2), although one recent study considered temperature and atmospheric environment in the context of ROS generation during bovine oocyte maturation [11]. This issue is important because it is believed that both bovine and porcine GCs cultured under low O_2 are more prolific, glycolytic and estrogenic than GCs cultured in 5% CO_2 in air [12,13]. A low O_2 culture environment probably better recapitulates intra-follicular atmospheric conditions as fractional O_2 concentrations in follicular fluid range between 2 and 9% [14,15].

Variable concentrations (10 to > 400 pg/mL) of the indole amine melatonin have been reported in follicular fluid of different species including the cow [16–20]. Melatonin is believed to exert protective effects on ovarian cells during thermal stress due to its capacity to act as a potent antioxidant [21]. However, as with the studies described earlier, *in vitro* culture experiments with melatonin have to date invariably been conducted under atmospheric O_2 conditions [17,22] so that the described modes of action and benefits of melatonin may be specific to these situations and less representative of intra-follicular processes. Culturing under low O_2 may provide a more physiologically relevant system to investigate the effects of this potent antioxidant in helping to attenuate thermal stress on somatic cells within the ovarian follicle. The current series of experiments, therefore, sought initially to characterise the effects of O_2 level (i.e. 5% vs 20% O_2) on GC viability and steroidogenesis and then to investigate the effects of standard (37.5 °C) vs high (40.0 °C) temperatures under low (i.e. 5%) O_2 levels in the presence of different concentrations of melatonin (0, 20, 200 and 2000 pg/mL).

2. Materials and methods

All reagents were obtained from Sigma–Aldrich unless otherwise stated.

2.1. Granulosa cell culture

Antral follicles (4–6 mm) were aspirated from abattoir derived ovaries using a 21 G needle and GCs prepared for serum-free culture. These GCs were therefore likely to represent a population of largely luminal GCs and cumulus cells. Compared to mural GCs (scraped from dissected follicles of comparable size) these cells are more estrogenic [12] and mitotically active [23]. Viable cells (determined by trypan blue exclusion [24] were re-suspended in 1 ml of pre-warmed M199 culture medium supplemented with (Penicillin (50 IU/ml), Streptomycin (50 μg/ml), bovine serum albumin free fatty acid (BSA; 1 mg/ml), testosterone (100 ng/ml), FSH (1 ng/ml; Cat. No. F2293), insulin (10 ng/ml), transferrin 2.5 (μg/ml), sodium selenite (4 ng/ml) and L-glutamine (365 μg/ml))) prior to plating in fibronectin coated wells (Nunclon Delta, Thermo Fisher, Denmark) at seeding densities depicted in Table S1. Fibronectin facilitates the attachment and proliferation of GCs [25], whilst low insulin (10 ng/mL) in serum-free media allows cells to form aggregates, proliferate and maintain a primary GC phenotype [26]; hence their responsiveness to trophic hormones [27].

2.2. Experimental designs

2.2.1. Experiment 1.A. effect of atmospheric vs physiological O_2 level on cell number, steroidogenesis and aromatase activity

This was a 2 × 3 factorial experiment with two O_2 levels (~5 vs 20%; using two humidified incubators (Model Innova CO-14, New Brunswick Scientific, Edison, NJ, USA) at 37.5 °C) and three culture endpoints (48, 96 and 144 h from seeding), replicated five times using a 6-well plate format (Table S1). 80% of media was replaced every 48 h during culture. Upon harvesting, spent media and cell pellets were snap frozen in liquid N and stored at −80 °C until analysis.

2.2.2. Experiment 1.B. effect of melatonin on cell number, steroidogenesis and gene expression under atmospheric vs physiological O_2 levels

This was a 4 × 2 x 3 factorial experiment with four levels of melatonin (0, 20, 200, and 2000 pg/ml), two O_2 levels (~5 vs 20%; using two humidified incubators at 37.5 °C) and three culture endpoints (48, 96 and 144 h from seeding), replicated four times using a 12-well plate format (Table S1). Melatonin levels for this and subsequent experiments were selected on the basis of concentrations reported previously in ovarian follicular fluids [16–20], and from a small pilot study where we determined melatonin concentrations by ELISA (MyBioSource.com; San Diego, CA, USA; Bovine kit - MBS743340) in follicular fluids from 15 heifers slaughtered at a local abattoir (Fig. S1). Simple and geometric means for ovarian follicular-fluid melatonin in that study were 1600 (95% CI = 173–3036) and 320 pg/mL respectively. Media were changed and cells harvested as described for Experiment 1A.

2.2.3. Experiment 1.C. effect of O_2 level and melatonin on ROS

This experiment adopted the factorial arrangement described for Experiment 1B but using a 96-well format (Table S1). Media were changed as described for Experiment 1A. Generation of ROS was assessed at 48, 96 and 144 h of culture (described later).

2.2.4. Experiment 2.A. effect of temperature and melatonin on cell number, steroidogenesis and gene expression under physiological O_2 levels

This experiment adopted a factorial arrangement similar to Experiment 1B but treatments (37.5 vs 40.0 °C; using two humidified incubators at 5% O_2 with four melatonin doses (0, 20, 200 and 2000 pg/ml)) commenced after 48 h of culture (Table S2). Incubator temperature was monitored using two thermometers (temperature loggers, EL-USB-1, Lascar Electronics, Salisbury, UK) in addition to that built into the incubator. Media were changed and cells harvested as described for Experiment 1A.

2.2.5. Experiment 2.B. effect of temperature and melatonin on ROS production under physiological O_2 levels

This experiment also adopted the factorial arrangement described for Experiment 1B but using a 96-well format (Table S2) and with treatments described for Experiment 2A. Media were changed as described for Experiment 1A. Generation of ROS was assessed at 96 and 144 h of culture (described later).

2.3. Hormone analyses

Progesterone and E_2 production by GCs after 48, 96 and 144 h of culture in Experiment 1A, 1B and 2A was assessed by ELISA using commercial kits provided by Ridgeway Research Ltd, Gloucestershire, UK (P_4 product code RIDGE-P), and DRG GmbH, Marburg,