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Effect of heat stress on the survival and development of *in vitro* cultured bovine preantral follicles and on *in vitro* maturation of cumulus–oocyte complex

V.M. Paes [a], L.A. Vieira [a], H.H.V. Correia [a], N.A.R. Sa [a], A.A.A. Moura [d], A.D. Sales [a], A.P.R. Rodrigues [a], D.M. Magalhães-Padilha [b], F.W. Santos [c], G.A. Apgar [f], C.C. Campello [a], L.S.A. Camargo [e], J.R. Figueiredo [a,*]

[a] *Laboratory of Manipulation of Oocyte and Preantral Follicles (LAMOFOPA), State University of Ceará, Fortaleza, CE, Brazil*
[b] *Postgraduate biotechnology, Potiguar University/Laureate International Universities, Natal, RN, Brazil*
[c] *Laboratory of Reproduction Biotechnology (Biotech), State of University of Pampa, Uruguaiana, RS, Brazil*
[d] *Laboratory of Animal Reproduction, Federal University Ceará, Fortaleza, CE, Brazil*
[e] *Dairy Cattle Research Center, EMBRAPA, Juiz de Fora, MG, Brazil*
[f] *Department of Animal Science, Food and Nutrition, Southern Illinois University, Carbondale, Illinois, USA*

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ABSTRACT

The deleterious effect of heat stress (HS) on competence of oocytes from antral follicles is well recognized, but there is a lack of data regarding its impact on the viability and growth of preantral follicles. In this study, we used *in vitro* preantral follicle cultures to investigate the effects of HS on the following parameters: survival and development of primordial follicles after *in vitro* culture of ovarian fragments (experiment I); growth and antrum formation of isolated advanced secondary follicles (experiment II); and maturation rates after *in vitro* maturation (IVM) of cumulus–oocyte complexes (COCs) from antral follicles (>2–6 mm) grown *in vivo* (experiment III). Furthermore, the following end points were evaluated in all experiments: follicle/oocyte survival, reactive oxygen species (ROS), estradiol (E2) and progesterone (P4) production, as well as mRNA expression for select genes related to stress (HSP70) and apoptosis (MCL1 and BAX). In all experiments, HS consisted of exposing the structures (ovarian fragments, isolated preantral follicles and COCs) to 41 °C for 12 hours and then to 38.5 °C until the end of the culture (7 days for experiments I and II and 24 hours for experiment III). The temperature for the control group was held at 38.5 °C for the entire culture period. Heat stress increased ($P < 0.05$) the percentage of developing follicles (intermediate, primary, and secondary follicles) at 12 hours and increased levels of ROS at all evaluated time points (12, 24 hours, and D7), when compared to the control (experiment I). Heat stress did not affect ($P > 0.05$) any identified end points when preantral follicles were cultured in their isolated form (experiment II). However, in experiment III, HS decreased ($P < 0.05$) both the rates of metaphase II after 24 hours and E2 production at 12 hours of IVM. Moreover, HS increased ($P < 0.0001$) levels of P4 after IVM and ROS production at every evaluated time point, compared with the control (12 and 24 hours). In conclusion, HS caused: (1) early activation of primordial follicles; (2) an increase in ROS production by early preantral follicles enclosed in ovarian tissue and by COCs; (3) a short-term reduction of E2 production by COCs; and (4) an increase in P4 secretion from COCs. However, HS did not affect *in vitro* culture of advanced isolated secondary follicles. Experimental evidence indicates that preantral follicles are less sensitive to HS than COC.

* Corresponding author. Tel.: +55 85 3101 9852; fax: +55 85 3101 9840.
E-mail address: jrf.lamofopapapers@gmail.com (J.R. Figueiredo).

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1. Introduction

Folliculogenesis is regulated by complex interactions between autocrine, paracrine, and endocrine factors [1] and can also be influenced by environmental factors such as high temperature [2,3].

In this regard, exposure to high temperatures is the main cause of decreasing fertility in cattle [4], affecting 60% of animal population during the warmest season of the year [5].

Cows under acute heat stress (HS) reported a reduction in follicle diameter, steroid production by theca and/or granulosa cells, as well as serum estradiol concentration [2,6]. A decrease in estradiol concentration has been associated with failures in luteolysis, due to an extended luteal phase with multiple follicular waves within the same estrous cycle, compromising the quality/competence of ovulated oocytes [2,6–8].

In the bovine, two to three estrous cycles are needed for full ovarian recovery after HS [9]. Moreover, previous studies conducted with cattle have shown that the same period of time (2–3 estrous cycles) is required for the follicle to grow from early antral to preovulatory stage [10]. On the basis of this information, it is suggested that HS affects at least antral follicles. In fact, oocytes originating from antral follicles grown *in vivo* are sensitive to HS either *in vivo* or *in vitro* [11,12]. To date, studies have shown that HS applied on bovine oocytes during IVM as well as on embryos during *in vitro* culture reduces both oocyte maturation [13–15] and embryo development rates [16,17], respectively. Although the impact of HS on antral follicles *in vivo* is well documented, the impact of HS on the preantral phase of folliculogenesis in the bovine remains unknown. Therefore, an appropriate manner to study the effect of HS on preantral follicles, which represent 90% of ovarian follicle population, is the use of *in vitro* preantral follicle culture [18].

Therefore, the novelty of the present study is to investigate the effect of HS on preantral follicles using bovine follicles cultured *in vitro*. The objective of this study was to investigate the influence of HS on *in vitro* folliculogenesis in bovine, evaluating: (1) survival and development of primordial follicles after *in vitro* culture of ovarian fragments; (2) growth and antrum formation of isolated advanced secondary follicles; and (3) maturation rates after *in vitro* maturation (IVM) of cumulus–oocyte complexes (COCs) from antral follicles grown *in vivo*. Furthermore, the following end points were evaluated in all experiments: follicle/oocyte survival, reactive oxygen species (ROS), estradiol (E2) and progesterone (P4) production, as well as quantitative expression of some genes related to stress (HSP70) and apoptosis (BAX and MCL1).

2. Materials and methods

Alginate was provided by FMC BioPolymers (Philadelphia, PA, USA). Culture media and other chemicals used in the present study were purchased from Sigma Chemical Co. (St Louis, MO, USA), unless mentioned otherwise.

2.1. Ovaries

Ovaries (n = 144) from 72 adult mixed-breed cows were collected from a local abattoir. Immediately after slaughter, ovaries (n = 96) were removed and washed once in 70% alcohol and twice in minimum essential medium supplemented with HEPES (HMEM) and antibiotics (100 μg/mL penicillin and 100 μg/mL streptomycin). The ovaries were transported to the laboratory within 1 hour at 4 °C [19] or 20 °C [20], for experiments I and II, respectively. For experiment III, the ovaries (n = 48) were washed and transported in saline solution (0.9% p/vol) containing 100 mg/L kanamycin sulfate at 33 °C [21,22].

2.2. Experimental design

The present study investigated the effect of HS on bovine early preantral follicles (primordial and primary follicles—experiment I), advanced preantral follicles (secondary–experiment II), and COCs (experiment III) (Fig. 1). For experiments I and II, preantral follicles were cultured enclosed in ovarian tissue (*in situ*) and in the isolated form, respectively. For experiment III, COCs from *in vivo* grown antral follicles (≥2–6 mm) were used.

In all experiments, HS consisted of exposing the structures (ovarian fragments, isolated preantral follicles and COCs) to 41 °C for 12 hours after which structures were held at 38.5 °C until the end of the culture (7 days for experiments I and II and 24 hours for experiment III). In the control group, temperature was set at 38.5 °C during the entire culture period.

The medium was partially (150 μL) or totally (1 mL) replaced every other day in experiments I and II, respectively. Culture media and structures (ovarian fragments, isolated preantral follicles, and COCs) obtained at time 0 (non-cultured), 12 hours, 1, and 7 days (experiment I and II); and 12 and 24 hours (experiment III) were stored at −80 °C for subsequent assays. Conditioned medium was used to assess hormone and ROS content. Structures from all experiments were evaluated for relative gene expression and viability. Morphological assessment of follicles was recorded at time 0, 12 hours, 1, and 7 days (experiment I) or on Days 0, 4, and 7 (experiment II). In experiment III, chromatin configuration was evaluated in the oocytes after 24 hours of IVM.

2.3. Experiment I: In vitro culture of primordial and primary follicles enclosed in ovarian tissue

At the laboratory, the cortex from each ovary (n = 12) was removed, avoiding regions of corpus luteum and large antral follicles, and fragments (3 × 3 × 1 mm) were obtained. For the *in vitro* culture, the cortex tissue samples were transferred to 24-well culture dishes containing 1 mL of the culture medium per well (TCM199 supplemented with 3 mM glutamine, 2 mM hypoxanthine, 1% BSA bovine serum albumin, 10 ng/mL insulin, 2.5 μg/mL transferrin, 4 ng/mL selenium, and 50 μg/mL ascorbic acid) [20]. The culture was performed in 5% CO_2 in a humidified incubator for 12 hours, for 1 or 7 days.

2.4. Experiment II: In vitro culture of isolated secondary follicles

Ovaries (n = 84) were submitted to a microdissection procedure [23] to recover preantral follicles of diameter