Delayed effect of heat stress on steroid production in medium-sized and preovulatory bovine follicles

Z. Roth¹, R. Meidan¹, A. Shaham-Albalancy¹, R. Braw-Tal² and D. Wolfenson¹*

¹Department of Animal Science, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, Rehovot 76100, Israel; and ²Institute of Animal Science, Agricultural Research Organization, The Volcani Center Bet Dagan 50250, Israel

Introduction

During the autumn, the conception rate of dairy cattle in warm countries is low although ambient temperatures have decreased and cows are no longer exposed to summer thermal stress, indicating that there may be a delayed effect of heat stress on cattle fertility. Two experiments were conducted to examine possible delayed effects of heat stress on follicular characteristics and steroid production at two distinct stages of follicular growth: medium-sized and preovulatory follicles, 20 and 26 days after heat exposure, respectively. Lactating cows were subjected to heat stress for 12 h a day in an environmental chamber, during days 2–6 of a synchronized oestrous cycle. In Expt 1, ovaries were collected on day 3 of the subsequent cycle, before selection of the dominant follicle, and medium-sized follicles were classified as atretic or healthy. In Expt 2, on day 7 of the subsequent cycle, PGF₂α was administered and preovulatory follicles were collected 40 h later. In both experiments, follicular fluid was aspirated, granulosa and thecal cells were incubated, and steroid production was determined. In healthy medium-sized follicles (Expt 1), oestradiol production by granulosa cells and androstenedione production by thecal cells were lower (P< 0.05) and the concentration of progesterone in the follicular fluid was higher in cows that had been previously heat-stressed than in control cows (P< 0.05). In preovulatory follicles (Expt 2), the viability of granulosa cells was lower (P< 0.05) and the concentration of androstenedione in the follicular fluid and its production by thecal cells were lower (P< 0.05) in cows that had been previously heat-stressed than in control cows. In both experiments, the oestradiol concentrations in the follicular fluids were not altered by heat stress. These results demonstrate a delayed effect of heat stress on steroid production and follicular characteristics in both medium-sized and preovulatory follicles; this effect could be related to the low fertility of cattle in the autumn.

*Correspondence
Email: wolf@agri.huji.ac.il
studied at two distinct stages of follicular growth: in medium-sized follicles before selection of the dominant follicle and in preovulatory follicles 3–4 weeks after heat exposure ended.

Materials and Methods

Two experiments were conducted in the winter. Experimental design, tissue collection and cell incubation were identical in both experiments. Delayed effects of 5 days of heat exposure on steroid production were examined in medium-sized (Expt 1) and preovulatory (Expt 2) follicles.

Animals

Mature, cyclic Holstein dairy cows were selected for the study. All experimental cows were housed in identical chambers in the same shelter under the same housing conditions from 1 week before cows were assigned to the experimental groups to eliminate a possible confinement effect. The cows were in late lactation, yielding an average of 25 kg milk per day. The cows were fed a complete mixed ration containing 16.5% (w/v) crude protein and 1.65 Mcal per kg dry matter. This study has been reviewed and approved by the appropriate institutional animal care and use committee.

Experimental protocol

For synchronization of oestrus, an intravaginal progesterone insert (CIDR; Eazi Breed, Hamilton) was inserted for 9 days and 500 µg cloprostenol, a PGF$_{2a}$ analogue (Estrumate; Coopers, Berkhamsted), was injected i.m. 7 days after insertion of the intravaginal progesterone implant. Cows were checked for signs of oestrus three times a day for 30 min each time. Cows expressing oestrous behaviour within 48 h after removal of the CIDR were assigned randomly to either control or heat-stressed groups. All cows were kept in the same shelter and were milked in their housing environment. Control cows were housed in the chambers under normothermic conditions and heat-stressed cows were kept in similar chambers in which air temperature and relative humidity were increased to 36°C and 60%, respectively. Heat-stressed cows were exposed to thermal stress for 12 h a day, between 07:00 h and 19:00 h, during days 2–6 of the cycle. This early stage of the cycle was chosen because the first follicular wave is considered to be highly predictable in terms of follicular dynamics. Although a 5 day duration of thermal stress was sufficient to induce a significant immediate effect on follicular steroidogenesis, a possible delayed effect was not determined (Wolfenson et al., 1997). Accordingly, it was decided to examine the delayed responses to such a short heat stress in the time frame of the subsequent cycle, within 4 weeks after the end of heat exposure. At the end of heat exposure, on day 7 of the oestrous cycle, control and heat-stressed cows were grouped together and housed under normothermic conditions. In both experiments, on day 18 of the treated cycle, PGF$_{2a}$ analogue (500 µg) was injected and cows showed signs of oestrus, on average, on day 21 of the cycle. This synchronization procedure enabled the interval between the end of heat exposure and the days in the subsequent cycle on which ovaries were collected to be set at a similar duration for all cows. Blood samples were collected once a day during days 21–19 in the treated cycle in both experiments, and during days 1–3 and days 1–7 of the subsequent cycle in Expt 1 and Expt 2, respectively.

Blood samples were centrifuged at 2000 g for 20 min and plasma was stored at −20°C for determination of oestradiol and progesterone concentrations.

In Expt 1, ovaries from control (n = 6) and heat-stressed (n = 5) cows were collected on day 3 of the subsequent cycle, 20 days after heat exposure was ended. The day of ovary collection was selected to obtain the maximal number of medium-sized follicles of the first follicular wave before the suppressive influence of the dominant follicle (Ginther et al., 1989; Fortune et al., 1991). Follicular development was examined at 2 day intervals, from day 15 of the treated cycle to day 3 of the subsequent cycle, using an ultrasond instrument (model SSD-210DXII; Aloka, Tokyo) equipped with a 7.5 MHz transducer. Positions and sizes of follicles and corpora lutea in the ovaries were traced at each scanning and the exact location of the follicles was recorded. This procedure enabled individual follicles to be identified accurately during dissection of the ovaries and selection of medium-sized follicles that had grown during the first follicular wave of the subsequent cycle and not those from the previous (treated) cycle.

In Expt 2, heat stress was applied on days 2–6 of the cycle, as described for Expt 1. On day 7 of the subsequent cycle, PGF$_{2a}$ analogue (500 µg) was injected to induce regression of the corpus luteum and development of the first wave preovulatory follicle and, after a further 40 h, ovaries were collected from both heat-stressed (n = 4) and control (n = 5) cows. Comparison of the morphology of the ovaries with the ultrasonography records obtained on days 2, 4, 6 and 8 of the subsequent cycle enabled easy identification of the preovulatory follicles.

Tissue collection and cell incubation

Ovaries from experimental cows were collected at the abattoir after the cows were killed. During dissection of the ovaries, the follicular diameter was measured with callipers and follicular fluid from each follicle was aspirated and stored separately at −20°C for determination of steroid concentrations. The granulosa and thecal cells were isolated from medium-sized follicles (6–9 mm in diameter; Expt 1) or preovulatory follicles (Expt 2), dispersed enzymatically and cultured separately. The viability of granulosa and thecal cells was determined with 0.1% (w/v) trypan blue as described previously (Meidan et al., 1990; Wolfenson et al., 1999, Shores et al., 2000). Long-term
experience in our laboratory has shown that low viability of granulosa cells, as assessed by trypan blue, is associated closely with oestradiol:progesterone concentration ratios < 1 in the follicular fluid. For example, Shaham-Albalancy et al. (2000) found that granulosa cell viability and oestradiol and progesterone concentrations in the follicular fluid of healthy and atretic follicles were 66%, 31 ng ml−1 and 16 ng ml−1 versus 35%, 1 ng ml−1 and 77 ng ml−1, respectively. Accordingly, in Expt 1, medium-sized follicles that expressed low viability of granulosa cells were classified as atretic follicles and were not subjected to further examination; the cell viability of these follicles was 21.3 ± 5.4 and 18.6 ± 2.8% in control and heat-stressed cows, respectively. Follicles that had > 50% viability of granulosa cells were classified as healthy follicles and were subjected to further examination. The status of the healthy medium-sized follicles was verified later by the observation of a > 1 ratio of oestradiol:progesterone concentration in the follicular fluid (Ireland and Roche, 1983). Cells (10^5 viable cells per well) from individual healthy follicles were incubated in a final volume of 0.5 ml in a 24-well plate (Nunc, Kampstrup) in Dulbecco’s minimum essential medium with Ham’s F-12 1:1 (v/v) nutrient mixture (Gibco, BRL Life Technologies, Gaithersburg, MD), containing 1% fetal calf serum (Biological Industries, Beit Ha’emek). Cells were incubated for 6 h to determine steroid production before spontaneous luteinization; a previous study in our laboratory had indicated that granulosa cells maintained aromatase activity after 6 h of incubation (R. Meidan and D. Wolfenson, unpublished). Cells were incubated at 38°C under 5% CO2. Granulosa cells were incubated in medium only or with the addition of testosterone (300 ng ml−1; Sigma, St Louis, MO). Thecal cells that had been isolated from preovulatory follicles were incubated in medium only or with the addition of forskolin (10 μmol l−1; Sigma) or LH (50 ng ml−1; USDA bLH-B-5, provided through the USDA Animal Hormone Program, Beltsville, MD). Thecal cells that had been isolated from medium-sized follicles were incubated in medium only or with forskolin (but not with LH, for technical reasons). Three replicate wells were used for each treatment. At the end of incubation, media were collected from all wells and stored separately at −20°C for determination of steroid concentrations.

Hormone analyses

Plasma samples were extracted with diethyl ether as described by Badinga et al. (1992). Concentrations of oestradiol, androstenedione and progesterone in follicular fluid and medium, as well as the concentrations of progesterone in extracted plasma, were determined by radioimmunooassays that had been validated previously in our laboratory (Meidan et al., 1990; Wolfenson et al., 1997, 1999) with specific antibodies (Sigma). The sensitivities of the assays were 1.92, 3.9 and 3.9 pg per tube for oestradiol, androstenedione and progesterone, respectively. Intra- and interassay coefficients of variation were 9.9, 13.3 and 4.6%, and 13.2, 13.9 and 8.6% for oestradiol, androstenedione and progesterone, respectively. Oestradiol concentrations in extracted plasma samples were determined by means of a radioimmunoassay described by Badinga et al. (1992) that had been validated in our laboratory (Shaham-Albalancy et al., 1997; Roth et al., 2000a). Assay sensitivity was 0.5 pg ml−1, and the intra- and interassay coefficients of variation were 3 and 5%, respectively.

Statistical analysis

The General Linear Models procedure of the Statistical Analysis System was used (SAS, 1987). Data relating to follicular characteristics and hormonal concentrations in the follicular fluid and media were analysed by one-way ANOVA, separately for each type of follicle and cell. The data of steroid hormone concentrations in the media of the three replicate wells for each treatment were averaged, and were analysed separately for basal and stimulated oestradiol and androstenedione production. Concentrations of steroid hormones in the media are expressed as ng per 10^5 viable cells. The statistical model for concentration of hormones in plasma included effects of treatment (control versus heat stress groups), cow (within treatment), day of oestrous cycle, and treatment by day interaction. Data are presented as mean ± SE.

Results

Body temperatures of heat-stressed cows increased gradually to a maximum of 40.7°C, which is a level that is maintained by high milk-yielding cows under natural conditions during hot summer months. Control cows maintained normothermia (body temperature of 38.7°C).

Experiment 1: delayed effect of heat stress on medium-sized follicles

On the day of examination (day 3 of the subsequent cycle) in both control and heat-stressed cows, a mean of one medium-sized follicle per cow was recognized as a follicle from the preceding cycle. These follicles had not been dissected. The number of medium-sized follicles that had emerged during the first follicular wave of the subsequent cycle was similar in control and heat-stressed cows (Table 1). More healthy medium-sized follicles were counted in control cows than in heat-stressed cows (56 and 38% of total follicles, respectively), but this difference was not significant. Healthy medium-sized follicles in control and heat-stressed cows did not differ in terms of follicular diameter, follicular fluid volume or number and viability of thecal and granulosa cells (Table 1). The concentration of progesterone in the follicular fluid was higher in heat-stressed cows than in control cows (P < 0.05; Table 2). Androstenedione and oestradiol concentrations in follicular fluid did not differ between groups. As expected, the oestradiol:progesterone concentration ratio and the oestradiol:androstenedione concentration ratio in the
The diameter, volume of follicular fluid, total number of granulosa cells and viability of thecal cells of the preovulatory follicles, collected on day 9 of the subsequent cycle, did not differ between control and previously heat-stressed cows (Table 3). However, the viability of granulosa cells was lower in heat-stressed cows than in control cows ($P < 0.05$). LH-stimulated androstenedione production was lower in thecal cells obtained from previously heat-stressed cows than in those from control cows (Fig. 2; $P < 0.05$). Forskolin-stimulated androstenedione production, although decreased, was not affected significantly by heat exposure. Oestradiol production by granulosa cells did not differ between previously heat-stressed and control cows in either the absence or the presence of testosterone (Fig. 2). Basal progesterone production by granulosa cells did not differ between previously heat-stressed and control cows ($2.3 \pm 1.2$ versus $4.4 \pm 1.1$ ng per $10^5$ cells, respectively). Basal, forskolin- or LH-stimulated production of progesterone by thecal cells ($0.4 \pm 0.1$, $2.7 \pm 0.5$ and $3.3 \pm 0.9$ versus $0.3 \pm 0.1$, $2.5 \pm 0.5$ and $3.7 \pm 0.8$ ng per $10^3$ cells, respectively) also did not differ between previously heat-stressed and control cows. The concentrations of progesterone and oestradiol in plasma did not differ between control and heat-stressed cows during heat exposure (days 2–6: $0.6 \pm 0.2$ versus $0.9 \pm 0.3$ ng progesterone ml$^{-1}$ and $1.3 \pm 0.5$ versus $1.5 \pm 0.3$ pg oestradiol ml$^{-1}$ in control and heat-stressed cows, respectively), after heat exposure (days 7–19 of the treated cycle: $1.7 \pm 0.4$ versus $1.5 \pm 0.4$ ng progesterone ml$^{-1}$ and $1.9 \pm 0.6$ and $1.6 \pm 0.4$ pg oestradiol ml$^{-1}$ in control and heat-stressed cows, respectively) and after the subsequent cycle (days 0–3: $0.06 \pm 0.03$ versus $0.08 \pm 0.03$ ng progesterone ml$^{-1}$ and $2.8 \pm 0.9$ versus $2.6 \pm 1.1$ pg oestradiol ml$^{-1}$ in control and heat-stressed cows, respectively).

**Experiment 2: delayed effect of heat stress on preovulatory follicles**

The diameter, volume of follicular fluid, total number of granulosa cells and viability of thecal cells of the preovulatory follicles, collected on day 9 of the subsequent cycle, did not differ between control and previously heat-stressed cows (Table 3). However, the viability of granulosa cells was lower ($P < 0.05$) in heat-stressed cows than in control cows. The similarly high concentrations of oestradiol (> $550$ ng ml$^{-1}$), together with the oestradiol: progesterone and oestradiol:androstenedione concentration ratios > 1 in the follicular fluid (Table 4), indicate that the follicles examined in both groups were oestrogenically active preovulatory follicles. The concentration of androstenedione in the follicular fluid was lower in heat-stressed cows than in control cows ($P < 0.05$). LH-stimulated androstenedione production was lower in thecal cells obtained from previously heat-stressed cows than in those from control cows (Fig. 2; $P < 0.05$). Forskolin-stimulated androstenedione production, although decreased, was not affected significantly by heat exposure. Oestradiol production by granulosa cells did not differ between previously heat-stressed and control cows in either the absence or the presence of testosterone (Fig. 2). Basal progesterone production by granulosa cells did not differ between previously heat-stressed and control cows ($2.3 \pm 1.2$ versus $4.4 \pm 1.1$ ng per $10^5$ cells, respectively). Basal, forskolin- or LH-stimulated production of progesterone by thecal cells ($0.4 \pm 0.1$, $2.7 \pm 0.5$ and $3.3 \pm 0.9$ versus $0.3 \pm 0.1$, $2.5 \pm 0.5$ and $3.7 \pm 0.8$ ng per $10^3$ cells, respectively) also did not differ between previously heat-stressed and control cows. The concentrations of progesterone and oestradiol in plasma did not differ between control and heat-stress groups during heat exposure (days 2–6: $1.8 \pm 0.9$ versus $0.7 \pm 0.9$ ng progesterone ml$^{-1}$ and $1.4 \pm 0.2$ versus $1.6 \pm 0.3$ pg oestradiol ml$^{-1}$ in control and heat-stressed cows, respectively), after heat exposure (days 7–19 of the treated cycle: $2.4 \pm 0.7$ versus $3.8 \pm 0.7$ ng progesterone ml$^{-1}$ and $1.4 \pm 0.2$ versus $1.4 \pm 0.2$ pg oestradiol ml$^{-1}$ in control and heat-stressed cows, respectively) and during the subsequent cycle (days 0–7: $0.4 \pm 0.2$ versus $0.6 \pm 0.1$ ng progesterone ml$^{-1}$ and $2.6 \pm 0.3$ versus $2.7 \pm 0.4$ pg oestradiol ml$^{-1}$ in control and heat-stressed cows, respectively).

**Discussion**

The present study provides, for the first time, evidence for a delayed effect of heat stress on follicular steroidogenesis. The delayed effect was detected in both medium-sized and preovulatory follicles, but was expressed differently in granulosa and thecal cells within each class of follicles.

Two experiments were performed during the winter to avoid any potential seasonal carry-over effects from the
In addition, the experimental design, in which heat-stressed cows were compared with contemporaneous control cows, enabled the acute heat exposure to be associated with alterations in follicular function in medium-sized and preovulatory follicles, 20 and 26 days later, respectively. It might be expected that during the processes of development and selection, the healthiest follicle from the cohort of impaired medium-sized follicles would be selected to become the dominant follicle. However, alterations in follicular function were also found in preovulatory follicles from previously heat-stressed cows. Calculations made according to Lussier et al. (1987) showed that the medium-sized or preovulatory follicles studied (7.7 mm or 15.2 mm in diameter, respectively) had diameters of about 0.5–1.0 mm when the cows were heat-stressed. Follicles in such an early stage of follicular growth are characterized by a high mitotic index of granulosa cells (Lussier et al., 1987) and might be particularly sensitive to environmental changes. It remains to be determined whether very small antral (< 0.5 mm in diameter) or pre-antral follicles are also susceptible to heat stress.

In terms of steroid production, the thecal cells were found to be more susceptible than granulosa cells to heat stress, and expressed a delayed effect of heat stress in both classes of follicle. The consistent decrease in androstenedione production in both medium-sized and preovulatory follicles in previously heat-stressed cows correlated with the decreased concentrations of androstenedione in their follicular fluid. This delayed effect of heat stress on

---

**Fig. 1.** Delayed effect of heat stress on steroid production in medium-sized follicles. (a) Basal or forskolin-stimulated (10 μmol l⁻¹) androstenedione production by thecal cells and (b) oestradiol production by granulosa cells in the presence or absence of testosterone (300 ng ml⁻¹). Columns represent production of cells obtained from follicles of control ( []; n = 6) or previously heat-stressed ( []; n = 5) cows on day 3 of the subsequent cycle, 20 days after heat exposure. Values are mean ± se. abDifferent letters indicate significant differences between groups (P < 0.05).

**Fig. 2.** Delayed effect of heat stress on steroid production in preovulatory follicles. (a) Basal, forskolin-stimulated (10 μmol l⁻¹) or LH-stimulated (50 ng ml⁻¹) androstenedione production by thecal cells, and (b) oestradiol production by granulosa cells in the presence or absence of testosterone (300 ng ml⁻¹). Production of steroids by cells obtained from follicles of control ( []; n = 5) or previously heat-stressed cows ( []; n = 4) on day 9 of the subsequent cycle 40 h after PGF₂α administration, 26 days after heat exposure. Values are mean ± se. abDifferent letters indicate significant differences between groups (P < 0.05).

---

**Table 3.** Characteristics of preovulatory follicles of control and previously heat-stressed cows at day 26 after heat exposure

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>Heat-stressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cows (one follicle per cow)</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td>18.2 ± 1.4</td>
<td>15.2 ± 1.5</td>
</tr>
<tr>
<td>Follicular fluid volume (ml)</td>
<td>2.6 ± 0.4</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>Number of granulosa cells × 10⁶ per follicles</td>
<td>12.3 ± 2.3</td>
<td>14.6 ± 1.6</td>
</tr>
<tr>
<td>Viability of granulosa cells (%)</td>
<td>42.0 ± 4.9</td>
<td>25.0 ± 5.4*</td>
</tr>
<tr>
<td>Viability of thecal cells (%)</td>
<td>&gt; 90</td>
<td>&gt; 90</td>
</tr>
</tbody>
</table>

Values are mean ± se.

*Significantly different from control group (P < 0.05).
Oestradiol:progesterone ratio 27.0 43.0
Androstenedione (ng ml–1) 10.2

Androgen production by thecal cells (McNatty et al., 1984). Atresia of bovine follicles is characterized by a decrease in androgen production by thecal cells observed in the autumn.

In conclusion, exposure of cows to heat stress resulted in impaired steroidogenesis 20 and 26 days later, in medium-sized and preovulatory follicles, respectively. The delayed effect was expressed in a different way in granulosa and thecal cells within each class of follicles. Granulosa cells expressed low oestradiol production in medium-sized follicles and low viability in the preovulatory follicles. In terms of steroid production, thecal cells appeared to be consistently susceptible to heat stress and expressed a carry-over effect on androgen production in both types of follicle. Delayed effects of heat stress on follicular steroidogenic capacity, together with its delayed effects on follicular dynamics (Roth et al., 2000a), as well as on oocyte quality and embryo development (Roth et al., 1999), could be responsible for the low fertility of dairy cows during the autumn.

Table 4. Steroid concentrations in the follicular fluid of preovulatory follicles of control and previously heat-stressed cows

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Control</th>
<th>Heat-stressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestradiol (ng ml–1)</td>
<td>600 ± 122</td>
<td>560 ± 136</td>
</tr>
<tr>
<td>Progesterone (ng ml–1)</td>
<td>22.3 ± 4.0</td>
<td>12.9 ± 3.6</td>
</tr>
<tr>
<td>Androstenedione (ng ml–1)</td>
<td>10.2 ± 1.6</td>
<td>4.8 ± 1.4*</td>
</tr>
<tr>
<td>Oestradiol:progesterone ratio</td>
<td>27.0</td>
<td>43.0</td>
</tr>
<tr>
<td>Oestradiol:androstenedione ratio</td>
<td>58.8</td>
<td>116.7</td>
</tr>
</tbody>
</table>

Values are mean ± SE (control: n = 5; heat-stressed: n = 4).
*Significantly different from control group (P < 0.05).
The authors would like to thank Y. Graber for technical assistance and the USDA for provision of bLH. This work was supported by a grant from the US-Israel Binational Agricultural Research and Development Fund (BARD).

References


Ireland JJ and Roche JF (1983) Development of nonovulatory antral follicles in heifers: changes in steroids in follicular fluid and receptors for gonadotropins Endocrinology 112 150–156


Accepted 30 January 2000.