The effect of high temperature on swine ovarian function *in vitro*

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ABSTRACT: The aim of the present study was to understand the hormonal mechanisms behind the effect of high temperatures on reproductive function. It was proposed that high temperatures can directly alter production of ovarian hormones and/or the response of ovarian cells to hormonal stimulators. To examine this hypothesis, in the 1st series of experiments, we compared the release of progesterone (P_4), estradiol (E_2) and expression of the leptin gene in whole ovarian follicles cultured in conditions of normal (37.5°C) and high (41.5°C) temperatures. In the 2nd series of experiments, we examined the release of P_4 and insulin-like growth factor I (IGF-I) by ovarian granulosa cells cultured in conditions of normal and high temperatures with and without IGF-I, leptin and FSH. The release of hormones was measured by RIA, while the expression of the leptin gene was evaluated by PCR. It was observed that high temperature significantly increased P_4 and E_2 release and reduced the accumulation of leptin DNA in ovarian follicles. In cultured ovarian granulosa cells, high temperatures promoted the release of both P_4 and IGF-I. The addition of IGF-I, leptin and FSH to granulosa cells cultured at normal temperature promoted the release of both P_4 and IGF-I. High temperature was able to prevent the stimulatory effect of leptin (but not of IGF-I or FSH) on P_4 output and the stimulatory action of both leptin and FSH on IGF-I release by granulosa cells. The present observations (1) demonstrate the possible production of leptin in the porcine ovary, (2) demonstrate for the first time the influence of high temperatures on ovarian P_4, E_2, IGF-I and leptin, and (3) suggest, that the negative effect of heat stress on reproductive processes can be due to high temperature-induced malproduction of ovarian hormones and a reduction in the response of ovarian cells to hormonal stimulators.

Keywords: temperature; IGF-I; leptin; FSH; progesterone; estradiol; ovaries; pig

The global warming which has occurred in recent decades can affect animal and human reproductive processes. Ambient temperatures and other environmental factors control and synchronise reproductive cycles, but the mechanisms of such action are insufficiently studied. The negative effect of high ambient temperatures on reproductive processes are well documented (Putney et al., 1989; Edwards and Hansen, 1997; Lawrence et al., 2004). A hot environment can increase blood, rectal and uterine temperatures, suppress ovarian folliculogenesis (Rensis and Scaramuzzi, 2003) and cyclicity (Christenson, 1980), suppress spermatogenesis and fertility (Kunavongkrit et al., 2005), oogenesis and embryogenesis (Putney et al., 1989; Edwards and Hansen, 1997; Lawrence et al., 2004) and reduce conception and pregnancy rates (Christenson, 1980; Putney et al., 1989; Rensis and Scaramuzzi, 2003) in different domestic animal species.

The mechanisms behind such effects remain unknown. Nevertheless, it is widely accepted that ovarian cell functions are under the control of hormones including growth factors and prostaglandins (Hillier, 1991; Erickson and Danforth, 1995; Berisha and Schams, 2005). In ovarian cells, increased temperatures induce the production of heat shock proteins, which is associated with changes in the production of prostaglandins (Narayansingh et al., 2004) and receptors for steroid hormones (Salvetti et al., 2008). Furthermore, heat stress-associated reproductive disorders are associated with altered progesterone and reduced estradiol production by ovarian follicles (Rensis and Scaramuzzi, 2003). Therefore, it cannot be ruled out that high tempera-

tures suppress ovarian functions through changes in the release of ovarian hormones. Nevertheless, there is no evidence for a direct effect of high temperatures on hormones (progestagens, estrogens and insulin-like growth factor I, IGF-I) produced in the ovary.

It is proposed that high temperatures can suppress gonadal functions also through reduction in food consumption (Rensis and Scaramuzzi, 2003; Kunavongkrit et al., 2005). Malnutrition can affect reproductive processes via changes in the secretion of the metabolic hormone leptin. Food restriction reduces the release of leptin, a product of adipose and some other tissues, which can affect reproduction through the hypothalamo-hypophysial system and by direct action on gonads leptin is able to regulate the growth of ovarian follicles, is involved in *corpus luteum* development, can suppress ovarian cell apoptosis and activate ovarian cell proliferation and can affect the release of the steroid hormones, oxytocin, prostaglandin and IGF-I and IGFBP-3 by ovarian cells (Spicer, 2001; Sirotkin et al., 2005; Zieba et al., 2005). Furthermore, external temperatures can potentially influence reproductive processes affecting (directly or through leptin) the release of gonadotropins (FSH, LH), the most known promoters of ovarian cell functions and stimulators of release of ovarian steroid and peptide hormones (Hillier, 1991; Erickson and Danforth, 1995; Berisha and Schams, 2005; Sirotkin et al., 2005). Both leptin (Spicer, 2001; Sirotkin et al., 2005; Zieba et al., 2005) and gonadotropins (Erickson and Danforth, 1995; Berisha and Schams, 2005) can control ovarian functions through stimulating the production of IGF-I, whose anti-apoptotic effects and stimulatory action on ovarian cell proliferation, follicullogenesis and hormone release are well known (Sirotkin et al., 1998, 2001, 2005; Makarevich et al., 2000; Berisha and Schams, 2005).

Therefore, it cannot be ruled out that heat stress can affect ovarian functions through changes in the production of leptin and leptin-regulated hormones including progestagens, estrogens and IGF-I or in the response of ovarian cells to hormonal stimulators (gonadotropins, leptin, IGF-I and others).

The release of steroid hormones and IGF-I by porcine ovarian tissues has been described previously (Hillier, 1991; Erickson and Danforth, 1995; Berisha and Schams, 2005; Sirotkin et al., 1998, 2001, 2005). The production of leptin by different tissues including ovarian cells has been proposed, but the synthesis of leptin in porcine ovaries has not been demonstrated yet.

The general aim of the present study was to understand the hormonal mechanisms behind the effects of high temperatures on reproductive functions. It was proposed that high temperatures can directly alter the production of ovarian hormones and/or the response of ovarian cells to hormonal stimulators. To examine this hypothesis, in the 1st series of experiments, we compared the release of P_4, E_2 and the expression of the leptin gene in whole ovarian follicles cultured in conditions of normal (37.5°C) and high (41.5°C) temperatures. In the 2nd series of experiments, we examined the release of P_4 and IGF-I by ovarian granulosa cells cultured in conditions of normal and high temperature with and without IGF-I, leptin and FSH.

MATERIAL AND METHODS

Isolation, culture, and processing of ovarian follicles

Non-cycling Slovakian white gilts (*Sus scrofa domestica* L.), 180 days of age and without visible reproductive abnormalities, were killed at a local slaughterhouse. Ovarian follicles (5 mm in diameter) were collected, processed and cultured for two days in Falcon 24 well plates (Becton Dickinson, Lincoln Park, USA), one follicle per well/2 ml culture medium DME/F-12 1 : 1 mixture supplemented with 1% antibiotic-antimycotic solution and 10% heat-inactivated foetal calf serum (all from Sigma, St. Louis, MO, USA) in conditions of normal (37.5°C) or high (41.5°C) temperature as described previously (Sirotkin et al., 1998). Immediately after culture, follicles were weighed and stored at –18°C to await RT-PCR. The culture medium was stored at –18°C to await RIA.

Preparation, culture and processing of granulosa cells

Granulosa cells were collected from the ovaries of non-cycling Slovakian white gilts, 200 days of age, after slaughter at a local abbatoir. They were processed and cultured as described previously (Sirotkin et al., 2001, 2008) in DMEM/F-12 1 : 1 mixture supplemented with 10% bovine foetal serum and 1% antibiotic-antimycotic solution (all from Sigma, St. Louis, MO, USA). Granulosa cells (1×10^6 cells/ml) were cultured in 2 ml culture medium in Falcon 24

well plates (Becton Dickinson). First the cells were precultured in medium at 37°C under 5% CO_2 in humidified air. After two days of preculture, cells were cultured for two days in fresh medium supplemented with foetal calf serum (10%, Sigma) at temperatures of 37.5°C or 41.5°C with or without hormones. Experimental groups received biological grade recombinant human leptin (Sigma, 100 ng/ml medium), immunological grade recombinant IGF-I (Calbiochem, Lucerne, Switzerland, 100 ng/ml) or biological grade porcine FSH (Sigma, 100 ng/ml). After 48 h culture, cell number and viability were determined by Trypan blue staining and counting with a haemocytometer. No statistically significant differences in these indices between the groups were observed.

Analysis

Concentrations of hormones were determined by RIA in 25 µl samples of incubation medium. P_4 and E_2 were assayed using RIA/IRMA kits from DSL (Webster, TX, USA) according to the instructions of the manufacturer. All RIAs were validated for use in samples of culture medium by dilution tests. The release of hormones was calculated per 10^6 cells or mg tissue/day. Characteristics of the assays were described previously (Sirotkin et al., 2005, 2008).

The concentration of DNA for leptin in whole follicles was evaluated using GenElute TM Mammalian Genomic DNA Miniprep Kit (Sigma). Up to 25 mg of tissue were used for preparation and the instructions provided in the user guide were followed. The concentration and quality of the genomic DNA prepared with the GenElute kit was determined by spectrophotometric analysis on WPA UV 1101 Biotech Photometer (Bichrom, UK). An absorbance of 1.0 at 260 nm corresponds to approximately 50 µg/ml of double stranded DNA. The PCR reaction was performed after verification of the concentration and quality of genomic DNA. Genus primers blep FOR 5' ATGCGCTGTGGACCCCTGTATC 3' and blep REV 5'TGGTGTCATCCTGGACCTTCC 3'were used in the reaction to confirm the presence of the leptin gene.

The basic master mix consisted of 5 µl PCR buffer, Fast Taq DNA polymerase 0.4 µl (Roche, USA), 1 µl Nucleotide mix, 5 µl each genus primer and H_2O (redistilled) to bring to a final volume of 50 µl. Reactions additionally contained 2.5 µl DNA template. Following an initial denaturation at 94°C for 4 min, products were amplified by 40 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s. Amplification was followed by a final extension at 72°C for 10 min. PCR products were electrophoresed on a 0.8% Tris-acetate-EDTA agarose gel and stained with Ethidium bromide.

Statistics

Each experiment was performed on ovaries obtained from 15–20 animals each. Each experimental group was represented by wells with ovarian follicles (one follicle per well) or four wells with granulosa cells. Each experiment was performed three times. Significant differences between the groups were determined by one-way ANOVA followed by Student's *t*-test using Sigma Plot 9.0 statistical software (Systat Software, GmbH, Erkrath, Germany). Differences from control at $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

RIA demonstrated the accumulation of both P_4 and E_2 in medium conditioned by cultured ovarian follicles, as well as the release of P_4 and IGF-I by cultured ovarian granulosa cells. This corresponds to our previous observations (Sirotkin et al., 1998, 2001, 2008) regarding the release of these steroid hormones and IGF-I by cultured porcine ovarian cells. RT-PCR demonstrated the presence of a substantial amount of leptin DNA in ovarian follicles. This is the first demonstration that leptin can be synthesised within the porcine ovary. The production of these two steroid hormones, IGF-I and leptin by ovarian cells, and the stimulatory action of IGF-I, leptin and FSH on the release of P_4 and IGF-I by granulosa cells observed in our experiments, together with the available data on the effect of steroid hormones, IGF-I, gonadotropins (Hillier, 1991; Erickson and Danforth, 1995; Berisha and Schams, 2005) and leptin (Sirotkin et al., 2001, 2005; Spicer, 2001; Zieba et al., 2005) on different reproductive functions suggest that these hormones can be involved in auto-, para- and endocrine regulation of porcine ovarian function.

It was also observed that these hormonal parameters could be influenced by temperature. A comparison of these parameters in follicles cultured in conditions of normal (37.5°C) and high (41.5°C) temperatures showed that exposure to high tem-

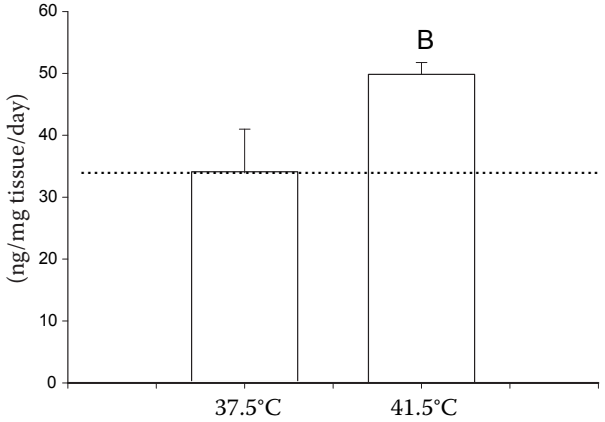

Figure 1. Release of progesterone (ng/mg tissue/day) by porcine ovarian follicles cultured at normal (37.5°C) and high (41.5°C) temperature. Values are means + S.E.M. B = significant ($P < 0.05$) differences between corresponding group follicles cultured at normal (37.5°C) and high (41.5°C) temperature

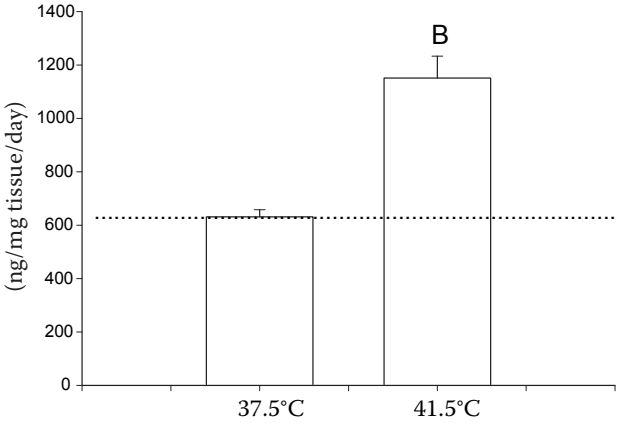

Figure 2. Release of estradiol (ng/mg tissue/day) by porcine ovarian follicles cultured at normal (37.5°C) and high (41.5°C) temperature. Values are means + S.E.M. B = significant ($P < 0.05$) differences between corresponding group follicles cultured at normal (37.5°C) and high (41.5°C) temperature

perature induced a significant increase in P_4 (Figure 1 and 4), E_2 (Figure 2) and IGF-I (Figure 5) release by ovarian cells. Furthermore, it inhibited the accumulation of leptin DNA in ovarian follicles (Figure 3). Our observations are the first demonstration of the influence of high temperatures on ovarian P_4, E_2, IGF-I and leptin. The mechanisms and functional interrelationships between temperature and these hormonal parameters remain to be studied, although it is not to be excluded, that heat stress-induced changes in steroid hormones can be due to changes in the production of IGF-I and leptin, known regulators of steroidogenesis (Erickson and Danford, 1995; Makarevich et al., 2000; Sirotkin et al., 2001; Spicer, 2001; Berisha and Schams, 2005; Zieba et al., 2005). It is less probable that changes in estradiol are due to changes in its precursor progesterone because in our experiments no similarity in action between high temperature and exogenous hormones on these steroids were observed. To gain a better understanding of the functional significance of the observed changes further studies are required. It is possible that the hormonal changes observed in our experiments in high temperature-treated follicles can be down to a neutralisation of the negative effect of overheating, although the adaptive and anti-stress actions of P_4, E_2, IGF-I and leptin have not been reported yet. A more likely hypothesis may be that the observed hormonal changes can mediate the effects of high temperatures on ovarian functions. This hypothesis is supported by the effect of high temperatures on both reproductive processes and hormones, and by the available data on the importance of P_4, E_2, IGF-I and leptin in the maintenance of reproductive processes (see above). Therefore, it is possible that the negative effects of high temperature on reproductive processes can be due to malproduction of these ovarian hormones.

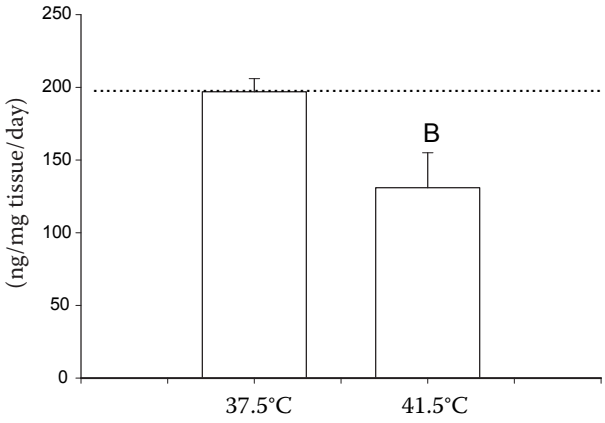

Figure 3. Accumulation of leptin DNA (ng/mg tissue/day) in porcine ovarian follicles cultured at normal (37.5°C) and high (41.5 °C) temperature. Values are means + S.E.M. B = significant ($P < 0.05$) differences between corresponding group follicles cultured at normal (37.5°C) and high (41.5°C) temperature

Furthermore, in our experiments high temperatures induced not only alterations in hormone release, but also in the response of ovarian cells to hormonal treatments. In cells cultured at normal temperature either IGF-I, leptin or FSH promoted release of their hormones, but hyperthermy prevented the stimulatory action of leptin on P_4 and of leptin and FSH on IGF-I output. This is the first

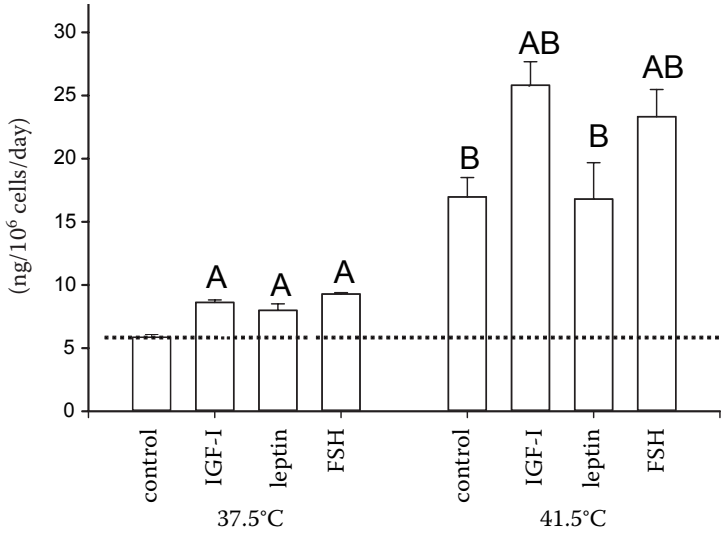

Figure 4. Release of progesterone (ng/10^6 cells/day) by porcine ovarian granulosa cells cultured at normal (37.5°C) and high (41.5°C) temperature with and without IGF-I, leptin and FSH (all 100 ng/ml). Values are means + S.E.M. A = significant ($P < 0.05$) differences between corresponding group of cells cultured with and without hormones, B = significant ($P < 0.05$) differences between corresponding group of cells cultured at normal (37.5°C) and high (41.5°C) temperature

demonstration of the ability of high temperatures not only to alter basal ovarian hormone production, but also to diminish the response of ovarian cells to physiological hormonal stimulators. The addition of IGF-I, leptin and FSH failed to prevent the reaction of ovarian granulosa cells to hyperthermy. This suggests that ovarian hormones and their response to endocrine regulators could be used as markers of optimal temperature conditions, but not for the elimination of the negative effects of high temperatures on ovarian cell function. Furthermore, it indicates that the physiological significance of the observed changes in ovarian hormones is not to alleviate the negative effect of stress on the ovary, but rather to mediate these effects on reproductive processes.

Taken together, our observations (1) confirm the release of P_4, E_2 and IGF-I by porcine ovarian cells, (2) demonstrate the possible production of leptin

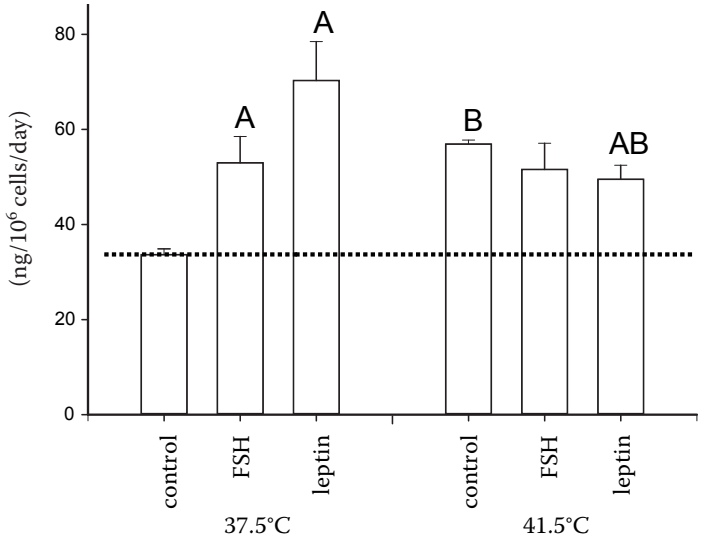

Figure 5. Release of IGF-I (ng/10^6 cells/day by porcine ovarian granulosa cells cultured at normal (37.5°C) and high (41.5°C) temperature with and without leptin and FSH (all 100 ng/ml). Values are means + S.E.M. A = significant ($P < 0.05$) differences between corresponding group of cells cultured with and without hormones, B = significant ($P < 0.05$) differences between corresponding group of cells cultured at normal (37.5°C) and high (41.5°C) temperature

in the porcine ovary, (3) demonstrate for the first time the influence of high temperatures on ovarian P_4, E_2, IGF-I and leptin, and (4) suggest that the negative effect of high temperatures on reproductive processes can be due to the malproduction of ovarian hormones and a reduced response of ovarian cells to hormonal stimulators.

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Received: 2010–05–17
Accepted after corrections: 2010–08–10

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