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Influence of thermal stress during in vitro maturation on the developmental competence of oocytes and embryos and the expression of Sirtuins in cumulus oocyte complexes in cattle

Influência do estresse térmico durante a maturação in vitro na competência de oócitos e desenvolvimento de embriões e na expressão de Sirtuínas em complexos cumulus-oócitos de bovinos

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Highlights

The first polar body extrusion was similar after oocyte maturation at 37, 38.5 and 40°C.
Lower blastocyst rate was observed in BCB+ COCs after IVM under cold and heat stress.
Cold and heat stress reduced SIRT1, but this was insufficient for stress protection.
1.5°C change for 24 h during oocyte maturation impaired embryo development.
SIRT1 may aid in vitro embryo development and stress protection.

Abstract

Sirtuins are of central importance in many cellular functions and promote cell survival under stress. However, little information is available regarding the relationship between sirtuins and female reproductive biology, especially in response to thermal stress. This study investigated the influence of moderately

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high (40°C) and low (37°C) thermal stress during *in vitro* maturation on the development competence of bovine oocytes and embryos. The expression and abundance of sirtuins and other proteins involved in stress response were also studied. The cumulus-oocyte complexes (COCs) of Simmental (*Bos taurus*) cows underwent *in vitro* maturation (IVM) at different temperatures (37°C, 38.5°C and 40°C). Before maturation, the oocytes were stained with Brilliant Cresyl Blue (BCB) and categorized as labeled (BCB+) or unlabeled (BCB-). Embryo production was analyzed at the different IVM temperatures. Polar body extrusion was evaluated following IVM, and the mRNA and protein abundance of sirtuins and P53 in oocytes and cumulus cells were analyzed. The differing temperatures during IVM did not significantly alter polar body extrusion and cleavage rates; however, significant differences in blastocyst production were observed. COCs matured at 38.5°C (control, 37.3%) had the highest blastocyst rate, in contrast to those matured at 37°C (33.2%) and 40°C (21.5%). In all groups, the blastocyst rates were higher for BCB+ oocytes than for BCB- oocytes. In BCB+ oocytes, the expression of *SIRT1, SIRT2, SIRT3,* and *SIRT5* genes was higher after maturation than that before maturation and in most of the cases, the expression was higher when IVM was performed at 38.5°C. In the cumulus cells of BCB+ COCs, only SIRT2 remained unaffected by the maturation temperature. In summary, the temperature change of ±1.5°C for 24 h during bovine oocyte maturation impaired *in vitro* embryo development. This lead to several cellular biochemical alterations in oocytes and granulosa cells from COCs with higher developmental competence (BCB+). Thus, SIRT1 is important for *in vitro* embryonic development and may protect against cold and heat stress.
Key words: Sirtuins. Temperature. IVP. *Bos taurus.* Heat stress.

Resumo

As sirtuínas são de importância central para muitas funções celulares e promovem a sobrevivência celular em células sob estresse. No entanto, apenas algumas informações sobre a relação da sirtuína e a biologia da reprodução feminina estão disponíveis, especialmente em resposta ao estresse térmico. O objetivo do presente estudo foi investigar a influência do estresse térmico moderado alto (40 °C) e baixo (37 °C) durante toda a maturação *in vitro* na competência de desenvolvimento de oócitos bovinos e no desenvolvimento embrionário, bem como a expressão e abundância de sirtuínas e proteínas envolvidas na resposta ao estresse. Os complexos cumulus-oócitos (COC) de vacas Simental (Bos taurus) foram maturados in vitro em diferentes temperaturas (37 °C, 38,5 °C e 40 °C). Antes da maturação, os oócitos foram corados com Azul Cresil Brilhante (BCB) e então categorizados como marcados (BCB+) e não marcados (BCB-). Primeiro, a produção de embriões foi analisada de acordo com as diferentes temperaturas de maturação in vitro (IVM). A extrusão do corpo polar foi avaliada após IVM e a abundância de mRNA e proteína de sirtuínas e P53 em oócitos e células cumulus foram analisadas. Diferentes temperaturas durante IVM não alteraram significativamente a extrusão do corpo polar e a taxa de clivagem, no entanto, houve diferenças significativas na produção de blastocistos. COC maturado a 38,5 °C (controle) teve a maior taxa de blastocisto, em contraste com aqueles maturados a 37 °C (33,2%) e 40 °C (21,5%). Em todos os grupos, as taxas de blastocisto foram maiores para oócitos BCB+ do que para oócitos BCB-. Em oócitos BCB+ a expressão gênica de SIRT1, SIRT2, SIRT3 e SIRT5 foi maior após a maturação do que antes da maturação e na maioria dos casos, sua expressão foi maior quando IVM foi realizada a 38,5 °C. Em células cumulus de BCB+ COC, apenas SIRT2 não foi afetado pela temperatura

de maturação. Em resumo, a mudança de temperatura de ±1,5°C por 24 horas durante a maturação do oócito bovino prejudica o desenvolvimento do embrião in vitro e leva a diversas alterações bioquímicas celulares no oócito e nas células da granulosa de COC com maior competência de desenvolvimento (BCB+). SIRT1 é importante para o desenvolvimento do embrião *in vitro* e parece estar relacionado ao processo de proteção contra o estresse pelo frio e calor.
Palavras-chave: Sirt1. Temperatura. PIVE. *Bos Taurus.*

Introduction

High temperatures during *in vitro* maturation (IVM) reduce the developmental competence of bovine oocytes as a function of stress intensity, which is measured by temperature and exposure time. However, the mechanisms by which heat shock affects oocyte physiology are not completely understood (Paula-Lopes et al., 2013). Additionally, studies investigating the effects of cold stress on bovine IVM are lacking.

The quality and competence of cumulus-oocyte complexes (COCs) obtained for *in vitro* production (IVP) can vary greatly due to factors such as origin, age, ovarian stage, and different kinds of stress. Brilliant Cresyl Blue (BCB) staining is a marker for glucose-6-phosphate dehydrogenase activity, indicating a link to energy metabolism, and consequently, viability (Bhojwani et al., 2007; Pujol et al., 2004; Rodríguez-González et al., 2002). Several studies have shown that BCB-stained COCs are more competent and provide better blastocyst rates (Alm et al., 2005; Bhojwani et al., 2007; Salviano et al., 2016); however, the manner in which they respond to thermal stress remains unknown.

Sirtuins are of central importance in many cellular functions, such as protein acetylation or histone and protein deacetylation, causing alterations in metabolism (Chen et al., 2015) . The sirtuin family (SIRT) is associated with antioxidant processes and functions, prevention of oxidative stress, mitochondrial function, repair mechanisms of damaged DNA, and longevity (Nakagawa & Guarente, 2011) . In addition, sirtuins act as switches between energy metabolism and other signaling pathways involved in specific cellular physiological processes (Singh et al., 2018). While SIRT1 and SIRT2 are mainly distributed in the cytoplasm and are involved in the regulation of enzyme activity and gene expression, SIRT3 and SIRT5 are located in the mitochondria and are directly linked to energy metabolism (Bai & Zhang, 2016; Grabowska et al., 2017). The relationship between SIRT1 and spermatogenesis has been demonstrated previously (Rato et al., 2016). However, little information is available on the female side (Tatone et al., 2015) of reproduction. Therefore, we hypothesized a relationship between sirtuins in the COCs and the response to thermal adaptation in female cattle. The mechanisms underlying the response can affect oocyte quality, particularly the developmental competence of embryos after in vitro fertilization.

To understand the mechanisms involved in cellular protection against *in vitro* cold and heat stress, we examined the possibility of cooperation between SIRT1 and P53 in bovine oocytes and cumulus cells. In most studies investigating heat stress during

IVM, the temperature increase was carried out for only a few hours (approximately 2–12 h) and in a non-physiological manner, using excessively high temperatures (Nabenishi et al., 2012a,b; Rispoli et al., 2013; Roth & Hansen, 2005). Therefore, we decided to test moderate temperature changes throughout the oocyte maturation period to simulate chronic stress. To assess the mechanisms involved in induced cold stress and compare with those involved in heat stress, we also examined the effects of IVM under low temperature (37 °C).

Thus, the present study aimed to investigate the influence of moderately high (40°C) and low (37°C) thermal stress on bovine oocyte developmental competence, consequently, embryo development, and sirtuin and P53 expression during IVM.

Material and Methods

Animals

The ovaries of Simmental cows (*Bos taurus*) from commercial properties in Germany were obtained from a local slaughterhouse between January and March. The collected ovaries were transported within 3 h to the laboratory in phosphate-buffered saline (PBS) containing antibiotics (30°C).

In vitro embryo production

The follicles, from the ovaries obtained, were aspirated using a syringe (18G needle) and transferred to Petri dishes (PBS, 0.3% w/v bovine serum albumin (BSA), pyruvate, heparin, penicillin, and streptomycin). We specifically used COC grades 1 or 2, as classified by stereomicroscopy (Blondin & Sirard, 1995; Leibfried & First, 1979; Madison et al., 1992).

The selected COCs were stained with BCB, as described by Alm et al. (2005) and Bhojwani et al. (2007), and categorized as labeled (BCB+) or unlabeled (BCB-). COC maturation was performed for 24 h on a 4-well plate with 420 µl of maturation medium (TCM199 with Earl salts, 5% v/v estrus cow serum, 0.5 ng/ml estradiol, 0.01 mIE/ml hCG, 200 mM L-glutamine, 0.01 mg/ml streptomycin, 10 U/ml penicillin) covered with mineral oil in incubators (Heracell™, ThermoScientific, Bonn, Germany) under 5% CO2. The COCs were divided into groups based on exposure to different temperatures: 37°C, 38.5°C (control), and 40°C. A group of immature oocytes was also used for comparison.

Following IVM, the COCs with extruded polar corpuscles were divided. One portion (n = 907) was used for cytogenetic studies and another (n = 635) for *in vitro* embryo production. To evaluate the maturation rate, the COCs were denuded by pipetting after IVM and washed twice with PBS. Polar body (PB) extrusion was observed using stereomicroscopy.

For IVP, the COCs were washed once and transferred to fertilization medium (supplemented with TALP medium containing heparin and 6 mg/ml BSA). Simmental cryopreserved semen was used for *in vitro* embryo production. The mobile spermatozoa were separated using the swim-up technique and fertilization was performed at a final concentration of $1x10^6$ sperm/ml. COCs and sperm were co-incubated for

22 h at 38.5°C and 5% CO_2 with maximum humidity. After washing, the zygotes were transferred to 4-well plates containing SOF medium (Minitüb, Tiefenbach, Germany; 10% v/v estrus cow serum). Embryo culture was performed at 39°C, 5% CO_2, and 5% O_2 with high humidity. The cleavage rate was evaluated at 72 h and the blastocyst rate was evaluated 7 d after fertilization.

Preparation of DNA, RNA and cDNA synthesis

Total oocyte and cumulus cell RNA were isolated using the Nucleus Spin RNA II kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. For this, oocytes and cumulus cells were mechanically separated (through frequent pipetting). Next, the oocytes were removed and washed thrice with PBS, and 30 oocytes per group (three replicates) were transferred with no more than 5 µl PBS into lysis buffer. The cumulus cells belonging to the respective oocytes were pooled and washed thrice again with PBS. The precipitates from the last washing step were re-suspended in the lysis buffer. The RNA concentration was measured using a NanoDrop1000 spectrophotometer (Thermo Scientific, Bonn, Germany). The cDNA synthesis was performed with M-ML reverse transcriptase and ribonuclease RNase inhibitor (M3683 and N2515, Promega Mannheim, Germany). Oligo-(dT) primers (2 ng/µl, #10814270001) mixed with random hexamer primers (4 ng/µl, #11034731001) were used, both sourced from Roche (Mannheim, Germany). The manufacturer's recommendations were followed. Subsequently, the cDNA was purified using a high-purity PCR purification kit (# 11732676001, Roche) and eluted in 115 µl of elution buffer.

Real-time quantitative PCR

Real-time PCR was performed using SensiFast™ SYBR No-ROX (#BIO-98020, Bioline, Luckenwalde, Germany) and gene-specific primers (Table 1). Each transcript was quantitated in triplicate on a LightCycler® 480 instrument (Roche) with the following cycle conditions: pre-incubation at 95°C for 5 min, 40 denaturation cycles at 95°C for 20 sec, annealing at 60°C for 15 sec, extension at 72°C for 15 sec and acquisition of single point fluorescence for 10 sec. The melting points of all samples were analyzed to ensure the amplification of the correct products. The lengths of the PCR products were checked after each run using agarose gel electrophoresis (3%, stained with ethidium bromide). All amplifications were sequenced at the initial stage to verify authenticity. Cloned PCR products from the studied transcripts were used as external standards. Fresh dilutions were used to obtain five different concentrations of standards (5 × 10-12–5 × 10-16 g DNA/reaction) that were co-amplified. Transcript abundance levels were normalized to the transcripts of RPS18.

Table 1
Primer sequences used for rtPCR

Name	Sequence	RefSeq	Bp
SIRT1F	TGGTTCCTTTGCAACAGCATCTTGC	NM_001192980.1	100
SIRT1R	AGGACATCGAGGAACCACCTGATT		
SIRT2F	ACTTACAGCTCTTTTCTGGGGAGCT	NM_001113531.1	400
SIRT2R	TTTCAGAGTTGGAGGCAGAGGCG		
SIRT3F	ACACCAGAGGTTACTTGGGAGTCAC	NM_001206669.1	100
SIRT3R	GTGTCCTGGTTCAGCCACAATTTGT		
SIRT5F	TGTGGATGGTGAGCCAAATACTGGA	NM_001034295.2	100
SIRT5R	CTGTTGCTTCCAAGTCCCCATCAA		
TP53F	TCTCCACAGCCAAAGAAGAAACCAC	NM_174201.2	100
TP53R	AGGCATCATTCAGCTCTCGGAACA		
RPS18F	GAGGTGGAACGTGTGATCACCATT	NM_001033614.2	
RPS18R	TGTATTTCCCGTCCTTCACGTCCT		

*F: forward; R: reverse gene sequence.

Immunocytochemistry – SIRT1, SIRT2, P53

After 24 h of maturation, the oocytes and cumulus cells were partially separated (through frequent pipetting) under stereomicroscopic control. The resulting complexes (20 per group, three replicates) were washed thrice in PBS and fixed in 3% v/v paraformaldehyde + 2% w/v sucrose in PBS at 4°C for 4 h.

The complexes were washed in wash buffer (Tris-buffered saline + 0.5% v/v + 0.05% v/v Triton X100) for 10 min at room temperature and permeabilized in permeabilization buffer (20 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, and 0.5% v/v Triton X100; pH 7.4, 10 min, 0°C adjustment). After washing in wash buffer (5 min), the non-specific binding was blocked by incubation with Roti® Block (Carl Roth, Karlsruhe, Germany) at a 1:50 dilution in H_2O for 2 h at room temperature. Immediately thereafter, incubation with the primary antibodies (Table 2) diluted in Tris-buffered saline (0.05% v/v Tween 20, 2% v/v Roti® Block) was carried out at 4°C overnight. The COCs were then washed in wash buffer (four times for 10 min each) and incubated with the appropriate secondary antibody (Table 2) for 5 h in the dark at room temperature. Subsequently, they were washed four times again in wash buffer at room temperature and the nuclei were stained with SYBR1 green (S-7563, Molecular Probes, Thermo Fisher, Dreieich, Germany) at a 1:500 dilution in PBS for 1 h at room temperature. After washing (wash buffer, 4×10 min, room temperature) and a second fixation (2% v/v paraformaldehyde in PBS, 4°C overnight) the complexes were mounted on cover sheets with glycerin gelatin, cooled, and stored in 4°C until use.

Table 2
Antibodies and dilutions of antibodies used for immunofluorescence staining

Target	Antibody ID	Description	Immunofluorescence
P53	ABIN184527	Primary mouse anti-bovine P53 (monoclonal)	1:100
SIRT1	ABIN729853	Primary rabbit anti-human SIRT1 (polyclonal)	1:100
SIRT2	ABIN493462	Primary rabbit anti-bovine SIRT2 (polyclonal)	1:100
2. Ab	A-11035	Secondary goat anti-rabbit Alexa® 546	1:100
2. Ab	A-32723	Secondary goat anti-mouse Alexa® 488	1:100

ABIN: Antibodies-Online, A: Invitrogene/Thermo Fisher Scientific.

Confocal microscopy

The stained COCs were analyzed using a confocal laser scanning microscope (LSM 5 Pascal) attached to an Axiovert 200M inverted microscope (Carl Zeiss, Jena, Germany). For the measurement of red and green fluorescence combinations, a multitrack procedure was used [Line 1:488 nm of an argon laser (458, 488, and 514 nm; mW), 505–530 nm filter; Lane 2:543 nm line of a HeNe laser (543 nm, 1 mW), 560 nm long pass filter]. Stacks of images (40 images, Δz = 4 µm, 1024×1024 pixels) were recorded using a 40× lens (oil immersion) and software provided by Carl Zeiss (Zeiss Zen 3.7). The distribution and intensity of fluorescent staining were studied in individual compartments, oocytes, and cumulus cells by sieving the stacks of images. For homogeneity, the cell level that revealed oocytes with the largest diameter (medium) was selected. At this level, computer-assisted analyses were performed using ImageJ (open source) with a macro adapted to create two regions of interest (ROIs), one involving exactly the oocyte and the second involving the cumulus cells. The intensities (per channel) and x/y coordinates for each pixel in the ROI were read.

Statistical analysis

The R software package (open source) was used to analyze the data and calculate the average intensities integrated into the ROI. First, the normality of the data was evaluated using the Shapiro–Wilk test. Analysis of variance was conducted for parametric data, and Tukey's test with 5% probability was performed if a significant difference was found. For non-parametric data, the Kruskal–Wallis test was performed with a 5% probability.

Results and Discussion

The different maturation temperatures did not significantly influence the proportion of oocytes which completed nuclear maturation, presenting the first polar body (91.9 (275/299)–96.9% (320/330), P= 0.7841; figure 1A). Even when the analysis considered the competence of COCs, BCB+ (p=0.8453), and BCB- (p=0.6543), the maturation temperature had no influence on the polar body extrusion rate.

No significant difference was found in the cleavage rates (figure 1B) among the different temperature groups, regardless

of whether BCB+ oocytes or BCB- were evaluated. However, blastocyst rates (figure 1C) of COCs in the 38.5°C group (control) were higher (37%; 115/310) than those of the 40°C group COCs (21.5%; 56/260). Blastocyst rates of the 37°C group COCs did not differ from those of the control group and 40°C group COCs. The competence of COCs showed a clearly impact on embryo development, since BCB+ COCs matured at 38.5°C showed the highest blastocyst rate (51.9%; 79/152), in contrast with BCB+ COCs matured at 37°C (37.8%; 62/164) and at 40°C (23.6%; 27/114). BCB- COCs matured at 40°C produced the lowest blastocyst rates (19.8%; 29/146; figure 1C).

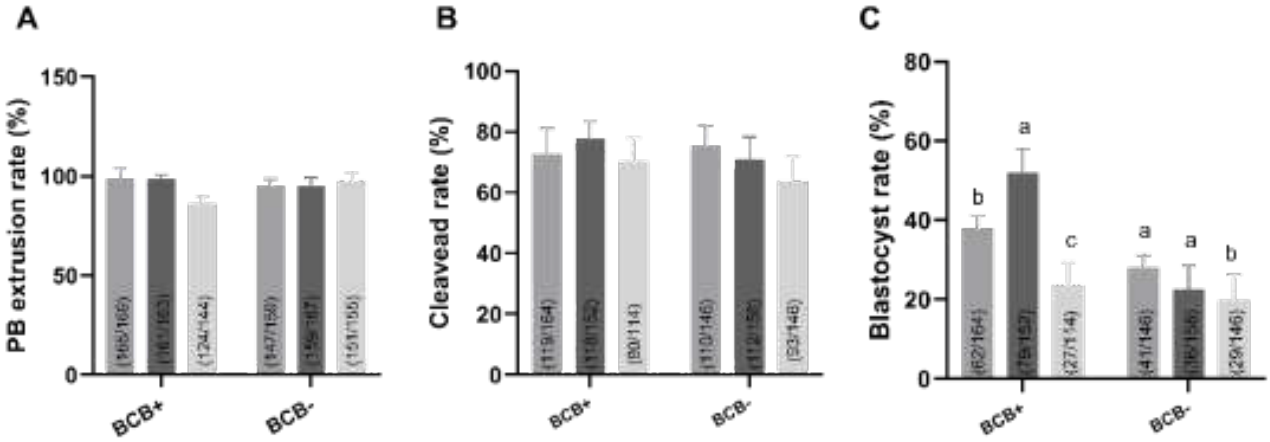

Figure 1. Effect of in vitro thermal stress on the percentage of oocytes with excluded first polar body (A), cleavage rates (B), and blastocyst rates (C) from *in vitro* fertilized *Bos taurus* oocytes matured for 24 h at different temperatures (37°C, 38.5°C, and 40°C) and BCB staining. Lowercase letters indicate significant differences. Values in parentheses indicate the number of structures found/total number of structures used. A) BCB+ P=0,8453; BCB- P=0,6543. B) BCB+ P=0,7869; BCB- P=0,7683. C) BCB+ P=0,076; BCB- P=0,0344.

COCs subjected to 24 h of IVM at lower (37°C) or higher (40°C) temperatures than the recommended temperature (38.5°C) exhibited successful nucleus maturation. While the extrusion of the first polar body can indicate the success of nuclear maturation (Halvaei et al., 2011), it does not provide information regarding cytoplasmic maturity. Many intracellular events, such as redistribution of cytoplasmic organelles, storage of messenger RNA (mRNA), transcription factors, and protein synthesis, are necessary for completing oocyte maturation (Conti & Franciosi, 2018; Reader et al., 2017). Successful embryonic development depends on the synchronization of these events (Conti & Franciosi, 2018). Cellular energy metabolism, lipid metabolism, and the accumulation of lipid reservoirs are of great importance for

the developmental competence of oocytes and early embryos (Andrade Melo-Sterza & Poehland, 2021). Although thermal stress does not prevent the nuclear maturation of COCs, it may impair cytoplasmic maturation, which is essential for proper embryonic development.

We performed IVM at a milder stress (40°C) considering that the temperature at which the follicles or COCs are exposed to in vivo is equal to or slightly below the body temperature of the animals, thus better representing the heat stress. The blastocyst formation rate was low during the entire IVM process under both cold and heat stress conditions. Reduction in IVP efficiency following different heat stress methods during IVM has been reported by many researchers. Varying, sometimes contradictory, results regarding the influence of temperature on oocyte maturation and blastocyst rate have been described when IVM was performed only for a short duration under elevated temperatures. Edwards and Hansen (1997) used 41□C or even higher temperatures, while Ju et al. (1999) used 40.5□C, 41.5□C, and 43□C, specifically at the zygote or 2-cell stage. Nabenishi et al. (2012a) used 38.5□C, 39.5□C, 40□C, and 40.5□C for their investigation. Zeng et al. (2016) showed that the duration of heat stress plays a relevant role on the viability of embryo development. The duration of exposure to high temperatures is probably the main reason for the differences between the present study and the studies cited earlier.

Corroborating our results, higher blastocyst rates and similar cleavage rates were observed in BCB+ COCs compared to BCB- COCs in previous studies (Alm et al., 2005; Silva et al., 2013). These findings confirm that embryo development is directly related to oocyte competence, though damage may not always be apparent in early cleavages.

In our study, the expression of most transcripts changed after cold and/or heat stress only in BCB+ oocytes and their corresponding cumulus cells, indicating that competent COCs are more likely to respond to thermal stress.

The results of gene expression studies of sirtuins and P53 in oocytes before and after maturation at different temperatures are shown in Figure 2. We observed that the expression of all studied sirtuin genes was greater after maturation than that before maturation. None of the maturation temperatures affected the expression of SIRT1, SIRT2, SIRT3, SIRT5, or P53 genes in BCB- oocytes. In BCB+ oocytes, the expression of the evaluated sirtuins was higher when matured under normal temperature conditions (38.5°C). Moreover, the expression of P53 was not influenced by the temperature at which BCB+ and BCB- oocytes were matured; only immature oocytes showed lower expression of this gene.

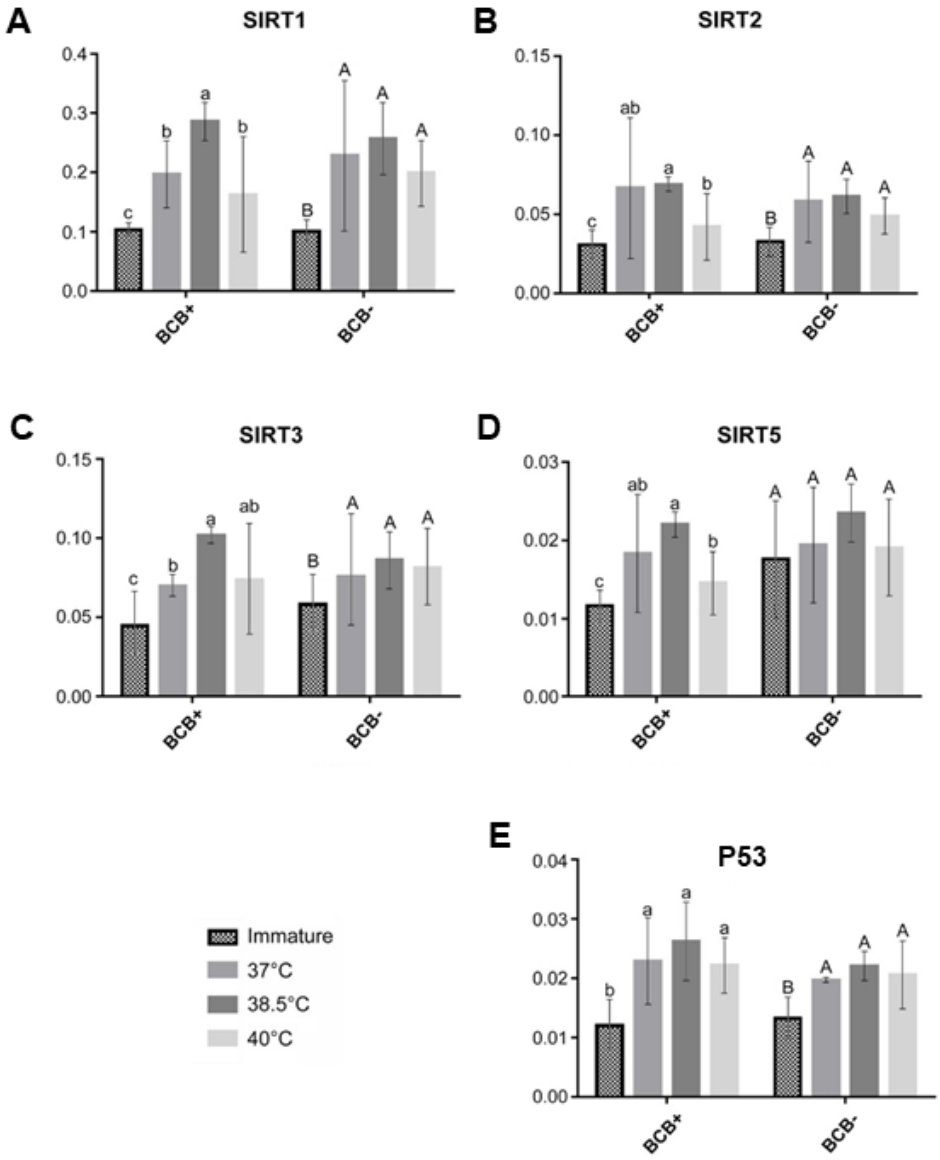

Figure 2. Effect of in vitro thermal stress on the transcripts of SIRT1 (A), SIRT2 (B), SIRT3 (C), SIRT5 (D), and P53 (E) genes in relation to the house keeping gene RPS18 measured using rtPCR. Samples consisted of 30 purified oocytes (three repeats) categorized by maturation temperature and BCB staining. Uppercase and lowercase letters indicate significant differences. A) BCB+ P=0,0021; BCB- P=0,0434. B) BCB+ P=0,0356; BCB- P=0,0455. C) BCB+ P=0,0231; BCB- P=0,0467. D) BCB+ P=0,0387; BCB- P=0,0943. E) BCB+ P=0,0495; BCB- P=0,0436.

SIRT1 expression was higher in cumulus cells (figure 3) from immature BCB+ and BCB- COCs than in cumulus cells of differing COC qualities matured at different temperatures. SIRT2, SIRT5, and P53 mRNA expression increased after maturation. In cumulus cells of BCB+ COC, a higher amount of SIRT1 transcripts was expressed when maturation occurred at 38.5°C and 40 °C. Temperature did not influence the expression

of SIRT2 in the cumulus cells of BCB+ COCs; however, SIRT3 expression increased with higher maturation temperatures. Decreased SIRT5 expression was observed in cumulus cells of BCB- COCs matured at 38.5°C. The expression of P53 was higher in cumulus cells of BCB+ COCs matured at 40°C.

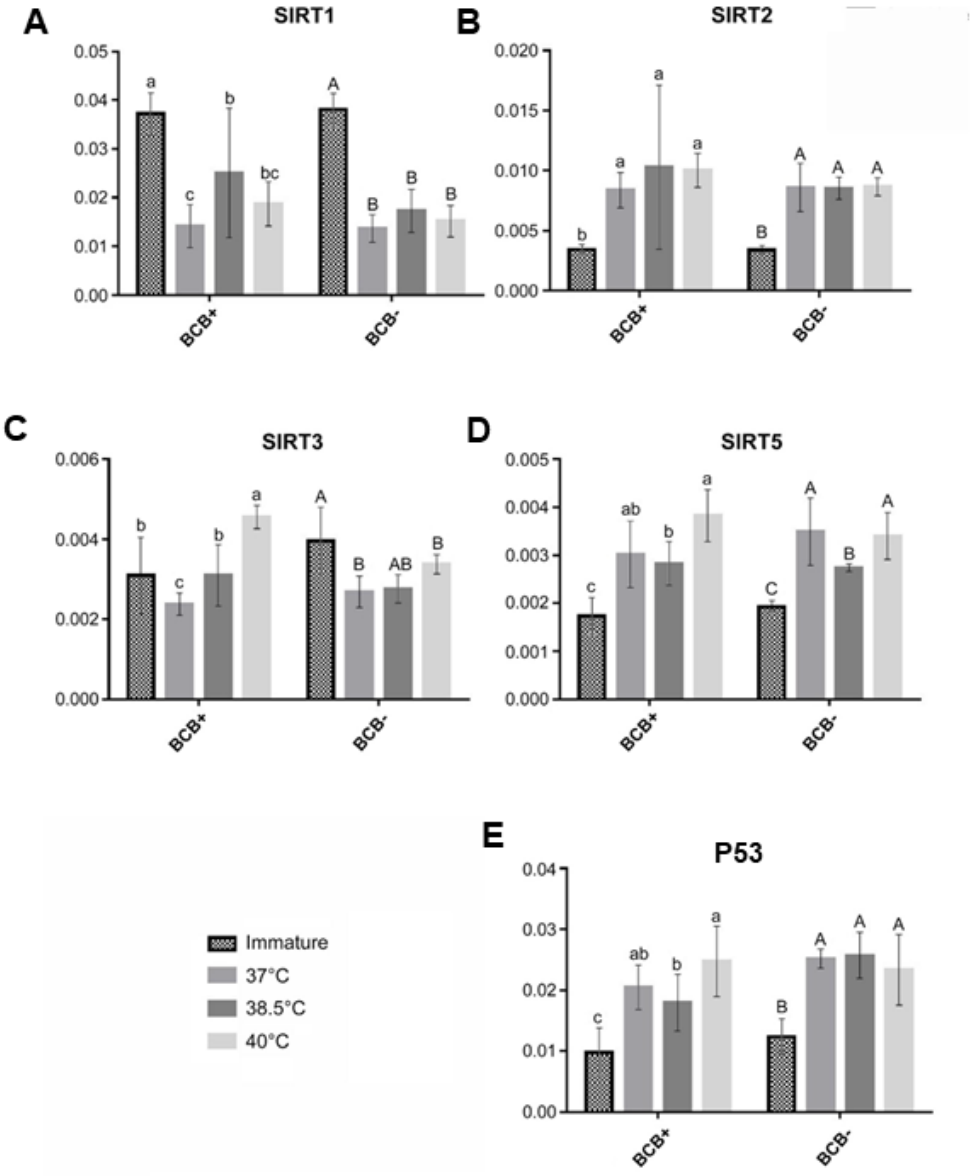

Figure 3. Effect of in vitro thermal stress on the transcripts of SIRT1 (A), SIRT2 (B), SIRT3 (C), SIRT5 (D), and P53 (E) genes in relation to the house keeping gene RPS18 measured using rtPCR. The samples consisted of cumulus cells recovered from 30 COCs (three repeats) categorized by maturation temperature and BCB staining. Uppercase and lowercase letters indicate significant differences. A) BCB+ P=0,0356; BCB- P=0,0411. B) BCB+ P=0,0387; BCB- P=0,0124. C) BCB+ P=0,0223; BCB- P=0,0187. D) BCB+ P=0,0278; BCB- P=0,0165. E) BCB+ P=0,0256; BCB- P=0,0165.

Confocal microscopy images after immunofluorescence staining are shown in figure 4, and the results of fluorescence intensity quantification are shown in figure 5. In oocytes, SIRT1 showed higher abundance at 38.5°C in BCB+ COCs, while no differences were observed in BCB- COCs from any group. SIRT2 and P53 abundances did not differ among IVM temperatures in oocytes or cumulus cells.

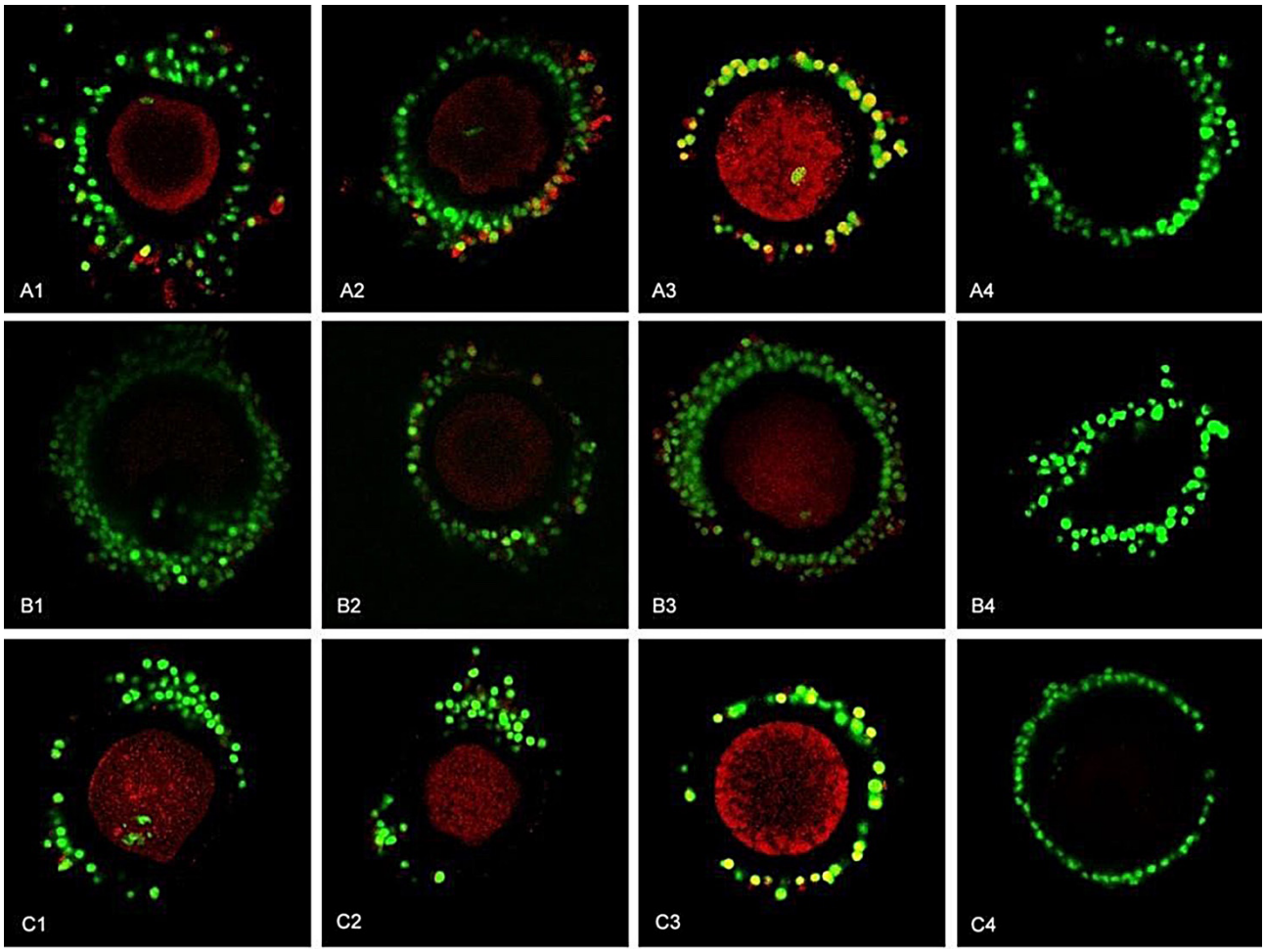

Figure 4. Confocal microscopic images of immunofluorescence-stained COCs after maturation at 37°C (1), 38.5°C (2), and 40°C (3). The stained proteins are SIRT1 (A), SIRT2 (B), and P53 (D) in red, with nuclear counter staining in green. Column 4 displays the unspecific control.

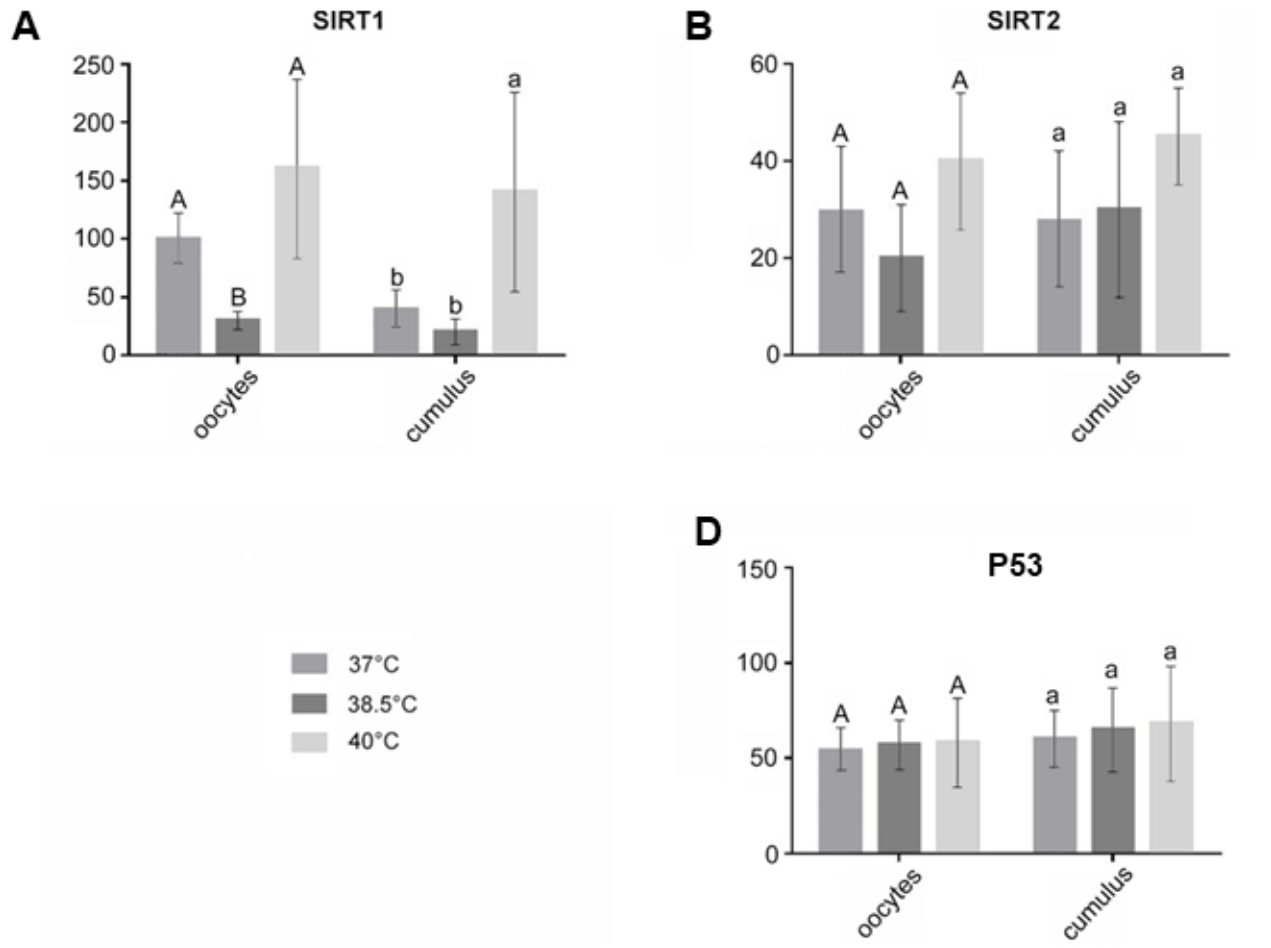

Figure 5. Effect of in vitro thermal stress on the densitometric evaluation (mean channels ± standard deviation; n = 10/group) of fluorescence intensities for stained SIRT1 (A), SIRT2 (B), and P53 (C) in COCs after maturation at 37°C, 38.5°C, and 40°C, measured using confocal microscopy. Uppercase and lowercase letters indicate significant differences (P<0.05). A) oocytes P=0,0334; cumulus P=0,0265. B) oocytes P=0,1440; cumulus P=0,0945. C) oocytes P=0,3468; cumulus P=0,4324.

Our results demonstrate an important role of sirtuins during IVM of bovine oocytes, as all studied sirtuins were highly expressed following the maturation of BCB+ oocytes. Similarly, in aged oocytes of mice, SIRT 1, 2, and 3 expression levels were maintained following treatment with the antioxidant quercetin (Wang et al., 2017). The treated oocytes preserved their spindle morphology and mitochondrial activity, reactive oxygen species (ROS) accumulation was prevented, and improvements were observed in the subsequent embryo development (Wang et al., 2017). Sirtuins are known to regulate cellular homeostasis, and their elevated expression in BCB+ oocytes may indicate

enhanced metabolic activity and resilience to oxidative stress, both of which are essential for successful maturation and embryonic development (Cao et al., 2020). These findings provide further insights into the molecular mechanisms underlying oocyte quality and suggest that sirtuin activity could be a potential marker or target for improving IVM outcomes in bovine reproduction.

The differential expression of sirtuins observed in the present study underscores their distinct roles in oocytes and cumulus cells during IVM under various thermal conditions. The increased expression of all the studied sirtuins in oocytes matured at thermoneutral temperatures suggests that optimal temperatures support balanced metabolic activity, efficient mitochondrial function, and reduced oxidative stress, fostering a conducive environment for oocyte maturation. In contrast, the increased expression of SIRT3 and SIRT5 in cumulus cells at 40°C indicates an adaptive cellular response to thermal stress. Given their mitochondrial localization and roles in energy metabolism and oxidative stress responses, SIRT3 and SIRT5 are likely to act as early sensors and mediators of thermal stress. Stress-induced metabolic shifts often lead to the generation of reactive oxygen species, and these sirtuins are critical for mitigating oxidative damage and maintaining mitochondrial integrity (Baur et al., 2012). Increased expression in cumulus cells may reflect their protective function, buffering the oocytes against the deleterious effects of thermal stress (Ren et al., 2024; Zhang et al., 2024). This observation aligns with the known role of cumulus cells as mediators of the oocyte microenvironment, suggesting that their metabolic responses are critical for safeguarding oocyte quality under suboptimal conditions. These findings emphasize the importance of maintaining thermoneutral conditions during IVM to promote optimal gene expression profiles, and indicate the potential of SIRT3 and SIRT5 as biomarkers for stress responses in cumulus cells. Further studies should explore therapeutic interventions targeting sirtuin pathways to mitigate the negative effects of thermal stress on oocyte maturation.

In general, sirtuins actively participate in the deacetylation of histone 3 (lysines 9 and 14) and histone 4 (lysine 16), which are important events regulating early embryo development and genome activation in swine (Adamkova et al., 2017). Thus, the reduction in blastocyst rates after thermal stress during IVM may be related to the downregulation of sirtuin under these conditions.

The mechanism underlying the exchange of different substances between oocytes and cumulus cells is important (Da Broi et al., 2018). P53 can induce apoptosis in cells under stress conditions by activating pro-apoptotic genes (Aubrey et al., 2018; Hori et al., 2013; Vousden, 2000). SIRT1, in turn, can bind directly to P53 in vivo and in vitro, allowing the survival of cells under stress by repressing the P53-dependent apoptotic response through its histone deacetylase (HDAC) function (Ong & Ramasamy, 2018; Yi & Luo, 2010). In the present study, higher expression of P53 was observed in cumulus cells when COCs matured under heat stress, which can be a signal of granulosa cell apoptosis. In fact, failures in bovine oocyte maturation may not be related to oocyte apoptosis or DNA damage, but rather to cumulus cell apoptosis (Nabenishi et al., 2012a,b). This leads to nutritional impairment

of the oocyte and disturbance of functions that are vital for embryo development (Thompson et al., 2007).

Cumulus cells may act as thermoprotectants and modulate the transfer of substances to the oocytes according to the demand. They may achieve this through cooperation mediated by junctions communicating between the oocyte and granulosa cells (Bhojwani et al., 2007; Nabenishi et al., 2012a). A significantly higher amount of SIRT1 protein was observed in oocytes and cumulus cells when COCs matured at 40°C. This may have occurred to control the accumulation of the P53 protein, which was probably the case, as the amount of this protein in oocytes and cumulus cells was similar across all groups.

SIRT1 appears to play a critical role in mediating cellular processes in oocytes and cumulus cells, as its transcription is high at the typically recommended temperature (38.5 °C) for IVM. Under stress conditions, the demand for SIRT1 translation is high, as can be seen in oocytes following IVM at 37°C and 40°C, and in cumulus cells corresponding to COCs matured at 40°C. Since many in vitro cultures of granulosa cells are successfully carried out at 37°C, this temperature may not be stressful for these cells (Baufeld et al., 2019; Yenuganti & Vanselow, 2017). Nevertheless, it is possible that cumulus cells did not trigger signals for oocyte protection at 37 °C, leading to impaired embryo development. At 40°C, despite the increased SIRT1 abundance after IVM, it may have been insufficient to prevent damages in vital processes, possibly due to chronic exposure to stress conditions throughout IVM.

Conclusion

In summary, temperature changes of ±1.5°C for 24 h during bovine oocyte maturation impair *in vitro* embryo development and lead to several cellular biochemical alterations in oocyte and granulosa cells. This is particularly seen in oocytes with higher developmental competence (BCB+). SIRT1 is important for *in vitro* embryonic development and appears to be associated with protective mechanisms against cold and heat stress.

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Competing interests

The authors have no conflict of interest to declare.

Ethical standards

Not applicable.

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