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Mild hypothermia and vitrification increase the mRNA expression of cold-inducible proteins in bovine oocytes and cumulus cells

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ABSTRACT

The cold-inducible RNA-binding protein (CIRBP) assists cells in adapting to new environmental conditions stabilizing specific mRNAs and promoting their translation. CIRBP participates in anti-apoptotic and anti-senescence processes, and its expression is induced by mild hypothermia, which may be advantageous to oocytes during vitrification. Several newly discovered small molecules, like zr17-2, mimic the effects of cold temperatures by increasing the expression of CIRBP at normothermia. This study aimed to evaluate the mRNA changes of a group of cold-inducible protein-encoding and apoptotic genes in response to exogenous supplementation of zr17-2 (experiment 1) or CIRBP (experiment 2) *in vitro* matured bovine oocytes and their cumulus cells. In experiment 1, cumulus-oocyte complexes (COCs) were randomly exposed to three concentrations of zr17-2 (1, 10, and 100 μM) during 24 h of *in vitro* maturation (IVM) under normothermia (38.5 °C) or mild hypothermia (34 °C) culture conditions. In experiment 2, COCs were randomly exposed to three concentrations of CIRBP (2, 4, and 6 μg/mL) or subjected to mild hypothermia (34 °C), followed by oocyte vitrification/warming after 20 h of IVM. The quantification of the selected gene transcript expression was performed separately in oocytes and cumulus cells by quantitative real-time PCR. We show that oocytes and cumulus cells exhibited similar mRNA expression responses to mild hypothermia and vitrification. However, minor differences were observed when COCs were exposed to exogenous supplementation with zr17-2 and CIRBP. In conclusion, the alterations observed in the mRNA expression in these experimental conditions may impact the quality of the cumulus-oocyte complexes in terms of vitrification and sublethal hypothermia challenges. In this sense, our results should complement other oocyte quality assessments for its application in future assisted reproductive techniques in the bovine species, including improving oocyte cryotolerance to vitrification.

1. Introduction

Oocyte cryopreservation is a valuable methodology for preserving genetic resources and contributes to developing assisted reproductive techniques [1]. On the other hand, cryopreservation may damage the structure of the oocyte and chromosomes, as well as the mitotic spindle [2–4]. During this process, the ultra-low temperatures affect mitochondrial activity, affecting apoptotic pathways [5], inducing premature cortical granule extrusion, and reducing successful fertilization chances [6].

In mammalian cells, exposure to mild hypothermia (34 °C) reduces the metabolism and the overall protein abundance, while the expression of a small group of homologous glycine-rich mRNA-binding proteins, known as cold-inducible proteins (CIPs), increases under this condition [7–12]. The CIPs bind to mRNA, acting as regulatory molecules that play an essential role in RNA post-

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transcriptional control [13]. They are upregulated in response to mild hypothermia and a variety of other cellular stressors, including osmotic stress, ultraviolet radiation, or hypoxia [14].

The cold-inducible RNA-binding protein (CIRBP) is a constitutively and ubiquitously expressed CIP considered a key component of the cellular response to moderate cold stress [15]. CIRBP has been identified in multiple tissues and species [16], including the bovine ovary and testicle [17]. Although only a few body tissues are exposed to hypothermia in physiological conditions, the widespread expression of CIRBP [16] may suggest that it is likely involved in other relevant cellular processes at normothermia. Primarily located in the nucleus, CIRBP controls RNA transcription and processing, while in the cytoplasm, CIRBP regulates mRNA translation and turnover [8,18]. Eventually, CIRBP can be released into the extracellular space [19]. Once extracellular CIRBP is present, it binds to toll-like receptor 4 (TLR4) and the myeloid differentiation factor 2 (MD2), activating several signaling pathways including the NF-κB pathway [20,21]. The activation of such pathways via CIRBP leads to an induction and release of proinflammatory cytokines and chemokines, including the IL-1β [19,22]. However, if IL-1β is modulated during the *in vitro* maturation (IVM) of bovine cumulus-oocyte complexes (COCs) in the presence of CIRBP is still unknown.

Other CIPs, as the RNA-binding motif protein 3 (RBM3) and the serine and arginine-rich splicing factor 5 (SRSF5) [7,12], also bind to particular mRNAs, stimulating their translation, regulating their half-life, controlling their expression potential, and determining their final functions [23,24]. Moreover, CIRBP and RBM3 bind to specific mRNAs involved in cell survival and anti-apoptotic cascades [14], being potential candidates to improve oocyte cryotolerance to vitrification. Because oocyte vitrification can induce apoptosis [25,26], the study of BCL2 (anti-apoptotic gene) [27], BAX (pro-apoptotic gene) [28], and its ratio is helpful to investigate the effects of vitrification on oocytes and cumulus cells.

A temporary increase in general adaptability generated by controlled exposure to sub-lethal stressors has been used to improve cryotolerance in mammalian oocytes and embryos using various strategies [29], such as heat stress [30] or high hydrostatic pressures [31]. However, little attention has been given to mild hypothermia as a sub-lethal stressor to enhance stress tolerance. In this sense, we have previously reported an increase in CIRBP expression on bovine COCs exposed to mild hypothermia (33.5 °C) [32]. However, detrimental effects on oocyte nuclear and cytoplasmic maturation rates were reported due to the non-optimal temperature conditions during the IVM [32]. Furthermore, some small molecules (including zr17-2) have been detected, capable of mimicking the effect of hypothermia in the absence of cold stimulus [33], being good candidates for their application to improve cell cryotolerance.

In the present study, we hypothesized that oocytes and cumulus cells might modify the expression of selected genes during the IVM in response to the exogenous supplementation with zr17-2 and CIRBP directly added to the culture medium. For this purpose, we aim to test the effect of exogenous supplementation with zr17-2 and CIRBP during the IVM of bovine COCs and whether this procedure implies upregulation of mRNA expression of the three CIPs included (*CIRBP*, *RBM3*, and *SRSF5*), *BCL2*, and *IL-1β*, and downregulation of *BAX* mRNA expression. In addition, we will determine the mRNA changes on three CIPs, apoptotic-related transcripts (*BAX* and *BCL2*, and their ratio), and the pro-inflammatory interleukin 1β (*IL-1β*) induced by mild hypothermia and vitrification in both oocytes and cumulus cells.

2. Materials and methods

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.1. Bovine COCs recovery and IVM

Bovine ovaries were obtained from a local slaughterhouse (*Escorxador de Sabadell,* Barcelona, Spain) and transported to the laboratory at 35-37 °C in 0.9% saline solution. The ovaries were collected from cycling non-gravid heifers in follicular phase. Immature COCs were aspirated from ovarian follicles between 6 and 8 mm in diameter using an 18-gauge needle attached to a low-pressure vacuum pump. Only COCs presenting more than three compact layers of cumulus cells and a homogeneous cytoplasm were selected for the study. The selected COCs were randomly allocated in groups of 50 oocytes in 500 μL of maturation medium in 4-well dishes (Nalge Nunc International, Rochester, NY, USA) and cultured following the experimental design. The maturation medium was composed of TCM199 with Earle's salts, L-glutamine, and sodium bicarbonate supplemented with 10% (v/v) fetal bovine serum (FBS), 10 ng/mL epidermal growth factor, and 50 μg/mL gentamicin.

2.2. Experiment 1: hypothermia mimetic zr17-2 supplementation

In experiment 1, COCs were randomly distributed in groups of 50 oocytes in 4 experimental groups: exposed to control conditions or exposed to three different concentrations of zr17-2 (1, 10, and 100 μM). These 4 experimental groups were incubated for 24 h in a humidified atmosphere containing 5% CO_2 at two different temperature conditions, 38.5 °C or 34 °C. After 24 h of IVM, COCs were washed and denuded by gently pipetting (PIPETMAN ® P100L, Gilson, Spain) in phosphate buffer saline medium. Oocytes were harvested and the remaining media after denudation was centrifuged (500×*g*, 3 min, room temperature) to obtain the cumulus cells of the oocytes on each experimental group. The supernatant was discarded and the cryotube containing the cell pellet of each group was immediately plunged in liquid nitrogen. Oocytes of each group (n = 25 oocytes/experimental group and replicate) were transferred to cryotubes using the minimum possible volume of media and directly immersed in liquid nitrogen. All samples were kept at −80 °C until further analysis. The experiment was performed three times.

2.3. Experiment 2: exogenous CIRBP supplementation

In experiment 2, COCs were randomly distributed in groups of 50 oocytes in 5 experimental groups: exposed to standard bovine IVM conditions (38.5 °C, 5% CO_2, in humidified atmosphere), exposed to three different concentrations of exogenous CIRBP (2, 4, and 6 μg/mL), or exposed to mild hypothermia (34 °C, 5% CO_2, in humidified atmosphere). After 20 h of IVM, half of the COCs on each experimental group were vitrified and ulteriorly warmed (n = 25 oocytes/experimental condition and replicate). Vitrified/warmed oocytes were returned for 4 h to the maturation dishes to complete 24 h of IVM. After finishing the 24 h of IVM, COCs (n = 25/experimental group and replicate) were denuded by gently pipetting to separate oocytes from cumulus cells, as before described. The experiment was performed three times.

2.4. Oocyte vitrification and warming

After 20 h of IVM, COCs were partially denuded by gentle pipetting until a few layers of cumulus cells remained. Following a modified vitrification protocol [34], oocytes were transferred into an equilibrium solution (TCM199 with HEPES supplemented with 20% (v/v) FBS, 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide (Me_2SO)) for 9 min at room temperature (20-22 °C). After being equilibrated, oocytes were transferred into a vitrification solution (TCM199 with HEPES supplemented with 20% (v/v) FBS, 15% (v/v) EG, 15% (v/v) Me_2SO, and 0.5 M sucrose) for 30 s. Groups of 5–6 oocytes were then picked up in a small volume of vitrification solution, placed on top of the polypropylene strip of a Cryotop device (Kitazato Supply Co, Fujinomiya, Japan), and immediately plunged in liquid nitrogen.

For warming, the polypropylene strip of the Cryotop device was immersed directly into TCM199 with HEPES supplemented with 20% (v/v) FBS containing 1 M sucrose at 37 °C for 5 min. Recovered oocytes were washed for 5 min in decreasing sucrose solutions (0.5 M, 0.25 M, and 0 M sucrose in TCM199 with HEPES). Once warming was finished, oocytes were returned to the maturation dishes allowing them to mature for four additional hours.

2.5. RNA extraction and cDNA synthesis

Total RNA from oocytes and cumulus cells was extracted using the commercial miRNeasy Mini Kit (Qiagen, Barcelona, Spain) following the manufacturer's instructions. The RNA concentration was determined using the Thermo Scientific NanoDrop™ 2000 (Fisher Scientific, Madrid, Spain). Synthesis of cDNA was performed using the High-Capacity RNA-to-cDNA™ Kit (Fisher Scientific, Madrid, Spain) according to the manufacturer's instructions. The cDNA obtained was stored at −20 °C for subsequent analysis.

2.6. Quantitative real-time PCR (qPCR)

Data analysis was performed using the CFX Maestro software version 3.3.3 (Bio-Rad Laboratories, Kabelsketal, Germany). Relative quantification of *CIRBP*, *RBM3*, *SRSF5*, *BCL2*, *BAX*, and *IL-1β* transcripts was performed using the $2^{-\Delta\Delta Ct}$ method [35] with *G3PDH* as a housekeeping gene for normalization. Commercial gene-specific qPCR primers for bovine species were used (PrimePCR™SYBR® Green Assay, Bio-Rad Laboratories, Kabelsketal, Germany). The product sizes for each primer pair were confirmed by loading the amplicons in an agarose gel using a gel imaging system (ChemiDoc XRS + System, BioRad Laboratories, Inc., Kabelsketal, Germany).

2.7. Data analyses

Each experiment was repeated three times. The relative mRNA expression data of the genes included in the study were analyzed by R software version 3.6.1 [36] with *nlme* [37] to develop linear mixed-effects (LME) models and *multcomp* [38] to perform pairwise comparisons. All data sets were analyzed for normal distribution and homoscedasticity using the Shapiro–Wilk Normality test and Levene's test, respectively. Non-normal distributed data were transformed using the log(x+1) transformation. The threshold of significance was set at $p < 0.05$.

For experiment 1, the experimental group and the temperature during IVM (34 °C or 38.5 °C) were included as fixed effects and the replicates as the random part of the LME. Pairwise comparisons were adjusted by the Sidak test.

For experiment 2, the experimental group and vitrification procedure (vitrified or non-vitrified) were included as fixed effects and the replicates as the random part of the LME. Pairwise comparisons were adjusted by the Sidak test.

3. Results

3.1. Experiment 1: hypothermia mimetic zr17-2 supplementation

The relative mRNA expression of selected transcripts showed that mild hypothermia induced most of the changes observed in *CIRBP*, *RBM3*, *SRSF5*, *BCL2*, and *BAX* mRNA expression, both in oocytes (Fig. 1a) and cumulus cells (Fig. 1b).

Mild hypothermia upregulated *CIRBP* and *RBM3* mRNA expression in oocytes and cumulus cells ($p < 0.05$). *SRSF5* mRNA expression was upregulated in cumulus cells exposed to mild hypothermia culture conditions ($p < 0.05$), but no differences were observed in oocytes. *RBM3* mRNA expression was upregulated in oocytes exposed to mild hypothermia at different concentrations of zr17-2 compared to the control group ($p < 0.05$). Besides, *RBM3* mRNA expression was upregulated in oocytes exposed to mild hypothermia at 100 μM zr17-2 compared to all the experimental groups at normothermia ($p < 0.05$). Additionally, *RBM3* mRNA expression was upregulated in oocytes exposed to mild hypothermia at 10 μM zr17-2 compared to oocytes exposed to normothermia at 10 μM zr17-2 and IVM control conditions ($p < 0.05$).

The mRNA expression of anti-apoptotic *BCL2* was downregulated in oocytes and cumulus cells cultured under mild hypothermia conditions ($p < 0.05$), whereas mRNA expression of the pro-apoptotic gene *BAX* was upregulated in cumulus cells cultured under mild hypothermia conditions ($p < 0.05$).

When cultured at normothermia, the relative mRNA expression of *IL-1β* was downregulated in oocytes by the 100 μM zr17-2 supplementation compared to the control group ($p < 0.05$).

3.2. Experiment 2: exogenous CIRBP supplementation

The relative mRNA expression of selected transcripts in Fig. 2 shows that mild hypothermia induced most of the changes observed in the *CIRBP* and *RBM3* mRNA expressions, whereas the vitrification procedure induced changes in *SRSF5* mRNA expression.

CIRBP mRNA expression was upregulated in oocytes exposed to mild hypothermia (vitrified and non-vitrified) compared to 6 μg/mL CIRBP (non-vitrified), 2 μg/mL CIRBP (vitrified and non-vitrified), and vitrified control oocytes ($p < 0.05$). For cumulus cells, the *CIRBP* mRNA expression was upregulated in non-vitrified cells exposed to mild hypothermia compared to vitrified cumulus cells exposed to 2 μg/mL CIRBP ($p < 0.05$).

RBM3 mRNA expression was upregulated in oocytes exposed to mild hypothermia (vitrified and non-vitrified) compared to the rest of the experimental groups ($p < 0.05$). In non-vitrified cumulus cells exposed to mild hypothermia, *RBM3* mRNA expression was upregulated compared to the rest of the non-vitrified experimental groups and the vitrified cumulus cells exposed to 2 and 6 μg/mL of exogenous CIRBP ($p < 0.05$). Additionally, *RMB3* mRNA expression was upregulated in vitrified cumulus cells exposed to mild hypothermia compared to the rest of vitrified and non-vitrified treatments, excluding the non-vitrified cumulus cells exposed to mild hypothermia ($p < 0.05$).

SRSF5 mRNA expression in oocytes and cumulus cells was increased by the vitrification procedure ($p < 0.05$). Besides, *SRSF5* mRNA expression was upregulated in vitrified oocytes exposed to mild hypothermia compared to non-vitrified oocytes exposed to 4, 2 μg/mL CIRBP, and control conditions ($p < 0.05$). Additionally, SRSF5 expression was upregulated in vitrified oocytes exposed to 4 μg/mL CIRBP compared to non-vitrified oocytes exposed to 2 μg/mL CIRBP and control conditions ($p < 0.05$).

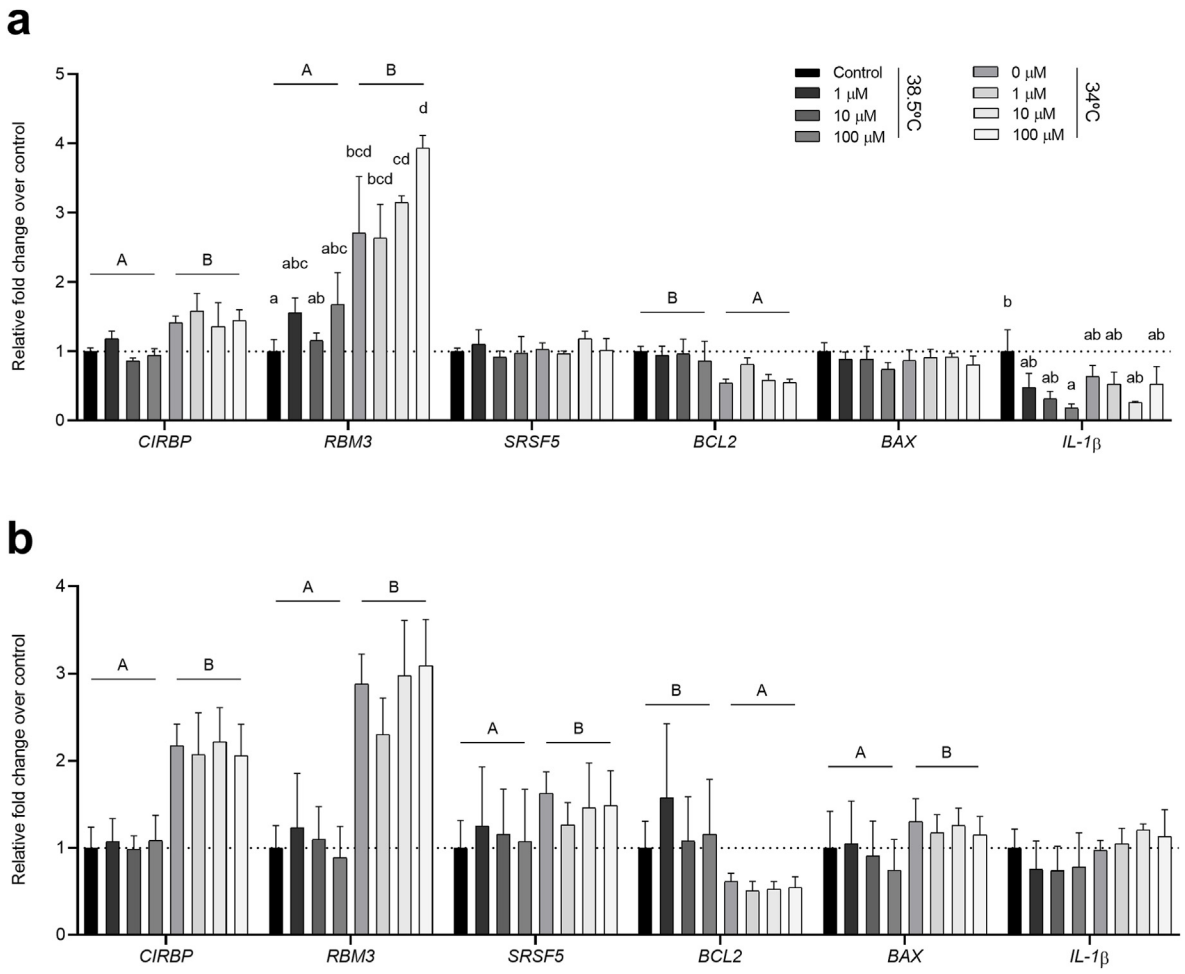

Fig. 1. Relative mRNA expression of selected genes in oocytes and cumulus cells of experiment 1. Relative mRNA expression ± SEM of *CIRBP*, *RBM3*, *SRSF5*, *BCL2*, *BAX*, and *IL-1β* in oocytes (**a**) and cumulus cells (**b**) of experimental groups in experiment 1. Supplementation with four concentrations of zr17-2 (0, 1, 10, 100 μM) incubated at 38.5 °C or 34 °C, 5% CO_2 in humidified atmosphere. Different lowercase-letters represent statistical differences between experimental groups. Different uppercase-letters represent statistical differences between culture temperature conditions. The control group (0 μM zr17-2 incubated at 38.5 °C, 5% CO_2 in humidified atmosphere) was established as the reference group.

Vitrification and mild hypothermia induced most of the changes observed in the *BCL2* mRNA of oocytes (Fig. 2a) and cumulus cells (Fig. 2b), respectively. In vitrified and non-vitrified cumulus cells exposed to mild hypothermia, the *BCL2* mRNA expression was downregulated compared to non-vitrified control cumulus cells and the vitrified and non-vitrified cumulus cells exposed to 4 μg/mL of exogenous CIRBP ($p < 0.05$). On the other hand, the vitrification procedure induced *BAX* and *IL-1β* mRNA expression changes in the cumulus cells. Additionally, *BAX* mRNA expression was upregulated in vitrified oocytes exposed to 6 μg/mL CIRBP compared to vitrified oocytes exposed to mild hypothermia and non-vitrified oocytes exposed to 4 μg/mL CIRBP ($p < 0.05$).

3.3. Apoptosis gene expression: BAX:BCL2 ratio

The relative *BAX:BCL2* ratios were increased by mild hypothermia in experiment 1 (Fig. 3a and b) and vitrification in experiment 2 (Fig. 3c and d) ($p < 0.05$).

Additionally, in experiment 2, the ratio was increased in non-vitrified oocytes exposed to mild hypothermia compared to non-vitrified oocytes exposed to 4 μg/mL CIRBP and to control conditions ($p < 0.05$) (Fig. 3c). On the other hand, the ratio was increased in vitrified and non-vitrified cumulus cells exposed to mild hypothermia compared to the non-vitrified treatments ($p < 0.05$) (Fig. 3d). Besides, the ratio was increased in non-vitrified cumulus cells exposed to mild hypothermia compared to the rest of vitrified cumulus cells ($p < 0.05$), except for the vitrified cumulus cells exposed to mild hypothermia (Fig. 3d).

4. Discussion

Different strategies have been used to improve cryotolerance in mammalian oocytes by increasing the general adaptability induced by sublethal stressors [29]. Mild hypothermia, on the other hand, has received little attention as an inductor of tolerance. Furthermore, the CIPs are potential candidates to mitigate the damage produced in oocytes during vitrification, mainly due to their involvement in cell survival and anti-apoptotic pathways [14]. This study has determined mRNA expression changes of CIPs and anti- and pro-apoptotic transcripts in oocytes and cumulus cells induced by mild hypothermia, vitrification, and exogenous zr17-2 and CIRBP supplementation.

Oocytes and cumulus cells exhibited similar mRNA expression responses to mild hypothermia and vitrification, but minor differences were observed when COCs were exposed to exogenous supplementation with zr17-2 and CIRBP. Our findings may suggest a common underlying mechanism of such observed response to mild hypothermia and vitrification in both types of cells. The

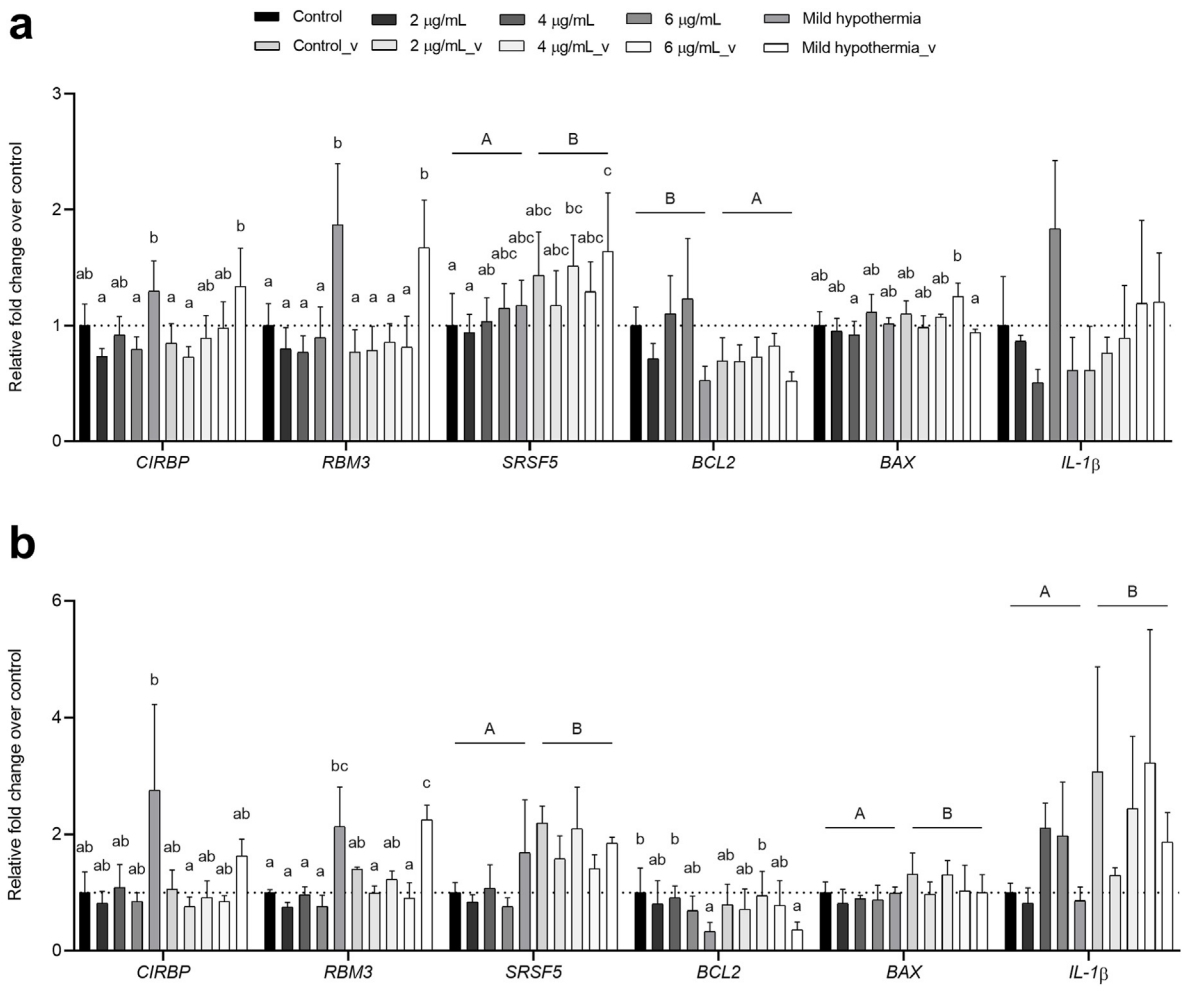

Fig. 2. Relative mRNA expression of selected genes related in oocytes and cumulus cells of experiment 2. Relative mRNA expression ± SEM of *CIRBP*, *RBM3*, *SRSF5*, *BCL2*, *BAX*, and *IL-1β* in oocytes (**a**) and cumulus cells (**b**) of treatments in experiment 2. Control (38.5 °C, 5% CO_2 in humidified atmosphere); three concentrations of exogenous CIRBP protein (2, 4, 6 μg/mL; 38.5 °C, 5% CO_2 in humidified atmosphere); and mild hypothermia (34 °C, 5% CO_2 in humidified atmosphere). Vitrified groups were represented with letter v. Different lowercase-letters represent statistical differences between treatments. Different uppercase-letters represent statistical differences between vitrified and non-vitrified groups. The control group was established as the reference group.

bidirectional communication between oocytes and cumulus cells is essential to establish the optimal intrafollicular microenvironment, which controls the growth and maturation of the follicles to produce viable and competent oocytes [39,40]. In addition, this complex communication may be involved in shared responses to ensure that both cells quickly adapt to the new environmental conditions. This fact suggests that the mRNA expression changes that occurred during the maturation of the oocyte may have potential effects during early embryo development [40]. In this sense, the regulation of CIRBP expression in mature yak oocytes before vitrification may explain the improvement in its *in vitro* developmental competence after vitrification/warming [41].

Several CIPs have been previously identified; yet, two of them (CIRBP and RBM3) have been well-characterized since their discovery [7,8]. Both proteins regulate gene expression at the transcription level by binding to different transcripts, allowing the cell to respond quickly to environmental changes [42]. Here, we confirmed that the mRNA expressions of *CIRBP* and *RBM3* were significantly induced by mild hypothermia in oocytes and cumulus cells, with or without an additional vitrification procedure. Even though oocytes were exposed to ultralow temperatures during vitrification, it seems that the brief exposure time to sub-physiological temperatures was not enough to induce the transcription of neither both CIPs when cells were previously long exposed to exogenous CIRBP and mild hypothermia. In contrast, previous studies have reported an increase of *CIRBP* mRNA expression after oocyte vitrification [43,44], using the expression of CIRBP as a marker of cryoinjury. Aside from its roles in hypothermia, CIRBP has been shown to play key roles during early development [45] and cell protection against endogenous and external stressors at normothermia [42]. Our study only analyzed an early event during oocyte maturation and no later events on development. Future research should focus on the effects of CIRBP and other CIPs during *in vitro* early embryo development and tests its impact on embryo competence after oocyte vitrification/warming.

Another recently described RNA-binding protein induced by mild hypothermia and other stressors is SRSF5 [12]. SRSF5 belongs to the serine-arginine rich splicing factor (SRSF) protein family, which is involved in alternative splicing and mRNA processing [46]. Additionally, SRSF5 seems to perform specific functions in pluripotent cells, managing the nuclear availability of splicing factors and cytoplasmic activities by responding quickly to changing cellular circumstances [47]. Our results showed that mild hypothermia increased *SRSF5* mRNA expression in cumulus cells but not in oocytes. Besides, *SRSF5* mRNA expression was increased by the vitrification procedure in both oocytes and cumulus cells. These results

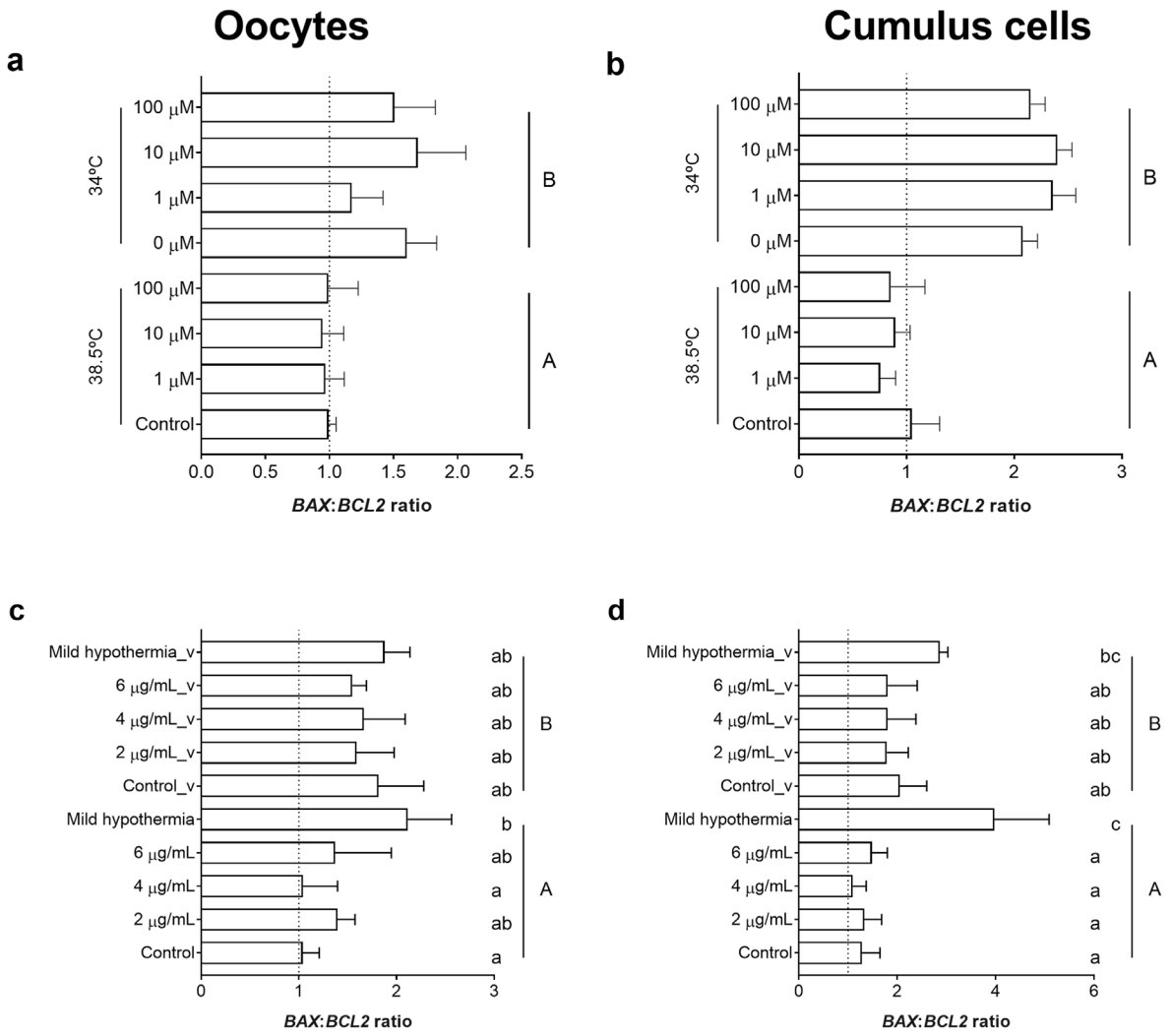

Fig. 3. Relative BAX:BCL2 ratios in oocytes and cumulus cells of experiment 1 and 2. Relative BAX:BCL2 ratio ± SEM in oocytes (**a, c**) and cumulus cells (**b, d**) of experiment 1 (**a, b**) and experiment 2 (**c, d**). Experiment 1: supplementation with four concentrations of zr17-2 (0, 1, 10, 100 μM) incubated at 38.5 °C or 34 °C, 5% CO_2 in humidified atmosphere. Experiment 2: control (38.5 °C, 5% CO_2 in humidified atmosphere); supplementation with three concentrations of exogenous CIRBP protein (2, 4, 6 μg/mL; 38.5 °C, 5% CO_2 in humidified atmosphere); and mild hypothermia (34 °C, 5% CO_2 in humidified atmosphere). Vitrified groups were represented with letter v. Different lowercase-letters represent statistical differences between experimental groups. Different uppercase-letters represent statistical differences between culture temperature conditions (experiment 1) or vitrified and non-vitrified groups (experiment 2). The control group was established as the reference group for each experiment.

contrast with what was observed in *CIRBP* and *RBM3* mRNA expression, where only mild hypothermia was responsible for most of the changes. Although SRSF5 has been recently described as a novel CIP [12], its mRNA expression responded differently from *CIRBP* and *RBM3*.

The use of mild hypothermia sub-lethal stress and the vitrification procedure lead to analyzing apoptotic-related transcripts in this study. BCL2 is an anti-apoptotic gene that promotes cells survival by releasing mitochondrial cytochrome *c* [27], whereas BAX is the first pro-apoptotic member activated during apoptotic conditions, accelerating cell death [28]. Other studies have investigated the effect of vitrification on the expression of both genes [25,26]; however, the results have been inconsistent. Our results showed that mild hypothermia and vitrification affected *BCL2* and *BAX* mRNA expression. Taken together, the detrimental effects caused by mild hypothermia and vitrification induced apoptotic pathways in oocytes and cumulus cells were independent of the zr17-2 and CIRBP exogenous supplementation. Consistent with this conclusion, our BAX:BCL2 ratio data confirmed this hypothesis.

CIRBP has an important role in regulating inflammatory molecules like IL-1β and the NF-κB pathway [22], and extracellularly, CIRBP acts as a damage-associated molecular signal [20]. Notably, IL-1β is a cytokine that plays a crucial role in inflammation, stimulating its production and other pro-inflammatory cytokines [48,49]. Cytokines operate as mediators between the immune and reproductive systems and paracrine and autocrine ovarian regulatory factors [50–52]. In addition to ovarian macrophages and monocytes, other ovarian cell types can produce cytokines, like the granulosa cells [53]. Besides, the supplementation with IL-1β and tumor necrosis factor alpha stimulates the growth and maturation of bovine oocytes during *in vitro* culture [54]. Our study showed an upregulation of *IL-1β* mRNA expression in cumulus cells after vitrification. However, our data cannot link the changes in *IL-1β* mRNA expression with the stimulation of the COCs growth after warming. Further research is needed to test if IL-1β could stimulate the COCs growth after warming to cope with the detrimental effects of vitrification. Somehow, increased concentrations of zr17-2 exogenous supplementation trigger the downregulation of *IL-1β* mRNA expression. These results suggest a link between the two molecules, perhaps via a regulatory function of CIRBP accumulation

[22], but further studies should be performed to define the exact mechanism behind these results.

5. Conclusions

In conclusion, the current study has identified differences in the mRNA expression of bovine oocytes and cumulus cells exposed to mild hypothermia and vitrification. In addition, the exogenous supplementation with zr17-2 to the maturation media has little impact on the CIPs mRNA expression of both oocytes and cumulus cells, but it affects *IL-1β* mRNA expression under physiological temperatures and at the higher concentration used. Mild hypothermia induced most of the mRNA expression changes observed in this study. CIPs mRNA expression increased in response to mild hypothermia. In addition, *SRSF5* mRNA expression among the CIPs included in this study was affected by vitrification. Vitrification also affected the mRNA expression of apoptotic-related genes and *IL-1β*. Future studies are needed to assess whether the identified mRNA expression changes might affect the overall oocyte quality and, perhaps, the subsequent embryo development. Understanding how CIPs influence oocyte maturation could help establish new protocols that prompt protection against vitrification-induced damage.

Declaration of interest

None.

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CRediT authorship contribution statement

Jaume Gardela: collected the samples, conducted the experiments, analyzed and interpreted the data, performed the statistical analyses, Formal analysis, Writing – original draft. **Mateo Ruiz-Conca:** helped with the experiments and sample collection. **Josune García-Sanmartín:** Supervision, helped with the experiments and sample collection. **Alfredo Martínez:** Supervision, Funding acquisition. **Teresa Mogas:** Supervision, Funding acquisition. **Manel López-Béjar:** Supervision, Funding acquisition. **Manuel Álvarez-Rodríguez:** Supervision, Funding acquisition, All authors revised and approved the final version of the manuscript.

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