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Relative transcript level of *BMP15, BAX* and *CASP3* with qRT-PCR in vitrified equine immature cumulus-oocytes complexes

Relative transcript level of *BMP15, BAX* and *CASP3* with qRT-PCR in vitrified equine immature cumulus-oocytes complexes

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Highlights

BAX is highly expressed in vitrified immature equine COCs.
BAX suggest the activation of apoptosis in equine cells exposed to vitrification process.
BMP15 and *CASP3* expression are detected in vitrified and non-vitrified equine COCs.

Abstract

Research focused on female gamete vitrification has increased attention to develop a reliable cryopreservation method to preserve immature equine oocytes. Despite the intensive implementation of biotechnological procedures for horse breeding, vitrification of immature equine cumulus-oocyte complexes (COCs) remain to be clearly elucidated. We aimed to determine the relative transcript level of target genes Bone morphogenetic protein 15 (*BMP15*); Bcl-2-associated X protein (*BAX*); and Caspase 3 (*CASP3*) in equine COCs prior to and after vitrification. Ovarian follicles were aspirated from ovaries collected from an abattoir. A total of 240 COCs were collected and distributed into vitrified COCs (VIT, n=120) and non-vitrified (Non-VIT, n=120) groups. Then, COCs were preserved and relative transcript expressions of *BMP15, BAX, CASP3* were measured and normalized against *GAPDH* performed by qRT-PCR. In addition, 38 COCs were evaluated to assess chromatin configuration of germinal vesicl e stage prior and after vitrification by

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exposure to 10 µg/ml of bisbenzimide. A difference was observed in the COCs' mRNA level of abundance for the *BAX* gene between the VIT (2.05 ± 0.47) and (0.85 ± 0.08) Non-VIT groups. There was no difference in mRNA relative transcript level of *CASP3* and *BMP15* in Non-VIT (0.63 ± 0.20 and 1.55 ± 0.73, respectively) compared to VIT (0.64 ± 0.01 and 2.84 ± 2.20, respectively) equine COCs. All COCs where considered at immature stage of development even though COCs in Non-VIT group showed higher condensed chromatin configuration compared to VIT (100% vs 60.7%, respectively). We demonstrate that *BMP15* and *CASP3* are detected in VIT and Non-VIT immature COCs. In conclusion, *BAX* is expressed highly in vitrified immature equine COCs and indicates that activation of apoptosis signaling cascades in cells exposed to vitrification.
Key words: Bcl-2-associated X protein. Bone morphogenetic protein 15. Gene expression. Caspase 3. Horse.

Resumo

Pesquisas sobre a vitrificação de gametas femininos estão sendo realizadas para o desenvolvimento de um método confiável de criopreservação dos complexos cumulus-oócitos (CCOs) na espécie equina. Apesar da implementação intensiva de biotecnologias reprodutivas em equinos, a vitrificação dos CCOs imaturos permanece em estágio experimental em relação à competência celular. O objetivo do estudo foi determinar o nível de transcrição relativo dos genes Proteína morfogenética óssea 15 (*BMP15*); Proteína X associada a Bcl-2 (*BAX*); e Caspase 3 (*CASP3*) em CCOs equinos antes e após a vitrificação. Folículos ovarianos foram aspirados de ovários coletados em matadouro. O total de 240 CCOs foi coletado e distribuído em grupos vitrificados (VIT, n=120) e não vitrificados (N-VIT, n=120). Os CCOs foram preservados e as expressões transcritas relativas de *BMP15, BAX, CASP3* foram determinadas pela técnica de qRT-PCR sendo normalizadas em relação ao GAPDH. Além disso, 38 CCOs foram avaliados para determinar a configuração da cromatina no estágio de vesícula germinativa antes e após a vitrificação pela exposição a 10 µg/ml de bisbenzimida. Os resultados mostraram uma diferença no nível de abundância de mRNA dos CCOs para o gene *BAX* entre os grupos VIT (2,05 ± 0,47) e N-VIT (0,85 ± 0,08). Não houve diferença no nível de transcrição relativa do mRNA de *CASP3* e *BMP15* nos CCOs do grupo N-VIT (0,63 ± 0,20 e 1,55 ± 0,73, respectivamente) em comparação com VIT (0,64 ± 0,01 e 2,84 ± 2,20, respectivamente). Todos os CCOs foram considerados em estágio imaturo de desenvolvimento, embora os CCOs no grupo N-VIT apresentaram a configuração de cromatina condensada em maior número de células avaliadas em comparação com VIT (100% vs 60,7%, respectivamente). Demonstramos que *BMP15* e *CASP3* são detectados em CCOs imaturos em VIT e N-VIT. Conclui-se que o *BAX* é altamente expresso em CCOs equinos imaturos vitrificados sendo relacionado à sinalização de apoptose em células expostas ao processo de vitrificação.
Palavras-chave: Proteína X associada a Bcl-2. Proteína morfogenética óssea 15. Expressão genética. Caspase 3. Equinos.

Introduction

The cryopreservation of gametes is considered an important tool to maximize genetic improvement and also to preserve female gametes from valuable horses and endangered breeds (Smits, Hoogewijs, Woelders, Daels, & Van Soom, 2012). Moreover, cryopreservation can be used to rescue gametes when females die unexpectedly to

ensure genetic maintenance after animal death and to improve oocyte cryopreservation at different stages of maturation (Hinrichs, 2018; De Coster, Velez, Van Soom, Woelders, & Smits, 2020). Vitrification technique is considered faster than slow-freezing that avoid intracellular ice formation which may result in higher embryo viability in several species (Zander-Fox, Lane, & Hamilton, 2013; Wong, Mastenbroek, & Repping, 2014; Araújo-Lemos et al., 2015). The vitrification of *in vitro* produced equine embryos using the intracytoplasmic sperm injection (ICSI) technique as well as *in vivo* retrieved embryos have good fertility rates in the horse industry, reaching post-transfer pregnancy rates of 45% to 67% (Eldridge-Panuska, Caracciolo di Brienza, Seidel, Squires, & Carnevale, 2005; Stout, 2012; Choi & Hinrichs, 2017).

Vitrification of equine oocytes surrounded only by corona radiata showed successfully blastocyst development after vitrification of immature equine oocytes fertilized by ICSI and did result in a healthy foal (Ortiz-Escribano et al., 2018). Despite the intensive implementation of biotechnological procedures for horse breeding, vitrification of equine oocytes remains at low efficiency due to low post-cryopreservation cell viability associated with poor embryo development in the horse (Tharasanit, Colleoni, Galli, Colenbrander, & Stout, 2009; Canesin et al., 2017, 2018). In addition, the uses of new ARTs to overcome fertility problems has been used to improve immature gamete acquisition by ovum pick-up (OPU) followed by ICSI methods that are applied to commercial programs in the horse industry (Galli, Colleoni, Duchi, Lagutina, |& Lazzari, 2013; Claes et al., 2016; Choi & Hinrichs, 2017).

Research focused on female gamete vitrification has increased attention to develop a reliable cryopreservation method to preserve immature equine oocytes due to the complex oocyte structure and sensitivity to chilling (Canesin et al., 2018; De Coster et al., 2020). Ducheyne, Rizzo, Daels, Stout and De Ruijter-Villani (2019) evaluated the effect of vitrifying horse oocytes at the germinal vesicle (GV) stage and subsequent metaphase-II (MII) spindle architecture and proposed that changes in spindle morphology damage are crucial to reduced developmental competence of vitrified equine oocytes. The use of immature oocytes is ideal for cryopreservation, because no meiotic spindle is present and the genetic material is condensed within the nucleus (Leibo, 2008). Interestingly, Purohit, Meena and Solanki (2012) demonstrated that immature cumulus compact goat oocytes better tolerate vitrification in terms of fertilization rate than do matured vitrified oocytes. Equine oocyte vitrification is limited to the uses of ethylene glycol, ficoll, and sucrose (Hurtt, Landim-Alvarenga, Seidel, & Squires, 2000), in addition to trehalose (Canesin et al., 2018) and dimethylsulfoxide (Maclellan et al., 2002) protocols. In the horse, the initial cumulus morphology of equine oocytes did not affect nuclear competence acquisition after vitrification, and immature oocytes can be used after vitrification/re-warming to obtain satisfactory M-II rates after IVM (Curcio et al., 2014). Perhaps, the vitrification technique is of fundamental importance to achieve high genetic cryopreservation and to improve fertilization rates in the equine species.

Data on the viability of equine cumulus-oocytes complexes (COCs) is of upmost importance in determining pro-survival and pro-apoptotic factors that may

interfere with development and competence acquisition of cell maturation. Studies of molecular biomarkers for assessing oocyte viability or apoptosis may help produce an understanding of the factors that act on female gamete viability prior and after the vitrification process. To that end, genes such as Bone Morphogenetic Protein 15 (*BMP15*) (Galloway et al., 2000), Cysteine Aspartate Protease 3 (*CASP3*) (Lin et al., 2016), and Bcl-2-associated X Protein (*BAX*) (Anchamparuthy, Pearson, & Gwazdauskas, 2010) are commonly used to evaluate oocyte competence in various species. The *BMP15* gene is described to be involved in the communication between oocyte and the adjacent cumulus cells via gap junctions and its known to improve subsequent quality of bovine embryos when the *in vitro* maturation (IVM) medium was supplemented with BMP15 (Machado et al., 2015; Sanfins, Rodrigues, & Albertini, 2018). Immature porcine COCs vitrified at the GV stage did not trigger mRNA levels for pro-apoptotic *BAX* and *CASP3* genes in oocytes and cumulus cells at the end of IVM and in cleavage stage embryos after *in vitro* fertilization (IVF) (Somfai et al., 2020). However, vitrification at the MII stage reportedly triggered the apoptotic cascade in porcine oocytes, which is believed to contribute to their low developmental performance (Vallorani et al., 2012). Therefore, additional studies on gene expression will certainly contribute to the optimalisation of more specific vitrification protocols that effectively cryopreserve equine oocytes.

Despite the intensive implementation of biotechnological procedures for horse breeding, vitrification of immature equine COCs remains at the experimental stage and it may contribute to the reduced viability of cryopreserved oocytes information regard cell competence. Thus, the aim of this study was to determine the relative transcript level of target genes *BMP15, BAX* and *CASP3* in equine COCs prior to and after vitrification.

Methods

Unless otherwise indicated, all chemicals used were purchased from Sigma Chemicals Company (St. Louis, MO, USA). The research was approved by the Research Ethics Committee from Universidade Federal de Pelotas (CODE CEEA: 40077). For this study, equine ovaries were obtained from an abattoir (30°25'31.5"S and 54°22'35.6"W) in Brazil during the physiological breeding season. Ovaries were collected and maintained at the laboratory facilities in a 0.9% NaCl solution at 25ºC until follicle aspiration occurred. Equine follicles from 5 to 30 mm in diameter were aspirated using an 18-G needle connected to a 20 mL syringe containing 2 mL PBS with 0.8% heparin. Then, COCs and follicular fluid were placed in 50 mL polypropylene tubes. The follicular contents were filtered, flushed using PBS and placed in 35-mm Petri dishes so that the contents could be examined under a stereomicroscope that COCs could be located.

The morphology and structural integrity of each COC were evaluated and classified as being compact (CP; having a tight, complete compact cumulus with a distinct, smooth hillock), expanded (EX; having a granular or expanded cumulus), or denuded (D; having a partial cumulus or having only corona radiata present) (Hinrichs & Schmidt, 2000). Only oocytes with a compact cumulus containing three or more cumulus cell layers and homogeneous cytoplasm were used in this experiment (Tharasanit et al., 2006). Following

morphological classification, selected COCs were washed four times in the holding medium (EquiHold®, Minitube®, Germany) held at room temperature (22-25ºC) for 2 h.

The experiment was carried out with 90 ovaries, from which 280 COCs were morphologically selected as previously described and distributed into non-vitrified (Non-VIT, n = 120) and vitrified (VIT, n = 120) groups of COCs. After group selection, COCs were immediately transferred to the vitrification solution (VIT) or immediately placed in TQC Complete Flush medium at room temperature for 6 h prior for relative transcript gene analysis (Non-VIT). In addition, 40 COCs were selected to assess the chromatin configuration status before and after vitrification. Of these, 02 COCs were lost during manipulation, 28 COCs were selected for evaluation of chromatin configuration after 6 h in TQC Complete Flush medium, and the remaining 10 COCs were vitrified and then kept in 6 h TQC Complete Flush before evaluation .

The COCs were subjected to chromatin configuration of GV stage oocyte assessment by exposure to 10 µg/mL of bisbenzimide (Hoechst 33342) for 15 min at 38°C. Afterward, the slides were mounted and the stained oocytes were classified under UV light (filter cube +A, with a wavelength of 340–380 nm) with a fluorescence microscope (Leica, DMI 4000B). Chromatin configurations of GV stage oocytes were classified as: Fluorescent Nucleus (FN), having fluorescence throughout the nucleus; Fibrillar (F), having chromatin strands throughout the estimated area of the nucleus; Intermediate (I), having chromatin strands or irregular masses of chromatin over approximately half the estimated nuclear area; or Condensed Chromatin (CC), having chromatin condensed into one small regular or irregular mass (Hinrichs, Schmidt, Friedman, Selgarth, & Martin, 1993; Martino et al., 2014).

The number of COCs submitted to vitrification ranged from 8 to 12 for each 200 µl drop of TQC Complete Flush (Nutricel®, Campinas, SP) at 37ºC. The vitrification of COCs was performed using EquiPro-VitKit commercial media (Eldridge-Panuska et al., 2005; Nowak, Kochan, Papis, & Okólski, 2014). COCs were exposed to vitrification solutions at room temperature (22-24°C). Briefly, COCs were placed in 200 µl of 1.4 M glycerol in PBS for 5 min. Afterward, COCs were exposed to 1.4 M glycerol + 3.6 M ethylene glycol for 5 min, and then transferred to 30 µl of the final vitrification solution containing 3.4 M glycerol + 4.6 M ethylene glycol. The final vitrification solution containing the COCs was loaded into a 250 µl non-irradiated, polyvinyl chloride straw and separated by two air bubbles. The straw ends were loaded with two columns of 60 µl 0.5 M galactose medium (Figure 1). The total time during which the COCs were exposed to the final vitrification solution and during which the COCs were loaded into the straw was less than 1 min. The straw was heat-sealed and placed for 1 min into a cooled plastic goblet 2.5 cm above the liquid nitrogen. The entire goblet containing the straw was then immersed in liquid nitrogen and stored until warming procedure. To warm the COCs, the straws were removed from the liquid nitrogen and held in the air for 10 s before being immersed in a 23ºC water bath for 10 s. Then, the contents of the straw were expelled into a Petri dish and gently stirred to facilitate the mixture of the vitrification and dilution solutions. After warming, VIT COCs were subjected to culture *in vitro* for 6 hours prior to quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis.

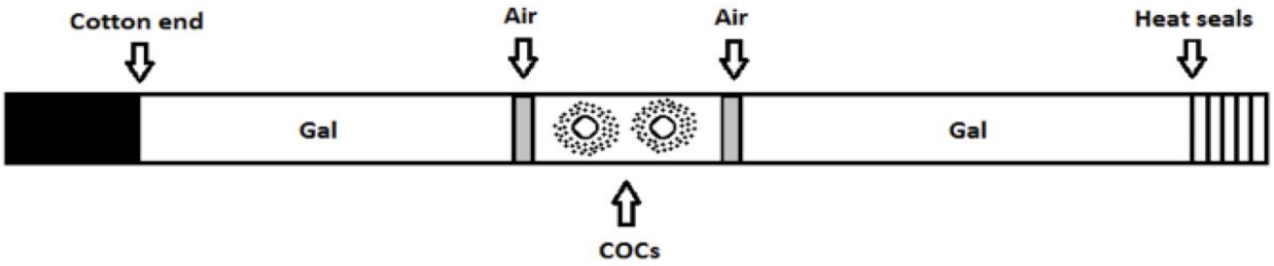

Figure 1. Straw containing two COCs loaded in the vitrification medium and hold with two columns of galactose (Gal) medium.

Total RNA was extracted from COCs using Trizol (Invitrogen; São Paulo, Brazil) according to the manufacturer's instructions. RNA quantity and purity were estimated using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, USA). Absorbance 260/280 nm ratios above 1.8 were considered pure. Total RNA was treated with 0.1 U DNase (Invitrogen) at 37°C for 5 min to digest the contaminating DNA. Reverse transcriptase reactions were performed with 500 ng RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Mississauga, ON, CA) in a final volume of 20 µl according to the manufacturer's protocol.

The qRT-PCR reactions were performed in a CFX 384 real-time PCR detection system using iQTM SYBR Green Supermix (BioRad). Primers were designed based on equine sequences available in GenBank (Table 1) and synthesized by Sintese Biotecnologia (Belo Horizonte, MG, Brazil). Three separate genes, *BMP15, CASP3, BAX* and plus an endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were amplified. Common thermal cycling parameters (3 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C) were used to amplify each transcript. Melting-curve analyses were performed to verify product identity. Samples were run in duplicates and the standard curve method was used to determine the abundance level of mRNA for each gene. The mRNA abundance was normalized to the mean abundance of the internal control GAPDH gene.

Comparative CT method was performed and $^{\Delta}$CT value was determined by subtracting the GAPDH-CT value of each threshold sample from the CT value of each target gene within the sample (Ebrahimi, Valojerdi, Eftekhari-Yazdi, Baharvand, & Farrokhi, 2010). Calculation of $^{\Delta\Delta}$CT involved using the highest sample $^{\Delta}$CT value as an arbitrary constant to subtract from all other $^{\Delta}$CT sample values. Fold differences in relative transcript abundance were calculated for target genes assuming an amplification efficiency of 100% and using the formula $2^{-\Delta\Delta CT}$ (Livak & Schmittgen, 2001). All reactions had efficiencies between 90 and 100%, $r^2 \geq 0.98$ and slope values from -3.6 to -3.1. Dissociation curve analyses were performed to validate the specificity of the amplification products.

Table 1
Chemical and physical composition of the commercial substrate Basaplant®, used in seedling production (SSP) and in the cultivation (SCV) of Physalis peruviana L. UFCG, Pombal, PB, 2019.

Gene symbol	Gene targeted	Primers sequence (5'- 3')	GenBank accession number
BMP15	Bone Morphogenetic Protein 15	F: TCCTAGAGAGAACCGCACCA R: GGTCTGTATATGCCAGGGGC	XM_001496223.2
CASP3	Caspase 3	F: AGGCAGACTTCCTGTATGCG R: GCGACTGGATGAACCAGGAT	NM_001163961.1
BAX	BCL-2 associated X Protein	F: TTCCGACGGCAACTTCAACT R: GGTGACCCAAAGTCGGAGAG	XM_005596728.1
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase	F: CAAGGCTGTGGGCAAGGT R: GCAGGTCAGATCCACGACTGA	NM_001163856.1

Statistical analysis

The differences in oocyte nuclear maturation were carried out by Fisher's exact test using Prism 8 (GraphPad Software, San Diego, CA). Differences in transcript levels were analyzed by a multi-comparison test using the LSMeans Student *t*-test. Data were tested for normal distribution using the Shapiro-Wilk test and were normalized when necessary. The results are presented as means ± SEM, and *P*-value < 0.05 indicated a statistically significant difference. All analyses were performed using the JMP software (SAS Institute Inc., Cary, NC). At least three individual gene replicates were conducted for each group of evaluated COCs.

Results and Discussion

The results of this study showed that *BAX, CASP3,* and *BMP15* relative transcript levels are detectable in equine COCs prior to and after vitrification. The differential expression of the apoptotic genes is related to oocytes' competence with advanced signs of atresia in different species (Livak & Schmittgen, 2001; Opiela et al., 2008; Ebrahimi et al., 2010; Leon et al., 2013; Nowak et al., 2014). In this study, we showed that *BAX* genes are highly expressed in equine COCs, indicating the possible marker of viability and cell competence in this specie. A difference was observed in our study between the mRNA levels of abundance for the *BAX* gene in VIT (2.05 ± 0.47) and Non-VIT (0.85 ± 0.08; P=0.03; Figure 2) groups, showing a higher expression of the pro-apoptotic gene *BAX* in VIT COCs. In contrast, Leon et al. (2013) reported no difference detected in the expression of *BAX* apoptotic genes analyzed in immature oocytes compared to oocytes submitted to IVM. Additional information about immature COCs vitrification is needed to ensure potential alternatives for gamete cryopreservation and oocyte development competence maintenance to increase fertilization rates in horses.

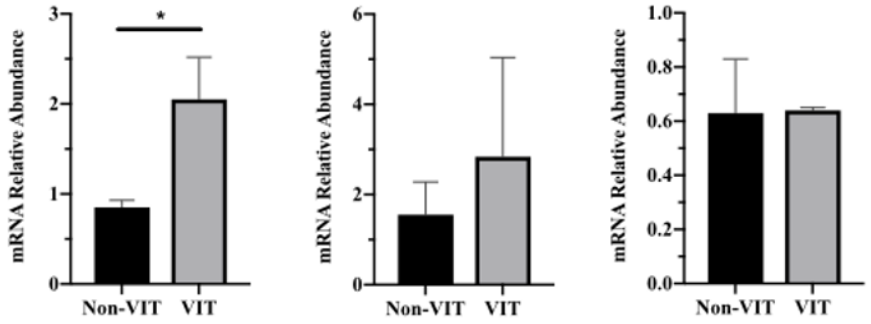

Figure 2. Relative abundance levels for the genes *BAX, BMP15, CASP3* that were analyzed by qRT-PCR from immature equine COCs: Vitrified (VIT) and non-Vitrified (Non-VIT). Data show the mean value ± SEM of five replicates for each stage (each replicate = pool of 30 oocytes). Asterisks indicate a significant difference in relative mRNA abundance (P<0.05) between the groups.

The presence of multiple layers of cumulus cells was also found to protect mature equine oocytes from vitrification-induced damage because cumulus-enclosed oocytes preserved their meiotic spindle integrity (Tharasanit et al., 2009). The process of the cryopreservation of mature and immature oocytes has several limitations, such as the sensitivity of oocytes at low temperatures (Aman & Parker, 1994). The cryopreservation of immature GV oocytes had the genetic material condensed within the nucleus; however, the success of immature oocyte cryopreservation depends largely on the ability to preserve the structural and functional integrity of the entire oocyte and the cumulus cells surrounding the oocytes (Rao et al., 2012). Due to the limiting process of equine oocytes availability for in vitro procedures, cryopreservation has focused on the use of immature GV oocytes stage of development.

We observed that 28.6% of COCs submitted to VIT were found in the GV state having fibrillar chromatin (Figure 3A), and only 10.7% of COCs submitted to VIT showed an intermediate stage having chromatin strands or irregular masses of chromatin spread over approximately half the area of the GV (Figure 3B). The prevalence of the fibrillar chromatin configuration in equine oocytes has been reported to increase with increased duration between slaughter and oocyte collection. These more diffuse configurations (fibrillar and intermediate) are labile and undergo chromatin degradation over time determining a significantly lower rate of maturation in vitro compared to condensed chromatin configurations (Hinrichs et al., 2005). However, in our study, we observed that the majority of COCs submitted to VIT group at GV were considered in the immature stage having the condensed chromatin configuration status (60.7%; Figure 3C). All COCs in the Non-VIT group were considered to be at an immature stage of development as they had a higher condensed chromatin configuration compared to VIT (Table 2). In vivo, the proportion of oocytes having condensed configuration increased with increasing follicle size and oocytes with fibrillar chromatin showed a significantly lower rate of maturation than did oocytes in the tightly condensed chromatin configuration (Hinrichs and Schmidt, 2000; Hinrichs et al., 2005). Thus, vitrification

of equine oocytes at immature GV stage bypasses the risk of chromosome aberrations due to the fact that chromatin is protected within a membrane-bound vesicle. Perhaps, vitrification of immature COCs would allow collection and preservation of cells without the requirement for culture facilities.

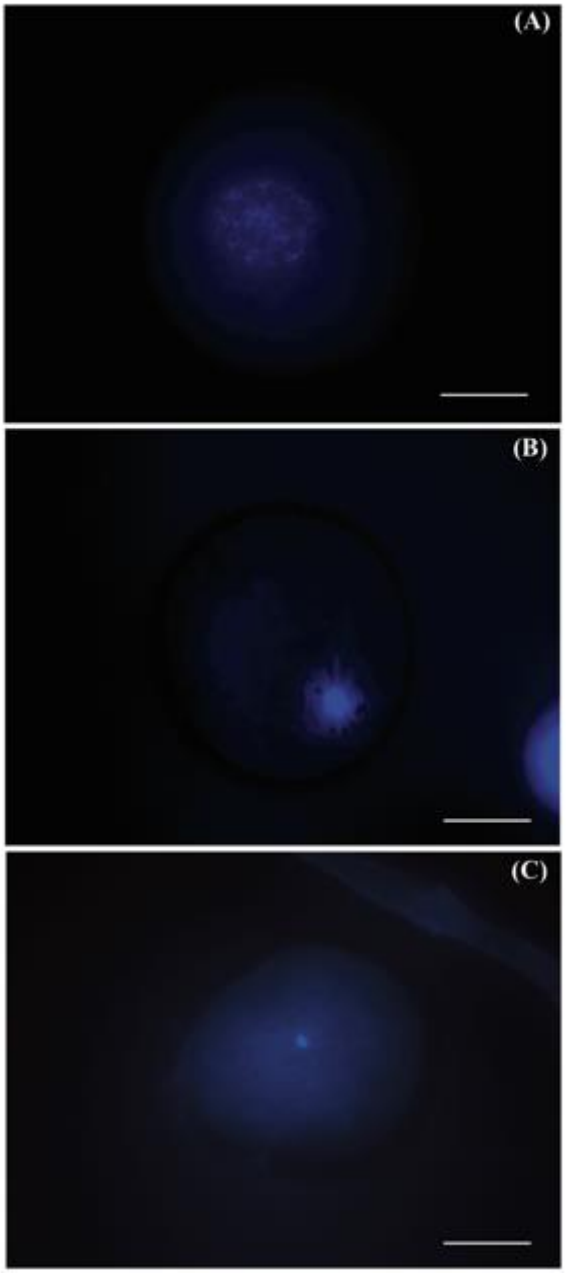

Figure 3. Photomicrographs of germinal vesicle (GV) chromatin configuration. (A) Vitrified oocyte showing fibrillar distinct strands of intertwined chromatin visible throughout the GV; (B) Vitrified oocyte showing an intermediate stage with chromatin strands or irregular masses of chromatin spread over approximately half the area of the GV; and (C) Non-vitrified showing a tightly condensed chromatin, whit one dense, circular to oval mass of chromatin. Scale bar represents 20 µm.

Table 2
Chromatin configurations status of equine cumulus oocyte complexes (COCs) submitted or not to vitrification

Treatments	COCs cultured (n)	COCs evaluated (n)	Germinal Vesicle n (%)	Intermediated Chromatin n (%)	Condensed Chromatin n (%)
VIT	30	28	8 (28.6)[a]	3 (10.7)[a]	17 (60.7)[a]
Non-VIT	10	10	0 (0.0)[b]	0 (0.0)[a]	10 (100.0)[b]

VIT: vitrified COCs; Non-VIT: not-vitrified COCs. Columns with different letters are significantly different (P<0.05; Fisher's exact test).

Studies of equine gamete competence are still scarce and gene expression in COCs and proteins involved in the apoptotic pathways have not been completely elucidated in this species. There was also no difference in mRNA expression of *CASP3* in Non-VIT (0.63 ± 0.20) and VIT (0.64 ± 0.01; P>0.05; Figure 2) COCs. *CASP3* protein expression has been described in equine oocytes and granulosa cells from pre-ovulatory follicles after induced ovulation; however, no difference in mRNA levels between young and old mares were observed (Rodrigues et al., 2010). To our knowledge, no previous work has quantified *CASP3* activity to identify equine immature oocyte atresia. It was found that *CASP3* activity does not increase in vitrified COCs and it was confirmed that *CASP3*, initiator of apoptosis, is expressed in equine COCs prior to and after vitrification. Thus, the *BAX* and *CASP3* expression profile of vitrified equine COCs can be used to predict oocyte viability and developmental competence.

The *BCL2* gene family, which includes the *BAX* (proapoptotic) and *BCL2* (anti-apoptotic) genes, is involved in the regulation of apoptosis in different cell types (Kim & Tilly, 2004; De Bem et al., 2014). These two genes, in particular, play a key role in the occurrence of apoptosis in female germ cells (De Bem et al., 2014) and are used in the analysis of apoptosis in oocytes and embryos (Opiela et al., 2008). The ratio of *BCL2:BAX* determines cell susceptibility to apoptosis; a lower ratio indicates a dominance of *BAX* over *BCL2*, and denotes accelerated apoptosis and a lower quality of oocytes and embryos development (Oltvai, Milliman, & Korsmeyer, 1993; Yang & Rajamahendran, 2002; Jang et al., 2014). Recently, a report showed a higher abundance of *BAX* expression in cumulus cells from mares >18 years of age, confirming the existence of age-related differences in gene expression in equine COCs; this may be associated with the lower quality and decreased developmental competence of oocytes from aged mares (Cox, Vanderwall, Parkinson, Sweat, & Isom, 2015). This study demonstrated a higher *BAX* relative abundance level in COCs after the vitrification procedure. Therefore, the evaluation of *BAX* transcripts may be used as a biomarker of the quality of the oocyte and the developmental potential in the horse. The expression of *BAX* may be constitutive, which suggests that the oocytes are under constant threat and that their development potential depends on their ability to inhibit pro-apoptotic activity as demonstrated in human oocytes (Boumela et al., 2011).

Recently, COCs in humans showed that the expression levels of *BMP15* mRNAs were significantly associated with oocyte maturation, normal fertilization, and cleavage rate; therefore, *BMP15* mRNAs in cumulus granulosa cells may be considered as new molecular markers for predicting oocyte developmental potential (Li et al., 2014). We determined that *BMP15* expression is present in VIT (2.84 ± 2.20) and Non-VIT (1.55 ± 0.73) equine COCs, although no differences observed between groups (P>0.05; Figure 2). In addition, in bovine ovaries, *BMP15* is involved in the regulation of cumulus cell apoptosis (Hussein, Froiland, Amato, Thompson, & Gilchrist, 2005). On the other hand, Ebrahimi et al. (2010) showed that the expression of the *BMP15* gene was retarded after the vitrification of sheep COCs. Thus, *BMP15* may help decrease the incidence of apoptosis within cumulus-oocyte complexes until the point of ovulation.

Similar to *GDF9*, the mRNA and protein of *BMP15* are found in oocytes during all stages of folliculogenesis. Indeed, these factors act as intraovarian regulators of primordial follicle activation, somatic cell proliferation, steroidogenesis, and oocyte maturation (Otsuka & Shimasaki, 2002; Knight & Glister, 2003). In our study, we detected that COCs showed no difference in the expression of *BMP15* because they have not developed competence at the stage of the COCs analysis. In equine oocytes, there has been a higher expression of BMP15 at the moment of cumulus cell expansion, which occurs subsequent to oocyte maturation 6 hours after the administration of equine luteinizing hormone (eLH) (Campos-Chillon, Farmerie, Bouma, Clay, & Carnevale, 2015). This allows *BMP15* to interact with *GDF9* and coordinate the expression of the genes involved in cumulus cells' expansion and proper follicular development (Lan et al., 2003).

In summary, the current study suggests that *BAX* relative transcript level is involved in the apoptotic cascade activation observed after our vitrification protocol using equine immature COCs. However, to obtain a complete gene expression profile and to better understand the mechanisms for regulating transcription and forming the mRNA stocks during oogenesis, more studies are needed that would evaluate the expression of proteins encoded by the genes in this study. However, it would be interesting to analyze the relationship between oocyte quality, follicle size, and apoptosis levels after IVM followed by ICSI fertilization rates, embryo development, pregnancy rates and the success of development to term in this species.

Conclusions

Based on the results, BAX relative transcript level is higher in VIT immature equine COCs. We also demonstrated that *BMP15* and *CASP3* were detected in VIT and Non-VIT COCs. These findings may provide insights for developing novel strategies to improve vitrification efficiency and offspring outcome via assisted reproductive techniques in the horse.

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