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Expression of apoptotic genes in immature and *in vitro* matured equine oocytes and cumulus cells

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Summary

The gene expression of Bax, Bcl-2, survivin and p53, following *in vitro* maturation of equine oocytes, was compared in morphologically distinct oocytes and cumulus cells. Cumulus–oocyte complexes (COC) were harvested and divided into two groups: G1 – morphologically healthy cells; and G2 – less viable cells or cells with some degree of atresia. Total RNA was isolated from both immature and *in vitro* matured COC and real-time reverse transcription polymerase chain reaction (qRT-PCR) was used to quantify gene expression. Our results showed there was significantly higher expression of survivin ($P < 0.05$) and lower expression of p53 ($P < 0.01$) in oocytes compared with cumulus cells in G1. No significant difference in gene expression was observed following *in vitro* maturation or in COC derived from G1 and G2. However, expression of the *Bax* gene was significantly higher in cumulus cells from G1 ($P < 0.02$).

Keywords: Apoptosis, Gene expression, *In vitro* maturation, qRT-PCR

Introduction

New knowledge about developmental competence, *in vitro* maturation (IVM) and cryopreservation of oocytes and embryos is critical for the *in vitro* production (IVP) of embryos. The techniques of assisted reproduction, *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) have been successfully performed with equine gametes, however low efficiency has limited their commercial application in this species (Squires *et al.*, 2003; Choi *et al.*, 2006; Hinrichs *et al.*, 2007). Progress in this area was initially slow as standard IVF is not reproducible in horses (Hinrichs, 2010). However, ICSI resulted in the birth of several foals, and has been used for the clinical production of foals (Galli *et al.*, 2007; Jacobson *et al.*, 2010; Mortensen *et al.*, 2010). Nevertheless, oocyte quality and culture conditions were found to be highly variable, with low levels of blastocyst formation *in vitro* and poor reproducibility between laboratories (Dell'Aquila *et al.*, 2003; Smits *et al.*, 2009).

The study of gene expression is emerging in applied embryology, those genes involved in biological processes such as apoptosis, oxidative stress and cryotolerance need to be studied in order to compare gene expression *in vivo, in vitro* and under different culture conditions (Wrenzycki *et al.*, 2005; Badr *et al.*, 2007; Rizos *et al.*, 2008). Oocyte quality can be assessed immediately following recovery by using several non-invasive, visual assessment parameters such as the morphology of the cumulus–oocyte complexes (COC). Oocytes with a compact cumulus composed of several layers of cells and a homogeneous cytoplasm are considered to be healthy; these oocytes can then be selected for IVM and IVF (Li *et al.*, 2009). However, it has been reported that equine oocytes with a compact cumulus exhibited a lower meiotic competence and lower fertilization rate after ICSI (Ambruosi *et al.*, 2009). Analysis of the expression of genes that are involved in early embryonic development is

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an important tool for the development of assisted reproduction biotechnology.

One of the main factors affecting the potential for embryonic development is apoptosis (Park *et al.*, 2006; Dhali *et al.*, 2007; Anguita *et al.*, 2009), or programmed cell death, which is an active and irreversible process of self-destruction of cells under physiological control (Parolin & Reason, 2001). The mechanisms of apoptosis may be activated by external stimuli, by binding to specific receptors on the cell surface, or by internal stimuli to intracellular stress, such as DNA damage, cell cycle alterations and in metabolic pathways and is controlled by several different gene families (Chang *et al.*, 2002). Previous studies on cumulus cell gene expression (McKenzie *et al.*, 2004) and apoptosis (Corn *et al.*, 2005) showed that cumulus cells can reflect the developmental potential of human embryos during IVF cycles. Previously, the apoptosis incidence in mare ovarian follicles, its relationship with cumulus expansion and increased meiotic competence was demonstrated, however, no association with cytoplasmic maturation was observed (Dell'Aquila *et al.*, 2003). The impact of apoptosis in the COC and its impact on oocyte development potential remains unclear, as contradictory reports from various species have failed to clarify the occurrence of apoptosis in the COC (Yuan *et al.*, 2005).

Thus, the aim of the current study was to evaluate the expression of the apoptosis-related genes *Bax*, *Bcl-2*, *survivin* and *p53* during IVM of equine oocytes and to compare gene expression between oocytes and cumulus cells isolated from COC with different morphological characteristics.

Materials and methods

Cumulus–oocyte complexes (COC) source

The biological material used in this experiment was obtained from a horse abattoir located in the city of Pelotas, RS, Brazil. The ovaries were collected randomly on the slaughter line, without identifying age, stage of the estrous cycle, clinical condition and nutritional status of mares. The time between slaughter and the collection was approximately 1 h, and the ovaries were transported in thermobottles in 0.9% NaCl sterile solution at 32–35°C to the Laboratory of Molecular Embryology, UFPel. The COC were aspirated from follicles ranging from 10 to 20 mm in diameter (follicles preceding follicle deviation). The contents were placed into a 50 ml conical tube and allowed to settle for 15 min. The sediment was evaluated using a stereomicroscope (Olympus) and suitable COC were selected. Degenerated or metaphase II (MII) stage oocytes were discarded.

Morphological characterization of the equine COC

The COC were evaluated morphologically using an inverted optical microscope (Olympus) for the number of layers and degree of compaction of the cumulus cells, cytoplasm homogeneity and integrity and were divided into two groups: G1 – considered morphologically healthy (oocytes with compact cumulus and more than three cell layers, intact cytoplasm, evenly granular and homogenous coloration); and G2 – considered morphologically less viable or with some degree of atresia (oocytes that presented less than three layers of cumulus cells and/or expanded cumulus, with cumulus granular appearance, heterogeneous or dense and/or shrunken cytoplasm).

In vitro maturation (IVM)

In vitro maturation of the oocytes was carried out in follicular fluid as described previously (Caillaud *et al.*, 2008). Briefly, equine follicular fluid was collected from follicles smaller than 30 mm, centrifuged at 14,000 rpm for 10 min, filtered through a 22 μm filter and heat-inactivated at 56°C for 30 min. For IVM, the COC were incubated for 36 h at 38.7°C and 5% CO_2. At the end of the incubation period the COC were denuded mechanically by repeated pipetting in a solution of 80 IU/ml of type-I hyaluronidase (Sigma-Aldrich). Groups of 60 oocytes and the corresponding cumulus cells were cryopreserved in 50 μl of TRIzol Reagent (Invitrogen) for RNA extraction and subsequent assessment of rates of gene expression by real-time reverse transcription PCR (qRT-PCR). As controls of the IVM process, the COC were classified into G1 and G2, denuded and total RNA was extracted immediately after collection. Nine hundred and sixty COC were divided among the experimental groups, four replicates were performed on pools of 60 COC.

To determine the stage of nuclear maturation of the oocytes upon collection and after IVM, 10% were assessed by staining. The maturation rate was determined by Hoechst 33342 staining (Sigma-Aldrich using a dye solution (10 μg/ml) and denuded oocytes were incubated for 10 min at 38.7°C. Slides were evaluated using an epifluorescence microscope (Olympus), with filter wavelength of BP330–385 nm. Immature oocytes were those oocytes presenting a germinal vesicle (GV) with a single condensed mass associated with the nucleolus; or germinal vesicle breakdown (GVBD) with an irregular envelope surrounding disperse condensed chromatin. Metaphase I (MI) oocytes were those presenting the first metaphase plate. In oocytes at MII the metaphase plate was located peripherally in the ooplasm and polar body in the perivitelline space. Oocytes with abnormal chromatin configurations or no chromatin visible were considered to be degenerating.

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Table 1 Primers used in qRT-PCR for equine oocytes

Gene	Accession number	Sequence	T_M (°C)	Fragment size (bp)	Efficiency (%)	Correlation (R^2)
Bcl-2 Eeq	XM_001499714.1	F 5′-GAGACCCCCAGTGCCATCAA-3′ R 5′-GGGATGTCAGGTCGCTGAAT-3′	57	146	99.9	0.97
BaxEq	XM_001489207.1	F 5′-TTTGCTTCAGGGTTTCATCC-3′ R 5′-ATCCTCTGCAGCTCCATGTT-3′	60	162	100	0.94
p53 Eq	XM_001918153.1	F 5′-AAAGGATGCCCATGCTACAGAGGA-3′ R 5′-AGTAGACTGGCCCTTCTTGGTCTT-3′	63	82	90.5	0.98
SurvivinEq	XM_001915400.1	F 5′-TTCATCCACTGTCCCACTGA-3′ R 5′-GTTCCTCTATGGGGTCGTCA-3′	57	98	70.5	0.98
GAPDH	NM_001163856.1	F 5′-GCCGTAACTTCTGTGCTGTG-3′ R 5′-AATGAAGGGGTCATTGATGG-3′	61	150	84.0	0.98

Total RNA isolation, reverse transcription and real-time PCR (qRT-PCR)

Total RNA was extracted from the COC stored in TRIzol as described by the manufacturer. The extracted RNA was quantified using the Qubit Fluorometer (Invitrogen), with the Quant-iT RNA BR Assay Kit following the manufacturer's instructions and standardized to a concentration of 4 ng/ml (equivalent to 60 oocytes). cDNA was produced using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. The cDNA was then used as template for the qRT-PCR, Stratagene Mx3005P Real-Time PCR System (Agilent Technologies), reaction using the Platinum Sybr Green Kit (Invitrogen). The primer pairs for the qRT-PCR were designed using Vector NTI 11 software (Invitrogen, USA) using the sequences for each of the target genes, Table 1. The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene (Smits *et al.*, 2009) was used for normalization of the target gene expression data.

Data processing and statistical analysis

Data processing and statistical analysis were performed using the software REST 2009. The rate of gene expression was calculated relative to the expression of the *GAPDH* gene, based on the method of Pfaffl and colleagues (Pfaffl *et al.*, 2002). The various groups were analysed using integrated randomization and bootstrapping methods to compare expression ratios, P-values ≤ 0.05 were considered to be significant.

Results

Differentiation and COC *in vitro* maturation

Some 1160 COC were recovered from 528 ovaries. Immediately after collection, 76% (76/100) of oocytes were at the germinal vesicle stage, 17% (17/100) were at the germinal vesicle breakdown stage and 7% (7/100) were at the degenerated stage. The COC were divided into two groups: G1 – considered morphologically healthy; and G2 – considered morphologically less viable or with some degree of atresia, see Fig. 1. The average rate of IVM was 51%, with a rate of 54% (27/50) for the G1 group and 48% (24/50) for the G2 group.

Relative expression of the *Bax, Bcl-2, survivin* and *p53* genes

Overall our results showed that survivin gene expression was significantly downregulated while p53 expression was significantly upregulated in cumulus cells in comparison with COC. Furthermore, p53 expression was higher in both immature and *in vitro* matured cumulus cells.

The survivin gene showed higher expression in *in vitro* matured oocytes from morphological group G1 than in cumulus cells from these oocytes ($P < 0.02$). The *p53* gene showed a lower expression in G1 *in vitro* matured oocytes compared to G1 *in vitro* matured cumulus cells ($P = 0.007$). However, the same relation was not observed between mature oocytes and cumulus cells mature G2. Figure 2 shows the data on survivin gene expression and the data on p53 gene expression in oocyte and cumulus cells of G1 and G2 groups. When the expression was compared between oocytes and cumulus cells according to morphology and maturation, differences for genes *Bcl-2* and *Bax* ($P > 0.05$) were not detected.

In this study, no difference was detected in the expression of apoptotic genes analysed in immature oocytes and after IVM ($P > 0.05$). In oocytes from different morphological groups, G1 and G2, there was no difference in Bcl-2, Bax, survivin and p53 gene expression in immature and *in vitro* maturated oocytes. Among the groups of oocytes according to morphological classification, G1 and G2, there was no difference in Bcl-2, Bax, survivin and p53 gene expression ($P > 0.05$).

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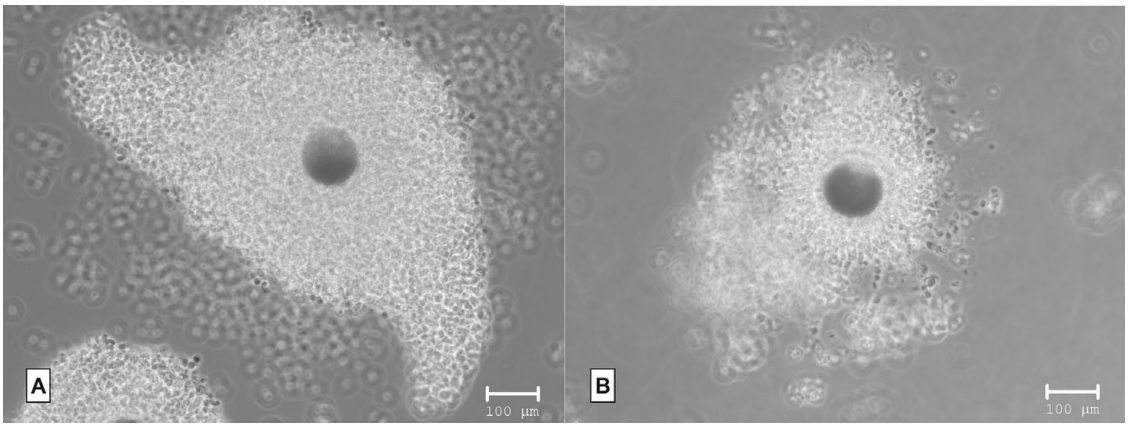

Figure 1 (*A*) Oocytes classified as healthy morphological group, G1. (*B*) Oocyte classified as morphological group with some signs of degeneration, G2.

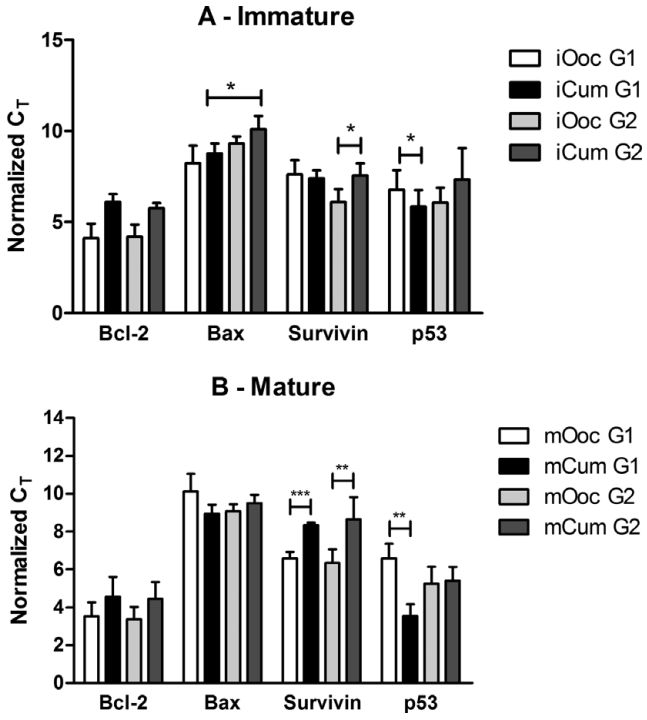

Figure 2 Normalized C_T values of the mRNA levels of the four apoptotic genes, *Bcl-2, Bax, survivin* and *p53*. (*A*) Immature oocytes and cumulus cells from groups G1 and G2. (*B*) *In vitro* matured oocytes and cumulus cells from groups G1 and G2. Significant differences are indicated $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. iCum: immature cumulus cells; iOoc: immature oocytes; mCum: matured cumulus cells; mOoc: matured oocytes.

Changes in gene expression of Bcl-2, survivin and p53 ($P > 0.05$) were not observed among cumulus cells. Between the morphological classification groups G1 and G2 there was observed difference in the expression of cumulus cells for the pro-apoptotic *Bax* gene. The expression of *Bax* was higher in cells of the morphological group G1 than G2 ($P = 0.02$) (Fig. 2).

Discussion

This study evaluated the expression of *Bax*, *Bcl-2*, *survivin* and *p53* genes during *IVM* of equine oocytes, to compare expression between different morphological parameters of oocytes and cumulus cells from these complexes. Gene expression studies in equine species are relatively scarce (Smits *et al.*, 2009), this was the first report of gene expression in equine COC, allowing us to elucidate the pattern of expression of apoptotic genes.

We observed different expression of anti-apoptotic gene *survivin* and pro-apoptotic gene *p53* between oocytes and cumulus cells. Equine morphologically viable oocytes expressed a higher rate of survivin and lower rate of p53 than cumulus cells. Gene expression in cumulus cells, including genes involved in the apoptotic process, provides evidence that embryo viability is reflected in differential gene expression in the cumulus cells (van Montfoort *et al.*, 2008). The bidirectional communication between the oocyte and companion somatic cells is essential for development of an egg competent to undergo fertilization and embryogenesis (Matzuk *et al.*, 2002). An increase in apoptotic cumulus cells has been related to a decrease of mature oocytes and to a decreased ability to be fertilized in stimulated IVF cycles (Host *et al.*, 2000). Our results indicated that the survivin and p53 proteins synthesized act on survival and early development of equine embryos.

Upon resumption of meiosis GVBD, oocytes become transcriptionally quiescent and must rely on pools of RNA accumulated during the growth phase for protein synthesis (Wassarman & Letourneau, 1976). In a study of oocytes and cumulus cells of bovine, ovine, porcine, canine, feline and murine, it was demonstrated that oocyte RNA features were repeatable whether maturation occurred *in vitro* or

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in vivo, and were similar between the phases of nuclear maturation of the germinal vesicle and MII oocytes (Payton *et al.*, 2010). The differences in features of total RNA from oocytes versus cumulus was reported, oocytes are contained within growing antral follicles which are in the large part transcriptionally inactive while cumulus cells are transcriptionally active. Furthermore, oocytes contain maternal pools of RNA, protein and energy stores that accumulated during the growth phase, a period during which features of oocyte RNA are more like somatic cells (Payton *et al.*, 2010).

Survivin expression was appointed as a possible marker for good quality *in vitro* developmental capacity of bovine follicular oocytes (Jeon *et al.*, 2008). The expression of survivin was related to the quality of COCs, their developmental competence and the quality of *in vitro* produced blastocysts in bovine (Jeon *et al.*, 2008). Survivin protein is known as a bifunctional protein that suppresses apoptosis and regulates cell division. Survivin acts as an inhibitor of apoptosis by linking directly to the caspases, it has been shown to inhibit the caspase 3 activity directly, preventing the formation of apoptosome (Kawamura *et al.*, 2003; Altieri, 2010).

The tumor suppressor gene *p53* is an important mediator in response to cellular stress (Dhali *et al.*, 2007). The transcriptional activity of p53 is required for cell death in some systems, its nuclear translocation is required for transcription of the gene *Bax* (Sabbatini *et al.*, 1995). However, p53 gene expression appears to have no correlation with morphological quality of bovine embryos (Melka *et al.*, 2009). A significantly higher expression of p53 from oocyte to four-cell stage as compared with that of the later pre-implantation stages was reported, suggesting p53-independent apoptosis in bovine embryos (Melka *et al.*, 2009). Equine oocytes are characterized by a large amount of lipid droplets in their cytoplasm, in association with the mitochondrion and smooth endoplasmic reticulum (Tremoleda *et al.*, 2003; Ambruosi *et al.*, 2009), suggesting that the oocytes and embryos of this species are more sensitive to oxidative stress and apoptosis (Ambruosi *et al.*, 2009). IVM of oocytes is an essential step for *in vitro* production of embryos, however the conditions under which the oocytes are subjected may result in changes in gene expression in these cells, as a response to injuries suffered.

Expression of the *Bax* gene has been identified in oocytes, granulosa cells and luteal cells of various species and levels of *Bax* expression appear to be positively correlated with apoptosis in each of these lineages. *Bax* and *p53* upregulation has been associated with apoptosis in granulosa cells (Zwain & Amato, 2001). In the morphological classification groups G1 and G2 difference in the expression of cumulus cells for the gene pro-apoptotic Bax was observed, the expression of Bax is higher in cells of the morphological group G1, and Bcl-2 has not changed. However, in the study by Filali *et al.* (2009) Bcl-2 mRNA expression was found to be significantly higher in cumulus cells associated with mature oocytes than those associated with immature oocytes, whereas Bax mRNA concentrations did not vary in cumulus cells from either source.

In this study, no difference was detected in the expression of apoptotic genes analysed in immature oocytes and after the IVM period, and in oocytes from different morphological groups, G1 and G2. There was no difference in gene expression of Bcl-2, Bax, survivin and p53 in immature oocytes and in *in vitro* maturated oocytes. Similar results were published comparing the expression levels of anti-apoptotic gene Bcl-2 in different morphological groups of bovine oocytes *in vivo* matured, and found no difference in the quantification of transcripts to this gene (Pretheeban *et al.*, 2009). The potential development of *in vitro* produced embryos depends mainly on the quality of oocytes from which they originate, with the selection of oocytes based primarily on morphological features. While, Yang & Rajamahendran (2002) reported higher levels of expression of the anti-apoptotic gene *Bcl-2* in oocytes considered morphologically healthy, Li *et al.* (2009) observed initial characteristics of apoptosis in immature COC, which was confirmed by qRT-PCR transcripts of Bax and Bcl-2 showing that the dynamic change in the transcriptional profile of Bax corresponded with the occurrence of apoptosis and early development of oocytes, while the pattern of Bcl-2 transcription showed a contrasting pattern.

In conclusion it was observed that equine morphologically viable oocytes expressed a greater rate of survivin and lower rate of p53 than cumulus cells. No difference was detected in the expression of apoptotic genes analysed in immature oocytes compared with after the IVM period, or in oocytes from different morphological groups, but higher expression of the pro-apoptotic gene *Bax* in G1 compared with the G2 group was observed in cumulus cells. In conclusion, other genes involved in cell survival and viability need to be studied, higher number of oocytes, corresponding to different stages of meiotic maturation, are targets for our subsequent studies. We believe that our data can contribute to identification of gene regulation during maturation and increase the knowledge of biology of the oocyte. It signals the progress in understanding the molecular events involved in communication and IVM of the COC, indicating possible markers of viability and competence.

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References

Altieri, D.C. (2010). Survivin and IAP proteins in cell-death mechanisms. *Biochem. J.* **430**, 199–205.

Ambruosi, B., Lacalandra, G.M., Iorga, A.I., De, S.T., Mugnier, S., Matarrese, R., Goudet, G. & Dell'Aquila, M.E. (2009). Cytoplasmic lipid droplets and mitochondrial distribution in equine oocytes: Implications on oocyte maturation, fertilization and developmental competence after ICSI. *Theriogenology* **71**, 1093–104.

Anguita, B., Paramio, M.T., Morato, R., Romaguera, R., Jimenez-Macedo, A.R., Mogas, T. & Izquierdo, D. (2009). Effect of the apoptosis rate observed in oocytes and cumulus cells on embryo development in prepubertal goats. *Anim. Reprod. Sci.* **116**, 95–106.

Badr, H., Bongioni, G., Abdoon, A.S., Kandil, O. & Puglisi, R. (2007). Gene expression in the *in vitro*-produced preimplantation bovine embryos. *Zygote* **15**, 355–67.

Caillaud, M., Dell'Aquila, M.E., De, S.T., Nicassio, M., Lacalandra, G.M., Goudet, G. & Gerard, N. (2008). *In vitro* equine oocyte maturation in pure follicular fluid plus interleukin-1 and fertilization following ICSI. *Anim. Reprod. Sci.* **106**, 431–9.

Chang, S.H., Cvetanovic, M., Harvey, K.J., Komoriya, A., Packard, B.Z. & Ucker, D.S. (2002). The effector phase of physiological cell death relies exclusively on the posttranslational activation of resident components. *Exp. Cell Res.* **277**, 15–30.

Choi, Y.H., Love, L.B., Varner, D.D. & Hinrichs, K. (2006). Blastocyst development in equine oocytes with low meiotic competence after suppression of meiosis with roscovitine prior to *in vitro* maturation. *Zygote* **14**, 1–8.

Corn, C.M., Hauser-Kronberger, C., Moser, M., Tews, G. & Ebner, T. (2005). Predictive value of cumulus cell apoptosis with regard to blastocyst development of corresponding gametes. *Fertil. Steril.* **84**, 627–33.

Dell'Aquila, M.E., Albrizio, M., Maritato, F., Minoia, P. & Hinrichs, K. (2003). Meiotic competence of equine oocytes and pronucleus formation after intracytoplasmic sperm injection (ICSI) as related to granulosa cell apoptosis. *Biol. Reprod.* **68**, 2065–72.

Dhali, A., Anchamparuthy, V.M., Butler, S.P., Pearson, R.E., Mullarky, I.K. & Gwazdauskas, F.C. (2007). Gene expression and development of mouse zygotes following droplet vitrification. *Theriogenology* **68**, 1292–8.

Filali, M., Frydman, N., Belot, M.P., Hesters, L., Gaudin, F., Tachdjian, G., Emilie, D., Frydman, R. & Machelon, V. (2009). Oocyte *in-vitro* maturation: BCL2 mRNA content in cumulus cells reflects oocyte competency. *Reprod. Biomed. Online* **19**, 4309.

Galli, C., Colleoni, S., Duchi, R., Lagutina, I. & Lazzari, G. (2007). Developmental competence of equine oocytes and embryos obtained by *in vitro* procedures ranging from *in vitro* maturation and ICSI to embryo culture, cryopreservation and somatic cell nuclear transfer. *Anim. Reprod. Sci.* **98**, 39–55.

Hinrichs, K. (2010). *In vitro* production of equine embryos: state of the art. *Reprod. Domest. Anim* **45**, 3–8.

Hinrichs, K., Choi, Y.H., Walckenaer, B.E., Varner, D.D. & Hartman, D.L. (2007). *In vitro*-produced equine embryos: production of foals after transfer, assessment by differential staining and effect of medium calcium concentrations during culture. *Theriogenology* **68**, 521–9.

Host, E., Mikkelsen, A.L., Lindenberg, S. & Smidt-Jensen, S. (2000). Apoptosis in human cumulus cells in relation to maturation stage and cleavage of the corresponding oocyte. *Acta Obstet. Gynecol. Scand.* **79**, 936–40.

Jacobson, C.C., Choi, Y.H., Hayden, S.S. & Hinrichs, K. (2010). Recovery of mare oocytes on a fixed biweekly schedule, and resulting blastocyst formation after intracytoplasmic sperm injection. *Theriogenology* **73**, 1116–26.

Jeon, K., Kim, E.Y., Tae, J.C., Lee, C.H., Lee, K.S., Kim, Y.O., Jeong, D.K., Cho, S.K., Kim, J.H., Lee, H.Y., Riu, K.Z., Cho, S.G. & Park, S.P. (2008). Survivin protein expression in bovine follicular oocytes and their *in vitro* developmental competence. *Anim. Reprod. Sci.* **108**, 319–33.

Kawamura, K., Sato, N., Fukuda, J., Kodama, H., Kumagai, J., Tanikawa, H., Nakamura, A., Honda, Y., Sato, T. & Tanaka, T. (2003). Ghrelin inhibits the development of mouse preimplantation embryos *in vitro*. *Endocrinology* **144**, 2623–33.

Li, H.J., Liu, D.J., Cang, M., Wang, L.M., Jin, M.Z., Ma, Y.Z. & Shorgan, B. (2009). Early apoptosis is associated with improved developmental potential in bovine oocytes. *Anim. Reprod. Sci.* **114**, 89–98.

Matzuk, M.M., Burns, K.H., Viveiros, M.M. & Eppig, J.J. (2002). Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science* **296**, 2178–80.

McKenzie, L.J., Pangas, S.A., Carson, S.A., Kovanci, E., Cisneros, P., Buster, J.E., Amato, P. & Matzuk, M.M. (2004). Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. *Hum. Reprod.* **19**, 2869–2874.

Melka, M., Rings, F., Holker, M., Tholen, E., Havlicek, V., Besenfelder, U., Schellander, K. & Tesfaye, D. (2009). Expression of apoptosis regulatory genes and incidence of apoptosis in different morphological quality groups of *in vitro*-produced bovine pre-implantation embryos. *Reprod. Domest. Anim.* **45**, 915–21.

Mortensen, C.J., Choi, Y.H., Ing, N.H., Kraemer, D.C., Vogelsang, M.M. & Hinrichs, K. (2010). Heat shock protein 70 gene expression in equine blastocysts after exposure of oocytes to high temperatures *in vitro* or *in vivo* after exercise of donor mares. *Theriogenology* **74**, 374–83.

Park, S.Y., Kim, E.Y., Cui, X.S., Tae, J.C., Lee, W.D., Kim, N.H., Park, S.P. & Lim, J.H. (2006). Increase in DNA fragmentation and apoptosis-related gene expression in frozen–thawed bovine blastocysts. *Zygote.* **14**, 125–31.

Parolin, M.B. & Reason, I.J. (2001). [Apoptosis as a mechanism of tissue injury in hepatobiliary diseases]. *Arq. Gastroenterol.* **38**, 138–44.

Payton, R.R., Rispoli, L.A. & Edwards, J.L. (2010). General features of certain RNA populations from gametes and cumulus cells. *J. Reprod. Dev.* **56**, 583–92.

Pfaffl, M.W., Horgan, G.W. & Dempfle, L. (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* **30**, e36.

Pretheeban, T., Gordon, M., Singh, R., Perera, R. & Rajamahendran, R. (2009). Differential mRNA expression in *in vivo* produced pre-implantation embryos of dairy heifers and mature cows. *Mol. Reprod. Dev.* **76**, 1165–72.

Rizos, D., Clemente, M., Bermejo-Alvarez, P., de La, F.J., Lonergan, P. & Gutierrez-Adan, A. (2008). Consequences of *in vitro* culture conditions on embryo development and quality. *Reprod. Domest. Anim.* **43**, 44–50.

Sabbatini, P., Lin, J., Levine, A.J. & White, E. (1995). Essential role for p53-mediated transcription in E1A-induced apoptosis. *Genes Dev.* **9**, 2184–92.

Smits, K., Goossens, K., Van, S.A., Govaere, J., Hoogewijs, M., Vanhaesebrouck, E., Galli, C., Colleoni, S., Vandesompele, J. & Peelman, L. (2009). Selection of reference genes for quantitative real-time PCR in equine *in vivo* and fresh and frozen–thawed *in vitro* blastocysts. *BMC. Res. Notes* **2**, 246.

Squires, E.L., Carnevale, E.M., McCue, P.M. & Bruemmer, J.E. (2003). Embryo technologies in the horse. *Theriogenology* **59**, 151–70.

Tremoleda, J.L., Stout, T.A., Lagutina, I., Lazzari, G., Bevers, M.M., Colenbrander, B. & Galli, C. (2003). Effects of *in vitro* production on horse embryo morphology, cytoskeletal characteristics, and blastocyst capsule formation. *Biol. Reprod.* **69**, 1895–906.

van Montfoort, A.P., Geraedts, J.P., Dumoulin, J.C., Stassen, A.P., Evers, J.L. & Ayoubi, T.A. (2008). Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis. *Mol. Hum. Reprod.* **14**, 157–68.

Wassarman, P.M. & Letourneau, G.E. (1976). RNA synthesis in fully grown mouse oocytes. *Nature* **261**, 73–4.

Wrenzycki, C., Herrmann, D., Lucas-Hahn, A., Korsawe, K., Lemme, E. & Niemann, H. (2005). Messenger RNA expression patterns in bovine embryos derived from *in vitro* procedures and their implications for development. *Reprod. Fertil. Dev.* **17**, 23–35.

Yang, M.Y. & Rajamahendran, R. (2002). Expression of Bcl-2 and Bax proteins in relation to quality of bovine oocytes and embryos produced *in vitro*. *Anim. Reprod. Sci.* **70**, 159–69.

Yuan, Y.Q., Van, S.A., Leroy, J.L., Dewulf, J., Van, Z.A., de, K.A. & Peelman, L.J. (2005). Apoptosis in cumulus cells, but not in oocytes, may influence bovine embryonic developmental competence. *Theriogenology* **63**, 2147–63.

Zwain, I.H. & Amato, P. (2001). cAMP-induced apoptosis in granulosa cells is associated with up-regulation of P53 and Bax and down-regulation of clusterin. *Endocr. Res.* **27**, 233–49.