Zygote

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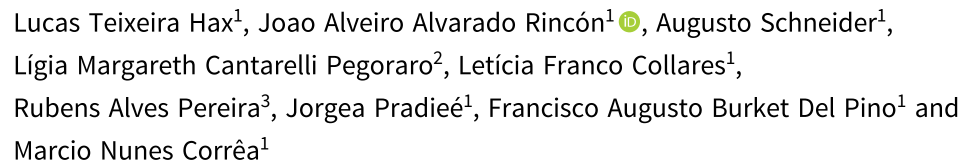
Effect of butafosfan supplementation during oocyte maturation on bovine embryo development



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Research Article

Cite this article: Teixeira Hax L *et al.* (2019) Effect of butafosfan supplementation during oocyte maturation on bovine embryo development. *Zygote* **27**: 321–328. doi: 10.1017/S0967199419000327

Received: 5 October 2018
Revised: 11 March 2019
Accepted: 8 May 2019
First published online: 15 August 2019

Keywords:
Blastocyst; Cattle; Gene expression; Oocyte competence; Phosphorus.

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Summary

Around 60–80% of oocytes maturated *in vivo* reached competence, while the proportion of maturation *in vitro* is rarely higher than 40%. In this sense, butafosfan has been used *in vivo* to improve metabolic condition of postpartum cows, and can represent an alternative to increase reproductive efficiency in cows. The aim of this study was to evaluate the addition of increasing doses of butafosfan during oocyte maturation *in vitro* on the initial embryo development in cattle. In total, 1400 cumulus–oocyte complexes (COCs) were distributed in four groups and maturated according to supplementation with increasing concentrations of butafosfan (0 mg/ml, 0.05 mg/ml, 0.1 mg/ml and 0.2 mg/ml). Then, 20 oocytes per group were collected to evaluate nuclear maturation and gene expression on cumulus cells and oocytes and the remaining oocytes were inseminated and cultured until day 7, when blastocysts were collected for gene expression analysis. A dose-dependent effect of butafosfan was observed, with decrease of cleavage rate and embryo development with higher doses. No difference between groups was observed in maturation rate and expression of genes related to oocyte quality. Our results suggest that butafosfan is prejudicial for oocytes, compromising cleavage and embryo development.

Introduction

In vitro production (IVP) of bovine embryos is a reproductive technique that allows improving the genetic gain by reducing the interval between generations and increasing offspring of individuals of high genetic merit (Andrade *et al.*, 2002). However, advances in IVP are compromised due to only 25–40% of *in vitro* maturated oocytes reaching competence, while rates of 60–80% are observed *in vivo* (Farin *et al.*, 2007). This difference is attributed to differences in the maturation process *in vitro* and *in vivo* (Combelles *et al.*, 2002). During maturation, oocytes should complete meiosis, chromosome segregation and cytoplasmic rearrangement (Ferreira *et al.*, 2009) to achieve competence and reach the blastocyst stage (Luciano *et al.*, 2011). Despite *in vitro* maturation (IVM) offering support to nuclear maturation, cytoplasmic maturation is often compromised (Combelles *et al.*, 2002).

Oocyte maturation is regulated by different processes including phosphorylation and dephosphorylation of proteins and cellular signalling molecules (Thach, 1992; Colgan *et al.*, 1996; Gavin and Schorderet-Slatkine, 1997). Moreover, ATP concentration is crucial for formation of the meiotic spindle (Zhang *et al.*, 2006), calcium oscillation (Dumollard *et al.*, 2006) and polar body extrusion during oocyte maturation (Stojkovic *et al.*, 2001; Hao *et al.*, 2009). Intracellular levels of ATP are associated with mitochondrial reorganization and total cell number in blastocysts. Embryos with low ATP concentrations have a slower development and have less cell numbers compared with embryos with higher ATP levels (Stojkovic *et al.*, 2001). Therefore, the use of supplements to improve mitochondrial metabolism and ATP levels during IVM represents an alternative to obtain better efficiency during IVP.

Butafosfan is an organic phosphorus molecule that has been used as a metabolic modulator in dairy cows (Pereira *et al.*, 2013), and may represent an alternative to improve *in vitro* oocyte competence. Some field reports have suggested beneficial results from butafosfan injection in cattle- and horse-assisted reproduction programmes, although no scientific evidence can be found in the published literature. When associated with cyanocobalamin, butafosfan increased the number of small follicles in cows (Lima *et al.*, 2017). Phosphorus is also essential for synthesis of nucleotides related to hormonal signalling (Cunningham, 2002) and for growth, differentiation and cellular integrity (Berg *et al.*, 2006). ATP concentration is higher in muscle and liver tissue of rats treated with butafosfan (Hasi Su-rong *et al.* (2004). Based on these

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Table 1. Primer sequence used for gene expression analysis of cumulus cells, oocyte and embryos by RT-qPCR

Gene	Primers sequence (5′–3′)	Fragment length (bp)	Reference
H2AFZ	F: GAGGAGCTGAACAAGCTGTTG	104	Portela *et al.* (2010)
	R: TTGTGGTGGCTCTCAGTCTTC		
BCL2	F: GAGTTCGGAGGGGTCATGTG	203	Boruszewska *et al.* (2015)
	R: GCCTTCAGAGACAGCCAGGA		
BAX	F: GTGCCCGAGTTGATCAGGAC	126	Boruszewska *et al.* (2015)
	R: CCATGTGGGTGTCCCAAAGT		
PLAC8	F: TTTACCGCTCTGTGCCCTTT	95	Boruszewska *et al.* (2015)
	R: CCATGTGAACTTGACCAAGCAT		
AREG	F: CTTTCGTCTCTGCCATGACCTT	100	Boruszewska *et al.* (2015)
	R: CGTTCTTCAGCGACACCTTCA		
EREG	F: TCACCGCGAGAAGGATGGAG	73	Boruszewska *et al.* (2015)
	R: GTACTGAAGACCAGGACGAGC		
SLC2A1	F: GATCCACAGAGCGCAGCC	90	Boruszewska *et al.* (2015)
	R: TGTCAGCTTCTTGCTGGTGG		
PFKP	F: TCAGAGAACCGTGCCTGGAAGAAA	112	Boruszewska *et al.* (2015)
	R: TGACCACAAGCTCCTTGATCTGCT		
GDF9	F: AGCGCCCTCACTGCTTCTATAT	80	Hosoe *et al.* (2011)
	R: TTCCTTTTAGGGTGGAGGGAA		
BMP15	F: ATCATGCCATCATCCAGAACC	72	Hosoe *et al.* (2011)
	R: TAAGGGACACAGGAAGGCTGA		

findings, the aim of this study was to evaluate the addition of increasing doses of butafosfan during oocyte maturation *in vitro* on the initial embryo development in cattle.

Materials and methods

Collection and selection of cumulus–oocyte complexes (COCs)

Bovine ovaries were collected from a local slaughterhouse and transported to the laboratory in NaCl 0.9% solution with gentamicin 0.5% at 30ºC. COCs were aspirated from follicles (3–8 mm in diameter) and COC grades 1 and 2 were selected according to Leibfried and First (1979). All culture media were purchased commercially (Biotecnologia Animal®, Brasília, DF, Brazil).

Experimental design and in vitro *maturation (IVM)*

Selected COCs were distributed randomly into four groups (60 COCs/group) according to butafosfan supplementation during IVM, in total six replicates ($n = 1400$ COCs). Butafosfan concentrations were determined through the available phosphorus blood levels of animals receiving butafosfan (Bayer Saúde Animal®, São Paulo, SP, Brazil) according to the manufacturer's recommendations (unpublished data). COCs were matured in 500 µl IVM medium (TCM199 supplemented with 0.2 mM sodium pyruvate, 0.2 µl/ml FSH, 75 µl/ml amikacin, 0.1 µl/ml estradiol and 10% of fetal bovine serum) supplemented with 0 mg/ml, 0.05 mg/ml, 0.1 mg/ml or 0.2 mg/ml of butafosfan (Bayer Saúde Animal®). IVM was performed at 39°C in an atmosphere of 5% CO_2 in air for 24 h.

In vitro *fertilization and* in vitro *culture*

In vitro fertilization (IVF) was performed with semen obtained from the same ejaculate from a *Bos taurus* bull. Semen was thawed at 35°C for 30 s and sperm selection was performed using a mini Percoll density gradient method (Parrish *et al.*, 1995). COCs (50 oocytes/group) were inseminated with sperm at the concentration of 1×10^6 cells/ml. IVF occurred in 400-µl drops of TALP-IVF medium supplemented with 6 mg/ml bovine serum albumin (BSA)-FAF, 0.2 mM sodium pyruvate, 30 µg/ml heparin, 20 µM penicillamine, 10 µM hypotaurine, 1 µM epinephrine and 75 µg/ml amikacin, at 39°C in an atmosphere of 5% CO_2 in air for 20 h.

After IVF, presumptive zygotes were denuded from cumulus cells by repeated pipetting. Denuded oocytes were transferred for 200-µl drops of *in vitro* culture (IVC) medium (SOFaa supplemented with 5% fetal bovine serum, 2.7 mM of myo-inositol, 0.2 mM sodium pyruvate, 5 mg/ml BSA and 75 µg/ml amikacin) under mineral oil. The culture was conducted at 39°C in an atmosphere of 5% CO_2 in air for 7 days after IVF (Day 0). After 72 h (Day 3) and 120 h (Day 5), 50% of the medium for IVC was replaced with fresh medium. The cleavage rate was evaluated at Day 2 and the blastocysts rate was recorded at Day 7 based on the number of inseminated oocytes.

Analysis of nuclear maturation

After IVM, five COCs/group were denuded by repeated pipetting, washed three times with phosphate-buffered saline (PBS) and fixed for 15 min in 4% paraformaldehyde. Then, a solution of PBS with

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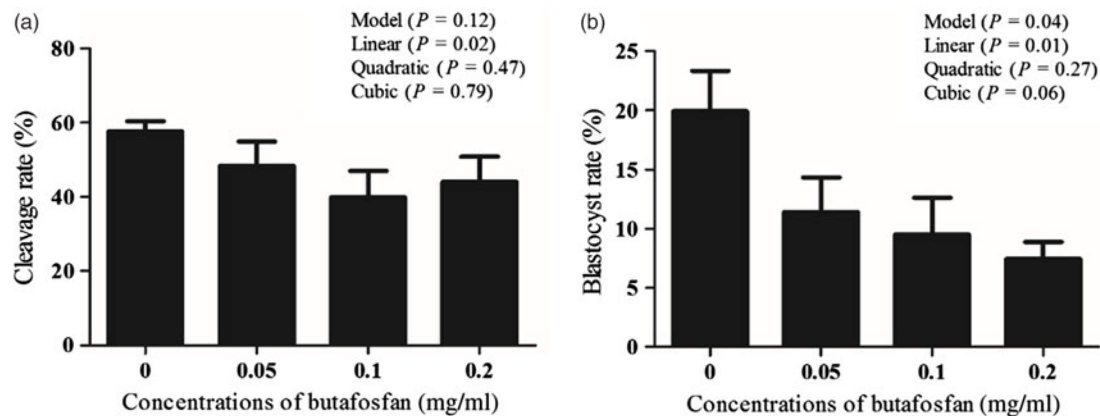

Figure 1. Cleavage rate D2 (*a*) and blastocyst rate on D7 (*b*) derived from oocytes supplemented with different doses of butafosfan during *in vitro* maturation.

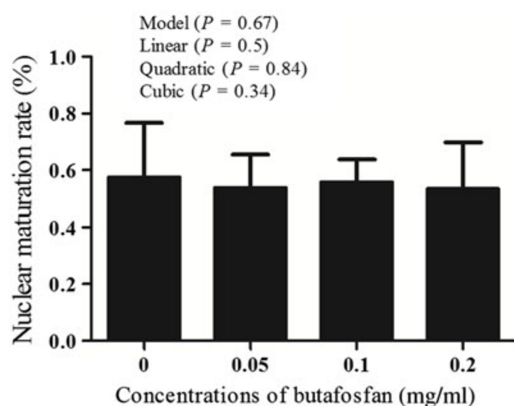

Figure 2. Nuclear maturation rate of oocytes analyzed by Hoechst staining after *in vitro* maturation with different doses of butafosfan supplementation.

0.5% Triton X-100 was used for short-term storage. These oocytes were stained in PBS supplemented with 15 µl/ml Hoechst 33342 stain (Sigma-Aldrich®, St. Louis, MO, USA), fixed in slides and evaluated using an inverted fluorescence microscope IX 71 (Olympus®, Shinjuku-ku, Tokyo, Japan), with ultraviolet (UV) light filters of 330–385 nm. Oocytes with polar body extrusion were classified as matured, indicating the metaphase II stage, while those with other chromatin configurations were classified as non-matured. In total 120 oocytes were analyzed (30 oocytes/group).

Analysis of gene expression

After IVM, 15 COCs/group were denuded by repeated pipetting. Oocytes were removed from the drop and cumulus cells were recovered by centrifugation. Oocytes and cumulus cells were transferred separately to microtubes containing 100 µl TRIzol (Invitrogen®, Carlsbad, CA, USA), homogenized and stored at −70°C. In total six replicates were performed, 360 oocytes in total for RNA extraction. In addition, at Day 7, all embryos were also collected and stored in 100 µl TRIzol (Invitrogen®) at −70°C, six replicates in total.

Total RNA was extracted from cumulus cells, oocytes and embryos using TRIzol reagent (Invitrogen®), according to the manufacturer's protocol. RNA concentration was measured on a spectrophotometer (NanoVue, Healthcare®, Amersham, UK) the 260/280 nm ratio of absorbance was determined. Reverse transcription was performed with total RNA in a 20-µl volume using the iScript Synthesis kit (Bio-Rad®, Hercules, CA, USA) according to the manufacturer's instructions.

Real-time polymerase chain reaction (PCR) reactions were run on an Applied Biosystems 7500 RT-PCR (Applied Biosystems®, Foster City, CA, USA) using SYBR Green PCR Master Mix (Applied Biosystems®) according to the manufacturer's instructions. H2A histone family, member Z gene (*H2AFZ*) used as the endogenous control for all evaluated structures.

The expression target genes was evaluated as follows: BCL2 associated X protein (*BAX*) and apoptosis regulator Bcl-2 (*BCL2*) as apoptosis markers; amphiregulin (*AREG*) and epiregulin (*EREG*) as genes related to cumulus cells expansion and resumption of meiosis; growth differentiation factor 9 (*GDF9*) and bone morphogenetic protein 15 (*BMP15*) as indicators of oocyte quality; facilitated glucose transporter member 1 (*SLC2A1*) and phosphofructokinase, platelet (*PFKP*) for analysis of energy metabolism in oocytes; and placenta-specific 8 (*PLAC8*) as indicator of embryo implantation. The coefficient of variation was less than 5% for all pairs of primers used (Table 1). Relative expression was calculated using the $2^{A-B}/2^{C-D}$ equation as described by Campos *et al.* (2017).

Statistical analysis

Analyses were carried out using SAS 9.0 software (SAS, Cary, NC, EUA) and the General Linear Model approach. The polynomial regression model was used to determine the linear, quadratic or cubic effect of supplementation with 0.0, 0.05, 0.1 and 0.2 mg/ml of butafosfan on cleavage and blastocysts rates. The same analysis was performed to determine the effect on the expression of genes *BAX*, *BCL2*, *AREG*, *EREG*, *GDF9*, *BMP15*, *SLC2A1*, *PFKP* in cumulus cells and oocytes, *BAX*, *BCL2* e *PLC8* in embryos, and the *BAX/BCL2* relationship in cumulus cells, oocytes and embryos. Results are shown as mean ± standard error of the mean.

Results

Increased butafosfan doses during IVM resulted in a reduction of cleavage and embryo development. The cleavage rate (%) was 57.67 ± 2.7, 48.38 ± 6.6, 39.92 ± 7.1 and 44.06 ± 6.8 and the blastocyst rate (%) was 19.93 ± 3.4, 11.43 ± 2.9, 9.52 ± 3.1 and 7.45 ± 1.4 in the groups supplemented with 0, 0.05, 0.1 and 0.2 mg/ml of butafosfan, respectively. A linear effect of butafosfan addition during IVM was observed for cleavage rate ($P = 0.02$;

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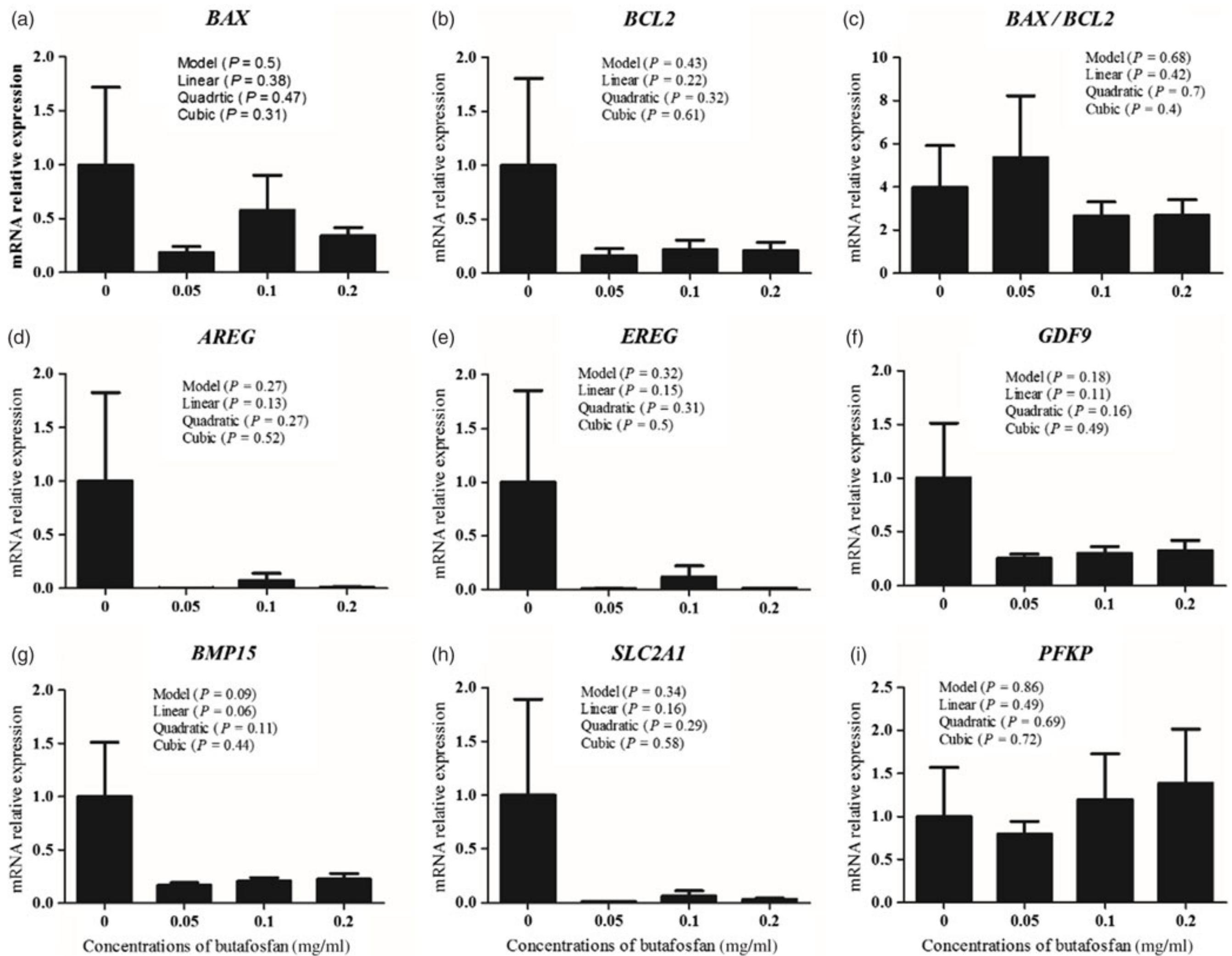

Figure 3. Relative expression of genes *BAX* (a), *BCL2* (*b*), *AREG* (*d*), *EREG* (*e*), *GDF9* (*f*), *BMP15* (g), *SLC2A1* (h), *PFKP* (i) and *BAX/BCL2* (c), in oocytes supplemented with different doses of butafosfan during *in vitro* maturation. Determined by real-time PCR using *H2AFZ* gene as internal control.

Fig. 1a) and blastocyst rate ($P = 0.01$; Fig. 1b). Nuclear maturation rate (%) of oocytes supplemented with 0, 0.05, 0.1 and 0.2 mg/ml of butafosfan was not different between groups (57.50 ± 19.2, 53.75 ± 11.7, 55.67 ± 8.1 and 53.40 ± 16.5, respectively, $P > 0.05$; Fig. 2).

Relative expression of the genes *BAX*, *BCL2*, *AREG*, *EREG*, *GDF9*, *BMP15*, *SLC2A1*, *PFKP* and *BAX/BCL2* in oocytes (Fig. 3) and cumulus cells (Fig. 4) was not different among groups ($P > 0.05$). In addition, no difference among groups was observed for relative expression of genes *BAX*, *BCL2* e *PLC8* and *BAX/BCL2* in Day 7 embryos ($P > 0.05$; Fig. 5).

Discussion

In the present study, we observed that increasing doses of butafosfan in IVM linearly decreased the cleavage rate. The same effect was observed for embryo development, which was lower as the concentration of butafosfan increased, suggesting that butafosfan is detrimental for oocyte maturation. It was proposed that butafosfan may interfere with phosphorylation of molecules involved in cellular signalling (Tabeleão *et al.*, 2017). To achieve competence, oocytes need to complete nuclear and cytoplasmic maturation (Ferreira *et al.*, 2009), a process dependent on several signalling pathways, such as promoting maturation factor (PMF) and regulated by different kinases and phosphatases (Belloc *et al.*, 2008).

Our results do not indicate any difference in oocyte maturation rate in groups supplemented with butafosfan during IVM, suggesting that its deleterious effects on cleavage rate and embryo development are not associated with nuclear maturation. Cytoplasmic maturation comprises the rearrangement of cytoplasmic organelles (Yamada and Isaji, 2011), increase of lipid content and reduction of the Golgi complex, leading the oocyte to interrupt transcription by nucleolus condensation (Sirard and Coenen, 2006; Wang and Sun, 2007). This process gives the oocyte the ability to prevent polyspermia, to allow decondensation of the sperm in the ooplasm and to maintain embryonic development in the early stages (van den Hurk and Zhao, 2005). Therefore, cytoplasmic maturation results in a cellular rearrangement that enables the oocyte to be fertilized (Ferreira *et al.*, 2009). Thereby, butafosfan may have affected cytoplasmic maturation, negatively affecting competence acquisition process. However, this study is the first using butafosfan during oocyte maturation, so doses tested here may not represent the best for beneficial potential of the butafosfan molecule *in vitro*.

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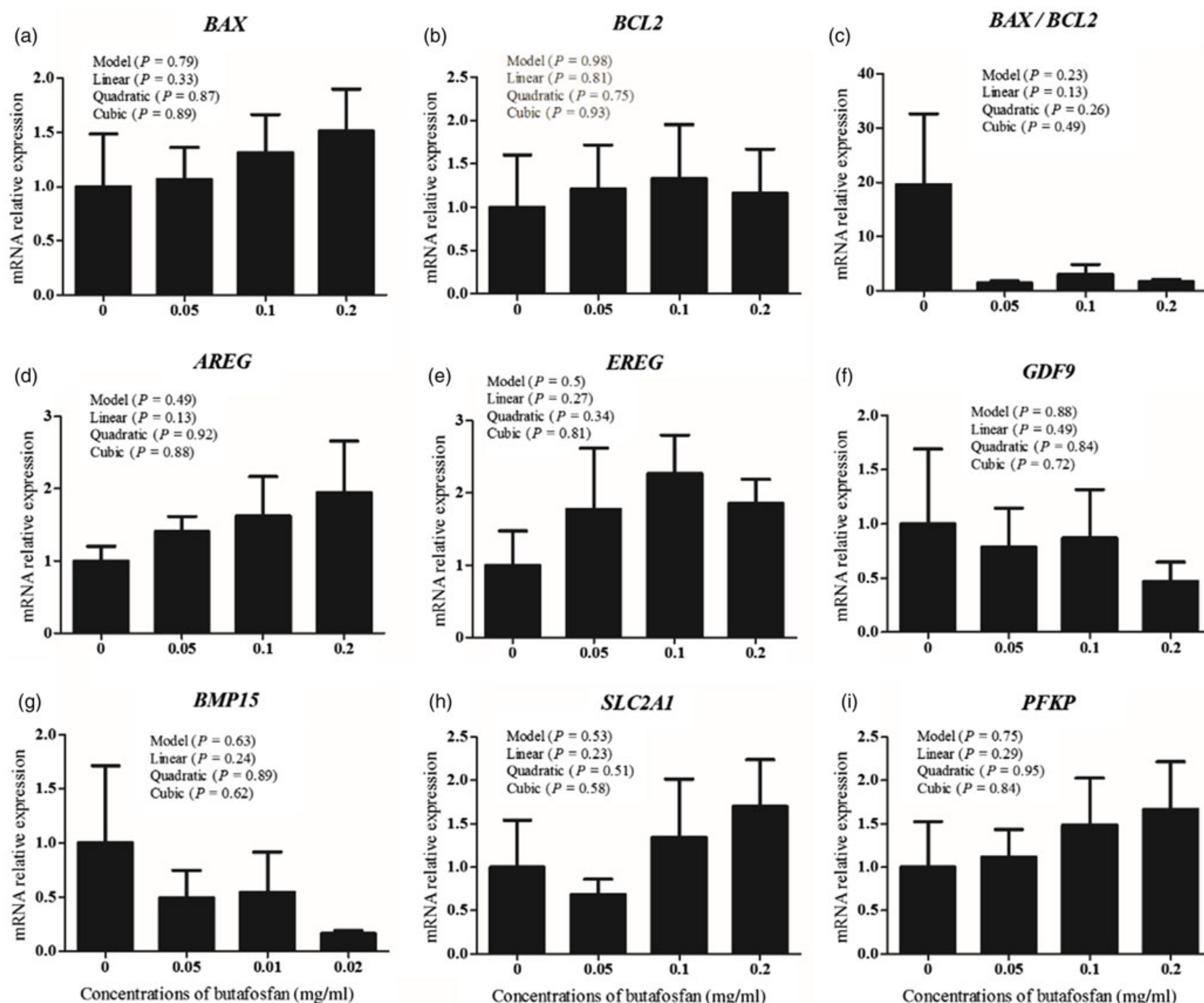

Figure 4. Relative expression of genes *BAX* (*a*), *BCL2* (*b*), *AREG* (*d*), *EREG* (*e*), *GDF9* (*f*), *BMP15* (*g*), *SLC2A1* (*h*), *PFKP* (*i*) and *BAX/BCL2* (*c*) in cumulus cells derived from oocytes supplemented with different doses of butafosfan during *in vitro* maturation. Determined by real-time PCR using *H2AFZ* gene as internal control.

Cumulus cells play a crucial role in oocyte maturation and competence acquisition (Tanghe *et al*., 2002). In our study, the effect of butafosfan on the expansion of cumulus cells was also evaluated by gene expression analysis. *AREG* and *EREG* play a central role in cumulus cell expansion (Ashkenazi *et al*., 2005; Park *et al*., 2004). These genes translate into proteins responsible for mediating the effect of luteinizing hormone (LH) on cumulus cells (Ashkenazi *et al*., 2005). However, no change was observed in relative expression of *AREG* and *EREG* in groups with lower cleavage rates and embryo development. Conversely, Boruszewska *et al*. (2015) reported an increase in relative expression of these genes in cumulus cells of oocytes maturated in medium supplemented with lysophosphatidic acid and a higher rate of embryo development. As we observed no effect on the level of transcription, it is possible that butafosfan acts post-transcriptionally or independent of these traditional markers of oocyte competence.

The effect of butafosfan in apoptosis of cumulus cells, oocytes and embryos was evaluated by relative expression analysis of *BAX* and *BCL2* genes and the *BAX/BCL2* ratio. The *BAX/BCL2* ratio is associated with oocyte and embryo quality (Yang and Rajamahendran, 2002) and higher mRNA levels of *BCL2*, an anti-apoptotic gene, in cumulus cells are associated with an increased embryo quality (Assou *et al*., 2008). However, when groups with higher cleavage rates and embryo development were compared with those with lower rates, no difference was observed in expression of *BCL2* and *BAX*, a pro-apoptotic gene, or in the *BAX/BCL2* relationship for cumulus cells, oocytes or embryos, suggesting that treatment with butafosfan did not increase apoptosis of these cells. In another study, IVM was supplemented with recombinant paraoxanase-1, a higher blastocyst rate was observed when compared with the control group, however no difference in the *BAX/BCL2* relationship was observed in cumulus cells, oocytes or embryos (Rincon *et al*., 2016). Therefore, the deleterious effects of butafosfan might not be directly related to the apoptotic pathway.

Expression of genes related to oocyte quality was also evaluated by real-time PCR. Several studies have demonstrated that *GDF9* and *BMP15* are markers for oocyte quality (Gendelman *et al*., 2010; Hussein *et al*., 2006; Gendelman and Roth, 2012). *GDF9* and *BMP15* regulate the crosstalk between oocytes and granulosa

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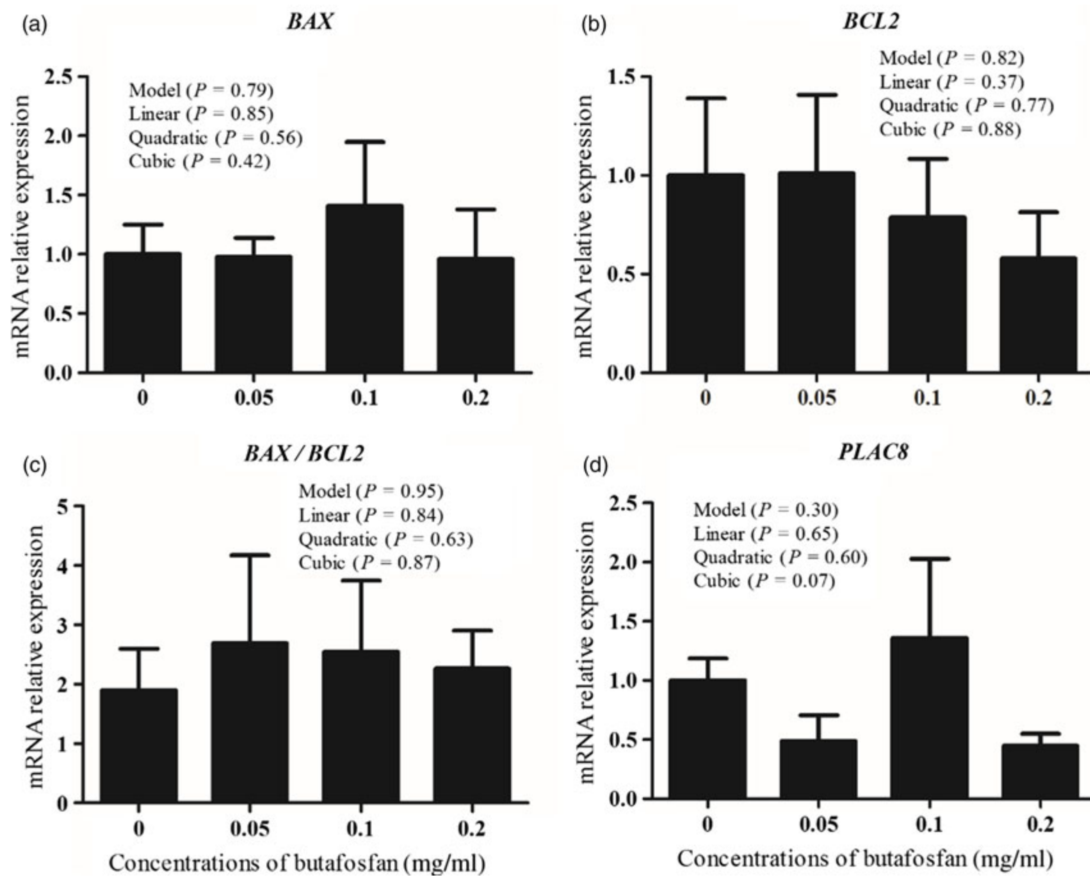

Figure 5. Relative expression of genes *BAX* (*a*), *BCL2* (*b*), *PLAC8* (*d*) and *BAX/BCL2* (*c*) in embryos derived from oocytes supplemented with different doses of butafosfan during *in vitro* maturation. Determined by real-time PCR using *H2AFZ* gene as internal control.

cells, as well as the metabolism of cumulus cells (Su *et al.*, 2008). *GDF9* expression is higher in oocytes with earlier cleavage than in oocytes with later cleavage (Gendelman *et al.*, 2010). However, we did not observe any significant difference in expression of these genes in butafosfan treated groups. Therefore, it is possible that there are other factors regulating the expression of *GDF9* and *BMP15* that are not directly associated with oocyte quality.

Genes involved in glucose metabolism were also evaluated in this study. Glucose is an important energetic substrate for cumulus cells expansion, oocyte maturation and embryonic development (Rose-Hellekant *et al.*, 1998). *SLC2A1* is the most abundant passive glucose transporter and its expression is detected in oocytes and embryos (Augustin *et al.*, 2001). During IVM, *SLC2A1* promotes the passive transport of glucose to COCs. Cumulus cells are responsible for glucose metabolism and transfer of pyruvate to the oocyte through gap junctions (Cetica *et al.*, 1999). In cumulus cells, glycolysis is mediated by the PFKP enzyme (Cetica *et al.*, 2002). Oocytes matured in the presence of lysophosphatidic acid had a high maturation rate and reduced apoptosis, in addition to a high expression of *SLC2A1* and *PFKP*, suggesting that the level of expression of these genes is associated with oocyte and embryonic quality (Boruszewska *et al.*, 2015). Studies have described that butafosfan increases ATP synthesis, improving the intracellular energetic status (Furll *et al.*, 2010; Rollin *et al.*, 2010; Pereira *et al.*, 2013). However, we did not observe any significant difference in *SLC2A1* and *PFKP* expression in cumulus cells and oocytes treated with different concentrations of butafosfan during IVM. Therefore, it is likely that the deleterious effect of butafosfan is not mediated by changes in energy metabolism.

In the present study, the effect of butafosfan on the improvement of embryo–maternal signalling ability was evaluated through expression analysis of *PLAC8*. *PLAC8* plays an important role in embryo–maternal signalling (Rekik *et al.*, 2011). Blastocysts produced *in vitro* that result in pregnancy have a higher expression of *PLAC8* (El-Sayed *et al.*, 2006). Despite that, no difference was observed in *PLAC8* mRNA levels in groups treated with butafosfan, indicating that implantation of these embryos is not compromised.

In conclusion, the supplementation of IVM with different doses of butafosfan decreases the cleavage rate and embryo development, with no difference in maturation rate and in expression of genes related to oocyte and embryo quality. Therefore, we suggest that butafosfan presents a deleterious effect to oocytes.

Ethics statement. All procedures were approved by the Ethics Committee in Animal Experimentation from Universidade Federal de Pelotas (Protocol 6936).

Conflict of interest. None of the authors of this study has any conflict of interest.

Financial support. This work was supported by FAPERGS, CNPq and CAPES.

https://doi.org/10.1017/S0967199419000327 Published online by Cambridge University Press

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