BCL2 AND BAX GENE EXPRESSION IN CUMULUS-OOCYTES COMPLEXES IN COW

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Abstract

In vitro maturation (IVM) is the first and the most important step for IVF technique because the success of in vitro embryos production highly depends on oocyte quality. In the present study it was aimed to identify the optimal time of maturation of cattle oocytes, in which IVF techniques are very important and frequently applied. One of the indicators of IVF failure is the apoptosis, occurring in the participating cells on the whole cycle of development. Therefore is essential to identify highly accurate the apoptotic moment. In this respect, the expression of two genes: Bax and Bcl2 was assessed in three stages of maturation of oocytes: time 0, 24h and 48h of cultivation on maturation culture media. These genes have been previously shown to play an essential role in apoptotic process of the cells. The genes expression was evaluated by Quantitative Revers-Transcription PCR, using SYBR Green reagents, having as target the total ARN isolated from the complex oocytes - cumulus cells. The value of genes expression was normalized using as control the Actin gene biomarker. Obtained data were interpreted using $2^{\Delta\Delta}C(T)$ method and the statistical analysis was performed using ANOVA algorithms. In the determinations, it was found that the expression of the two : Bax and Bcl2 genes are somewhat antagonist, so that at 24 hours of maturation time are both over-expressed comparing to the 0 moment. By comparison, Bcl2 expression is low, which indicates that the apoptotic process has not started and that the stress of in vitro cultivation has not reached the maximum level. Bax gene expression indicates the existence of high stress factors, but not the entry of a cell into apoptosis process. Therefore, it can be considered that the optimum time interval for maturation in culture media is 24h. At 48h the Bax gene overexpression compared to the Bcl2 gene expression indicates the entry of cells into apoptotic process, knowing that a very high level of the Bax gene may have an inhibitory effect on Bcl2 gene experssion.

***Key words** : polimorphism screening, variabile number of tandem repeats*

In vitro maturation (IVM) is the process by which immature oocytes are harvested and subject to artificial maturation conditions in the laboratory before being fertilized. Is a breeding technique that generates the mature oocytes capable of supporting embryonic development before and after implantation. Harvesting oocytes can be done on living animal, transvaginally by OPU technique (Ovum Pick-up) or post mortem after slaughter, by aspiration of ovarian follicles. IVM involves the removal of cumulus-oocyte complexes artificially (COC) of antral follicles and their cultivation in standard essential conditions for 24-28 hours (depending on species) to reach metaphase II (MII). (Chankitisakul et al., 2013, Coyral-Castela et al., 2012, Ferre et al.,2016, Gilchrist and Thompson, 2007).

Proper maturation of oocytes is necessary for them to develop their competences necessary in embryonic development. During maturation in vitro, the release of the first polar globe, in cows, corresponds with metaphase stage II and is reached at 18-24 hours after initiation of oocyte cultivation. Sperm can fertilize the ovum even after 16 hours of cultivation, in metaphase II, but the best is that oocyte meiosis is completed. Even if oocyte releases the polar globe after 16 hours of cultivation, it needs a longer period (24 hours) to accumulate the necessary competence for embryonic development.(Ward et al., 2002).

Gene expression is the process by which the gene information is transcribed to synthesize a functional gene product. Quantifying the expression level of a gene in a cell, tissue or organism, provides the necessary information to better understand the functioning of living organisms. The most commonly used method for quantifying gene expression is PCR (Polymerase Chain Reaction) which investigates changes, decreases or increases, of gene

expression or of a set of genes by measuring the amount of the resulting product (Mircu et al., 2015).

Apoptosis is a genetically controlled act, which can be initiated in two major ways, the dead receptor and mitochondrial pathway. The members of the cell protein family Bcl-2 (B-cell leukaemia / lymphoma-2) participate in the regulation pathway of mitochondrial apoptosis and represents one of the most relevant class of regulators of apoptosis by acting in the effector stage of the of apoptosis (Reed and Hematol, 1997).

Bcl-2 gene family comprises around 20 homologues regulators, pro and anti-apoptotic, of programmed cell death. They either disturb or preserve the integrity of mitochondria, thus induce or prevent the release of apoptotic factors such as *cytochrome c*. (Chakravarthi et al., 2015, Kirikin et al., 2013, Sanchez and Smitz, 2012).

The protein Bcl-2 prevents apoptosis and maintains the survival of cell by influencing the release of *cytochrome C* from mitochondria (Yang et al., 1997). BAX (also known as BCL-2-like protein 4), identified in co-immunoprecipitation with Bcl-2 protein, can suppress the Bcl-2 ability to block apoptosis (Jurgensmeier et al., 1998). Cell vulnerability to apoptotic stimulus was determined by the relative ratio between pro- and anti-apoptotic members of the Bcl-2 family (Chao and Korsmeyer, 1998).

Intrinsic mechanism of apoptosis is present in ovarian granulosa cells, and the ratio of Bcl-2 / BAX appears to play an important role in their apoptosis (Senbon et al., 2003, Tilly, 1996).

The evolution of the follicle, regarding the atresia, degeneration or development until ovulation is determined by complex interactions between the members of the Bcl-2 family of proteins. Granulosa cell survival promoted by the gonadotropic hormones is correlated with a reduction in the level of expression of the Bax gene, with no observed changes in the levels of Bcl-2 and Bcl-X_L. High levels of BAX are correlated with granular cell death and follicular atresia. BAX encoding gene expression is found in germ cells and an increased level is correlated with apoptosis of fetal germ cells, but without any change in the level of Bcl-2 (Kim and Tilly, 2004). It was observed that in the absence of BAX protein, granulosa cells are not able to enter into apoptosis, leading to the extension of their reproductive life in mice that were genetically engineered with this deficiency (Kim and Tilly, 2004, Sanchez and Smitz, 2012). Bcl-2 gene expression in the mouse oocyte both during fetal development and in postnatal life and suppress apoptosis indicating that Bcl-2 can promote the survival of germinal cells (Kim and Tilly, 2004, Kirikin et al., 2013).

In cattle, the wave of gonadotropin hormones in granulosa cells induced progesterone receptor expression, developing their resistance to apoptosis (Vintilă,2005). This relationship was also found to cumulus cell complex of bovine origin, in whom treatment for 24 hours with progesterone ratio decreased Bcl-2 gene transcription (Marek et al., 2014, Goovaerts et al., 2011).

The aim of this study was to identify the optimal time of maturation of oocytes collected from cattle by assessment and quantification of Bcl2/ BAX genes expression biomarkers which are recommended in the literature. The genes included in this study indicate the maturation and fertilization in vitro preparation, their expression is correlated with the time of oocyte entrance into the cell apoptosis process.

Material and Methods

The ovaries were obtained from Agrocompany slaughterhouse, Nojag, Romania, from where were transported within one hour in a saline solution supplemented with antibiotics (NaCl 0,9% şi streptomicyn) at 35-37º C. Oocytes were harvested through puncturing the

ovaries with 18G needles, the follicular liquid being aspired into the 5 ml syringe and then introduced in sterile 50 ml tubes containing PBS. After 10 minutes the sediment was removed using sterile pipettes and transferred in Petri dishes containing PBS, for the two cycles of washing. In this manner 75 cow oocytes were obtained.

Oocytes were cultivated on TCM maturation media. For this, oocytes are placed in a Petri dish containing and 10ml TCM and 1ml ECS, this representing the first TCM wash. The second wash was carried in the same conditions and finally, the oocytes are placed into 400 µl TCM media added with 15µl of FSH. Mineral oil is poured drop wise in order to prevent evaporation while placed at incubation. The incubation conditions are as follows: temperature of 38.5°C, with 5% CO_2, for equilibration, for 48 hours.

Biological samples consisted of oocytes harvested before maturation (the 0 moment), after 24 hours and 48 hours of maturation.

The oocytes along with cumulus cells were transferred in another Ependorf tube and centrifugated for 5 minutes at 200 x g (1060 rpm). The sediment was recovered, suspended in PBS and again centrifugated using same parameters. The supernatant was removed and the samples were deeped in liquid nitrogen for 1 minute and after that stored at -80º C till total RNA isolation. For each harvesting moment six biological samples were used according to Table 1.

Table 1

Experimental variants used for gene expression quantification

Bovine oocytes/ ,,0'' moment (**D**)	Bovine oocytes/ ,,24 h'' moment (**E**)	Bovine oocytes/ ,,48 h'' moment (**F**)
D1	E1	F1
D2	E2	F2
D3	E3	F3
D4	E4	F4
D5	E5	F5
D6	E6	F6

Prior to proceding with RNA isolation the cell samples were washed with PBS buffer and sedimented by centrifugation at 3000 x g for 5 minutes. For each sample aproximative 1,5x 103 cells were harvested, this corresponding to a 50 mg quantity as requiered by the protocol. Total ARN was isolated and from sedimented cells using *SV Total RNA Isolation System* (*Promega, US*) commercial kit according to manufacturer's protocol.
Quantity and quality of extracted RNA was assessed by measurements with *NanoDrop 8000* spectrophotometer (*Thermo Scientific*).
From isolated RNA, the cDNA was synthesized using *Hight-capacity cDNA Reverse Transcription* kit (Applied Biosystems) following the producer indications and oligo dT(8) primer, also provided with the kit.

Obtained cDNA was used as template in qPCR reactions using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific), according to provided protocol with a *Stratagene Mx3000P* (*Agilent*) real time PCR equipment. The primers sequences (Table 2) used in this study were obtained from the reference literature and were synthesized by *Eurogentec* (Belgium).

Table 2

Sequences of primers used in this study

Expression marker	**Primer sense 5'-3'**	**Primer antisense 3'-5'**
BAX	TCTCCCCGAGAGGTCTTTTT	TGATGGTCCTGATCAACTCG
BCL2	ATGTGTGTGGAGAGCGTCAA	CTAGGGCCATACAGCTCCAC
β-actin	GAGCGGGAAATCGTCCGTGAC	GTGTTGGCGTAGAGGTCCTTGC

Each sample was analyzed in duplicate. For normalization of gene expression in terms of number of copies β - Actin gene was used. For each primer a sample without DNA template considered as negative control was run. For the relative quantification the Δ (ΔCt) method was used (Livak and Schmittgen, 2001). For all of the samples the number of cycle's threshold (Ct) were determined. For relative quantification the Δ (ΔCt) method was used. According to this method the R (the relative ratio between the control and stressed variant) is calculated with the following formula: $R = 2^{-\Delta\Delta Ct}$. The obtained data were interpreted with ANOVA software (Table 3 and 4).

Results and discussions

Reproductive biotechnologies are widely used in animal breeding. Methods like artificial insemination, super ovulation and embryo transfer are allowing the genetic potential of parental forms to be harnessed effectively. In vitro fertilization permits the use of biological material derived from valuable parental forms, but however little used because the survival rate of embryos obtained is very small. The main cause of low survival rate appears to be cell apoptosis induced by the stress of *in vitro* cultivation. In this frame, quantification of apoptosis gene expression in different moment of oocytes maturation can be used for the improving of IVF protocols.

Total RNA was successfully isolated from all biological samples, the quantity and quality being considered optimum for the following qPCR experiments.

The Real-Time qPCR results were analyzed and presented in graphical form (Figure 1 and 2).

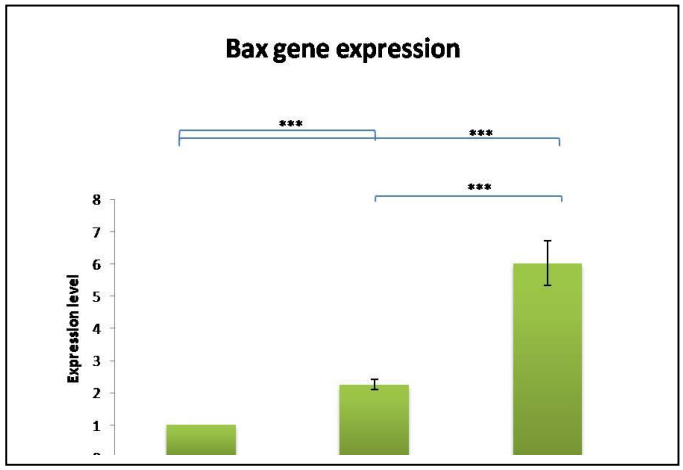

Fig. 1. Expression levels of BAX gene scored in three time intervals.

Gene expression BAX in the case of in vitro matured oocytes with cumulus cells increases proportional with the passage of time from the moment 0 of sowing in culture medium, up to 48 hours, thus showing that the mechanisms of cell protection against stress factors are overwhelmed, heading toward cells apoptosis, a phenomenon closely related to survival of cells (Kirikin et al., 2013). Increasing the level of expression of these genes is very high statistically significant, progressive in all times of observation of cells evolution.

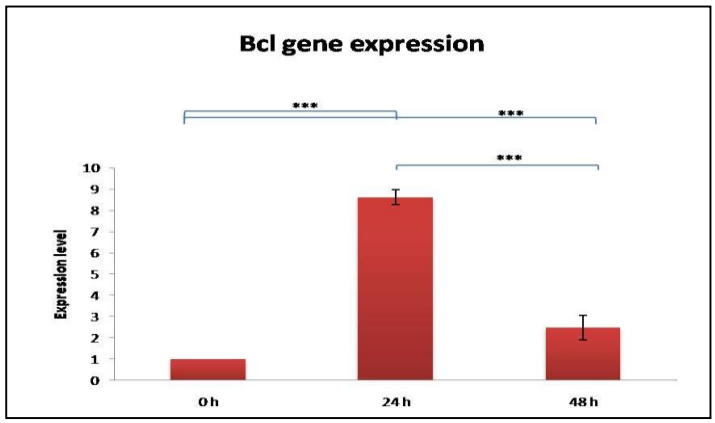

Fig. 2. Expression levels of Bcl 2 gene scored in three time intervals.

Gene Bcl2 is over expressed at 24 h from inoculation, proofing that cells subjected to stress factors, are attempting to adapt to new conditions. Decreasing the expression of this gene at 48 h correlated to enhanced BAX gene expression indicates exceeding the compensatory mechanisms of cell. Also indicates that these cells entered earlier than 48 h into apoptosis, correlated with decreasing viability time related.

The same pattern was observed when the culture medium is added with cysteine, except that this addition results in a period of viability of the oocyte over 48 hours by an almost double expression compared to cells of the cumulus cultured in medium culture without the addition of cysteine (Mircu et al, 2015). Differences in the expression of this gene between all three moments of examination is very high statistically significant.

Gene expression ratio of BAX / Bcl2 is subunitary after the passage of 24 hours of cell culture indicates their ability to adapt to their new environment and preserving the sustainability of these cells at this studied interval. At 48 hours after the passage this ratio is reversed becoming supra unitary, being an indicator of apoptotic processes through which cells lose their viability and thus the ability to be used for in vitro fertilization.

Conclusions

Gene expressions in Bcl2 and BAX evolve somehow antagonistic, so that in 24 hours of maturation both have increased levels of expression compared to moment 0.

At 48 hours the over expression of the Bax gene as compared to the Bcl2 gene expression indicates the entry of cells into apoptotic process.

According to obtained data the apoptotic process begins after 24 hours in cattle. It was found that after 48 hours of cultivation the oocytes are found in the apoptotic process.

Given the correlations between the experimental data and the applicative model it can be stated that identifying the moment of entry into apoptosis of oocytes is indicated accurately by genes BAX and Bcl2. Moreover, these genes evaluated together can indicate the homeostatic status of oocyte.

Aknowlegdements

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References

1. Chakravarthi V.P., Kona S.S.R., Siva Kumar A.V.N., Bhaskar M., Rao V.H. (2015) - *Quantitative expression of antiapoptotic and proapoptotic genes in sheep ovarian follicles grown in vivo or cultured in vitro*, Theriogenology 83, 590–595
2. Chankitisakul V., Somfai T., Inaba Y., Techakumphu M., Nagai T. (2013) - *Supplementation of maturation medium with L-carnitine improves cryo-tolerance of bovine in vitro matured oocytes*, Theriogenology 79, 590–598
3. Chao D.T., Korsmeyer S.J. (1998) - *BCL-2 family: regulators of cell death*, Annu Rev Immunol., 1998;16:395-419.
4. Coyral-Castela S., Brisarda D., Touzéa J.-L., Dupont M., Raméa C., Uzbekovaa S., Dupont J. (2012) - *Analysis of in vivo oocyte maturation, in vitro embryo development and gene expression in cumulus cells of dairy cows and heifers selected for one fertility quantitative trait loci (QTL) located on BTA3*, Theriogenology 77, 1822–1833
5. Ferré P., Bui Tmt., Wakai T., Funahashi H. (2016)- *Effect of removing cumulus cells from porcine cumulus-oocyte complexes derived from small and medium follicles during in vitro maturation on the apoptotic status and meiotic progression of the oocytes*, Theriogenology, doi: 10.1016/j.theriogenology.2016.05.024.
6. Gilchrist R. B., Thompson J. G. (2007) - *Oocyte maturation: Emerging concepts and technologies to improve developmental potential in vitro*, Theriogenology 67, 6–15
7. Goovaerts I.G.F., Leroy J.L.M.R., Rizos D., Bermejo-Alvarez P., Gutierrez-Adan A., Jorssen E.P.A., Bols P.E.J. (2011) - *Single in vitro bovine embryo production: Coculture with autologous cumulus cells, developmental competence, embryo quality and gene expression profiles,* Theriogenology 76, 1293–1303
8. Jürgensmeier J.M., Xie Z., Deveraux Q., Ellerby L., Bredesen D., Reed J.C. (1998) - *Bax directly induces release of cytochrome c from isolated mitochondria,* Proc Natl Acad Sci U S A. 1998 Apr 28; 95(9): 4997–5002.
9. Kim M.-R., Tilly J. L. (2004) - *Current concepts in Bcl-2 family member regulation of female germ cell development and survival*, Biochimica et Biophysica Acta 1644, 205– 210
10. Kirkin V., Joos S., Zornig M. (2004)- *The role of Bcl-2 family members in tumorigenesis*, Biochimica et Biophysica Acta 1644, 229– 249
11. Marek B., Marta W., Anna K., Iwona B., Beenu M. (2014)- *The effect of hormonale estrus induction on maternal effect and apoptosis-related genes expression in porcine cumulus-oocyte complexes,* Reproductive Biology and Endocrinology, 12:32, p2-11, December 2014
12. C.Mircu, Oana Boldura, Aikaterini Ignatiadi, Ana-Maria Raţiu, I.Huţu, Sorina Popescu, Camelia Tulcan, Mirela Ahmadi, Gh.Bonca, Cornelia Milovanov (2015) - *Effect of cysteine supplementation on sow cumulus cells and on BCL2 gene expression during in vitro maturation, Lucrari Stiintifice, seria ,,Medicina Veterinara"* Vol. 58(2), p.292-297, ISSN 1454-7406.
13. Reed J.C., Hematol S. (1997) - *Bcl-2 family proteins: regulators of apoptosis and chemoresistance in hematologic malignancies*, Oct; 34(4 Suppl 5):9-19.
14. Sánchez F., Smitz J. (2012) - *Molecular control of oogenesis*, Biochimica et Biophysica Acta 1822, 1896–1912
15. Senbon S., Ota A., Tachibana M., Miyano T. (2003) - *Bovine oocytes in secondary follicles grow and acquire meiotic competence in severe combined immunodeficient mice*, Zygote., 2003 May;11(2):139-49.
16. Tilly J.L (1996) - *Apoptosis and ovarian function,* Rev Reprod. 1996 Sep;1(3):162-72.
17. Vintilă I. (2005) – *Transferul de embrioni şi biotehnologii asociate*, Editura Orizonturi Universitare, Timişoara
18. Ward F., Enright B, Rizos D., Boland M, Lonergan P. (2002) – *Optimization of in vitro bovine embryo production: effect of duration of maturation, lenht of gamet co-incubation, sperm concentration and sire*, Theriogenology, 57 (2002), 2105-2117
19. Yang J., Liu X., Bhalla K., Kim C.N., Ibrado A.M., Cai J., Peng T.I., Jones D.P., Wang X. (1997) - *Prevention of apoptosis by Bcl-2: release of cytochrome C from mitochondria blocked*, Science 21 Feb 1997: Vol. 275, Issue 5303, pp. 1129-1132