In vitro bovine embryos evaluation based on OCT4, SOX2, IGF1R and IGF2R expression level

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Abstract
In vitro production of bovine embryos comprises a lot of factors that can influence the successful of this technique, oxidative stress being one of them. These factors can influence the evolution of important development processes such as the maternal to zygotic transition and the embryonic genome activation. Adding antioxidants to in vitro culture media exerts the key role to reduce the effects of reactive oxidative species produced during assisted reproduction technique, influencing in a positive way also the early embryonic developement. The objective of this study was to determine the effect of antioxidant rosmarinic acid (105 µM), added to in vitro bovine oocytes maturation media, on the quality of embryo produced based on gene expression level of OCT4, SOX2, IGF1R and IGF2R. For this purpose, we used 35 bovine ovaries taken from slougtherhouse from which we obtain 202 cumulus-oocyte-complexes and 127 of them were maturated in vitro based on morphological aspects. The cumulus-oocyte-complexes were divided in two groups: control (M1, M2, M3) and with acid rozmarinic (AR1, AR2 and AR3). The levels of OCT4, SOX2, IGF1R and IGF2R were the highest in group AR1, embryos obtain from oocytes class I supplemented with rozmarinic acid, where OCT4 expression was 4.08, SOX2 was 27.66, IGF1R and IGF2R were 53.44 and 25.10.
***Key words**: cumulus-oocyte-complexes, antioxidants, gene expression, bovine*

Introduction

Curently the *in vitro* fertilization technique has become a routine technique with good results, but nevertheless there are a large number of oocytes matured *in vitro*, about 60-70% which are lost due to the inability to reach the stage of blastocyst after they have been fertilized (Meirelles et al., 2004). There are a lot of factors that interfere with the development of bovine embryos *in vitro* and cause them to stop dividing between cell cycle 4 and 5 (Betts and King, 2001). This is a very important moment because in bovine specie the transition from the maternal genome to the embryonic genome takes place in this perios, in other words the activation of the embryonic genome, and the embryo must rely on its own mRNA transcripts to continue its development. Activation of the embryonic genome is a process that takes place gradually. Embryos must go beyond the stage of transcriptional repression to initiate transcriptional activation of the genome. The impossibility of some embryos to overcome the transcriptional blockade in the fourth cell cycle is also demonstrated by the existence of nuclear fragmentation and blastomeres after this state of arrest (Betts and King, 2001). The incriminated mechanisms in blocking embryonic development are: the inability to overcome chromatin repression and activation of transcripts of important genes in development, the inability to react to the influences of the development environment.

When discuss about the influence of medium on embryonic development we have to discuss also about reactive oxygen species (ROS). Sources of ROS during *in vitro* fertilization procedures could be either endogenously or exogenous environmental factors (Agarwal et al., 2014). Although ROS are produced during cell metabolism, excessive quantities can cause DNA damage, mitochondrial dysfunction and others negative effects. Reducing the ROS activity during ART can be done by using antioxidants and rosmarinic acid is one of them. Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid derived from hydroxycinnamic acid, that belongs to polyphenols group and is found as an active compound in several medicinal plants

(*Rosmarinus officinalis, Salvia officinalis, Mentha arvense, Ocimum basilicum, Thymus vulgaris* etc)(Krajcovicova et al, 2013). In *Rosmarinus officinalis* the antioxidant activity is support by rosmarinic acid, carnasol and diphenol rosemary and other phenolic compounds that have various biological functions such as oxidation inhibitory function, preventing DNA from mutation and oxidation and anti-thrombotic effects (Huang and Zheng, 2006). Antioxidant activity of rosmarinic acid is supporting by the enhancement of superoxide and hydroxyl scavenging (Krajcovicova et al, 2013) and also by its chemical structure where the carboxylic acid group together with the catechol elements in the aromatic ring are responsible for neutralizing free radicals (Borjizadeh et al., 2019). The rosmarinic acid has a preventive effect on Sertoli cells apoptosis caused by electromagnetic fields (Hajhosseini et al, 2013). Other researchers observed that rosmarinic acid used together with ascorbic acid in the vitrification solution, improved significantly the survavial, maturation, fertilization rate and development to 4 cell stage in mice studies (Borjizadeh et al., 2019). Possitive effects on bovine oocytes maturated *in vitro* based on their morphological examination was observed also in our previous experiments (Marc et al., 2017).

There are molecular markers that can predict the developmental competence of oocytes and also the quality in embryos. Most of them are related to the functions of cell cycle regulation (CCNBI), transcription control (OCT4, YEAF1), DNA packaging (H2A, H3A), glucose transport (GLUT1), signaling (BMP15, IGF2, IGF1R, IFNT), oxidative stress (SOD2) etc (Orozco-Lucero and Sirard, 2014). With great importance are SOX2, OCT4, IGF1R and IGF2R. SOX2 is part of the SOX protein family which has 20 genes, genes that contain a DNA-binding HMG domain, nuclear import-export signals and act as transcription factors (Wegner, 2010). In bovine blastocysts SOX2 is localized in the inner cell mass (ICM) and its downregulation negatively impacts preimplantation development (Goissis et al., 2014). OCT4, a transcription factor from POU (Pit-Oct-Unc) class with gene CDX2 are essential for early development in bovine embryos (Sakurai et al., 2016). IVM is very important step for further development of the oocytes and for early bovine embryos development. Buruszewska et al. (2015) based on evaluation of embryos developmental competence-related factors (OCT4, SOX2, IGF2R), apoptosis genes (BAX, BCL2) and other genes involved in ovulatory and oocytes competence (PFKP, GLUT1, AREG, EREG and others) observed that lysophosphatidic acid, a transmembrane phospholipid, supplementation sustains the expression of developmental competence at the blastocyst stage. Other factors that are important for growth potential of the embryos and not only, are the insulin-like growth factor (IGF). The insulin-like growth factor (IGF) system is essential for pre- and postnatal growth and development and consists of two growth factors (IGF1, IGF2), type 1 and 2 receptors (IGF1R, IGF2R), the insulin receptor (IR) with short and long isoforms (IR-A, IR-B), six major IGF binding proteins (IGFBP1–6) and several lower-affinity binding proteins (IGFBP7 to IGFBP10) with increased expression from embryo to fetal stage and decreased expression from fetal to juvenile stage (Ghanipoor-Samami et al., 2018).

The main purpose of this research was to observe if rosmarinic acid added to the maturation environment of cow oocytes can improve *in vitro* fertilization results and for this we quantified gene expression of OCT4, SOX2, IGF1R and IGF2R. These genes are particularly important in the early stages of embryo development because they have roles in differentiating cell lines, maintaining the pluripotency of embryonic germ cells (OCT4, SOX2), regulating cell survival, proliferation and differentiation (IGF1, IGF2).

Materials and methods

Chemicals reagents

Culture media for *in vitro* production of bovine embryos were purchased from Minitube (Germany) and these were: TCM199 maturation medium (19990/0010), TL fertilization medium (19990/0030) and TL sperm capacitation medium (19990/0020). All chemical reagents for *in vitro* culture were purchased from Sigma Aldrich unless otherwise stated. Plastic dishes and four well plates were obtained from Nunc (Thermo Scientific).

Cumulus-oocyte complexes (COCs) collections

Bovine ovaries (n=35) were collected from slaughterhouse and transported within two hours to the laboratory in containers consisting of 0.9% NaCl solution supplied with PenStrep (17-602F, Lonza), at 35^0C. The handling medium for COC was Dulbecco-PBS (100 ml) (D8662) supplemented with 100 µl Pen/Strep; 3.6 mg sodium piruvate, 30 mg BSA (A9647), 100 mg glucose (G7021). Cumulus-oocyte complexes (COCs) were aspirated by puncturing the follicles with 3-8 mm diameter with a 18G needle attached to a 5 ml syringe.

The classification of COC's based on morphological aspects was made with stereomicroscope (Stemi 2000-C, ZEISS) with hot plate (33.4^0C) after the criteria of Hawk and Wall, (1994) as follows : *class I* - CI (COCs with cumulus compact and unexpanded, with full or at least 5 layers of cumulus cells, cytoplasm clearly seen, dense and homogenous), *class II* – CII (COCs with cumulus compact, thick, 2-4 layers of cumulus cells, covering all zona pellucida, cytoplasm dense, with uniform granulation) and *class III* - CIII (oocytes partially denuded of cumulus cells, or with 1-2 complete layers of cumulus cells and/or with irregular shrunken cytoplasm)(Figure 1). COC were washed one time in Dulbecco-PBS and three times in TCM199 maturation medium.

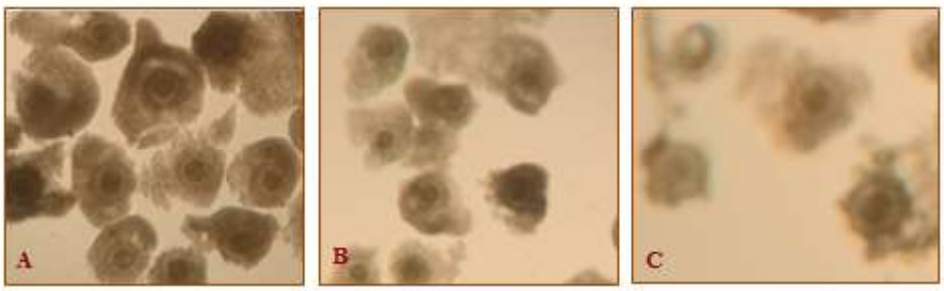

Fig 1. Classes of bovine COCs based on their morphological aspects (2.5X)
(A - class 1, B –class 2, C- class 3)

In vitro embryo production

Brefiely the TCM199 maturation medium (400 µl) was supplemented with 10% fetal bovine serum (FBS, S1400-100, Biowest, France), 0.02UI/ml PMSG (Folligon, Intervet), 0.01 UI/ml hCG (Chorulon, Intervet), covered with mineral oil and allowed to equilibrate for 6h in the incubator at 38.5^0C, 5% CO_2, humidified air atmosphere, then the oocytes (15-20/well) were cultivated for 24h in these 4 well dishes (Nunc, Germany) prepared. After 24h since *in vitro* maturation, all oocytes were examined for maturation and signs like expansion and presence of mucus in cumulus cells were observed, then the COCs were fertilized *in vitro* and cultured. TL fetilization medium (19990/0030) was supplemented with 10µg/ml heparin, 20 µM sodium

piruvate and 0.6% BSA. For *in vitro* fertilization frozen-thawed semen from different bulls was used. After thawing, motile spermatozoa were isolated with Swim-up method using TL sperm capacitation medium (19990/0020) supplemented with 1µM sodium piruvate, 0.6% BSA and 0.6 mg/ml gentamicin and incubated for 1h at 38.5^0C, 5% CO_2, humidified air atmosphere. After incubation, the upper two-thirds of the capacitation medium were recovered, centrifugated at 1000 RPM for 10 min, the supernatant removed. Above the sediment was added 1 ml TL sperm capacitation medium and one more time centrifigated at 1000 RPM for 10 min. The sperm pellet was diluted with 40µl TL fetilization medium. Groups of 10-15 COCs were co-incubated with 10 µl spermatozoa in 60 µl of fertilization medium under mineral oil for 18-22h at 38.5^0C, 5% CO_2, humidified air atmosphere. The day of *in vitro* fertilization was considered the Day 0. After fertilization the embryos were washed two times in culture medium (TCM199 supplemented with 10%FCS) and cultured under mineral oil at 38.5^0C, 5% CO_2, humidified air atmosphere. Every 48 hours the embryos were feeded with 200 µl TCM199, 20% FCS, till the Day 7.

Rosmarinic acid antioxidant for COCs *in vitro* maturation

6 groups of 12-15 COCs each were used to establish all experimental groups: control (C1, C2 and C3) and rosmarinic acid (AR1, AR2, AR3). Using of rosmarinic acid as antioxidant in bovine oocyte maturation medium was based on previously studies on boar spermatozoa (Luno et al., 2014; Luno et al., 2015), on ram spermatozoa (Olaciregui et al., 2017) and on sow oocytes (Zhang et al., 2019). The conentration used of rozmarinic acid was 105 µM.

RNA isolation reverse transcription and real time PCR

Prior to proceeding with RNA isolation, the cell samples were washed with PBS buffer and sediment by centrifugation at 3000 x g for 5 minutes. For each sample approximately $1,5x\ 10^3$ cells were harvested, this corresponding to a 50 mg quantity as required 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.

From isolated RNA, the cDNA was synthesized using High-capacity cDNA Reverse Transcription (Thermo Scientific, Lituania) following the manufacturer indications and oligo dT(8) primer, also provided with the kit. Obtained cDNA was used as template in qPCR reactions using GoTaq qPCR Master Mix Kit (Promega, U.S.A). 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 for real-time PCR

Gene	Primer sequence
OCT4 fw	GAGAAAGACGTGGTCCGAGTG
OCT4 rew	GACCCAGCAGCCTCAAAATC
SOX2 fw	TGGATCGGCCAGAAGAGGAG
SOX2 rev	CAGGCGAAGAATAATTTGGGGG
IGF1R fw	GAGTGGAGAAATCTGCGGG
IGF1R rev	AAATGAGCAGGATGTGGAGGT
IGF2R fw	ACCTCCGATCCTCAATCCCA
IGF2R rev	TGTAGTTGAAGTGCCGGTCC
Reference gene β-actin fw	GTCACCAACTGGGACGACA
Reference gene β-actin rew	AGGCGTACAGGTGACAGCA

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. For all the samples the number of cycle's threshold (Ct) was 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: $R = 2^{-\Delta\Delta Ct}$. (Livak and Schmittgen, 2001).

Results and discussions

Following the experiment, we observed a high value of OCT4 gene expression in group AR1 compared to all other groups, namely 27.66. High values were also recorded in groups AR2 and AR3 (Figure 2), which highlights the positive effect of rosmarinic acid added in IVM on quality of bovine embryos . Similar possitive effects of acid rosmarinic were observed by researchers on sow oocytes that were maturated in medium supplemented with 5 µM acid rosmarinic based on measurement of intracellular ROS levels in cumulus cells and oocytes and of intracellular free thiols levels, one marker of cytoplasmatic maturation of the oocytes at the end of IVM (Zhang et al., 2019). Our previous good results observed by quantifying other genes, such as PTX3, p53, BAX2, BCL2, genes involved in the regulation of cellular apoptosis in both sow and cow oocytes emphasize the importance of this antioxidant in *in vitro* fertilization technique (data being published).

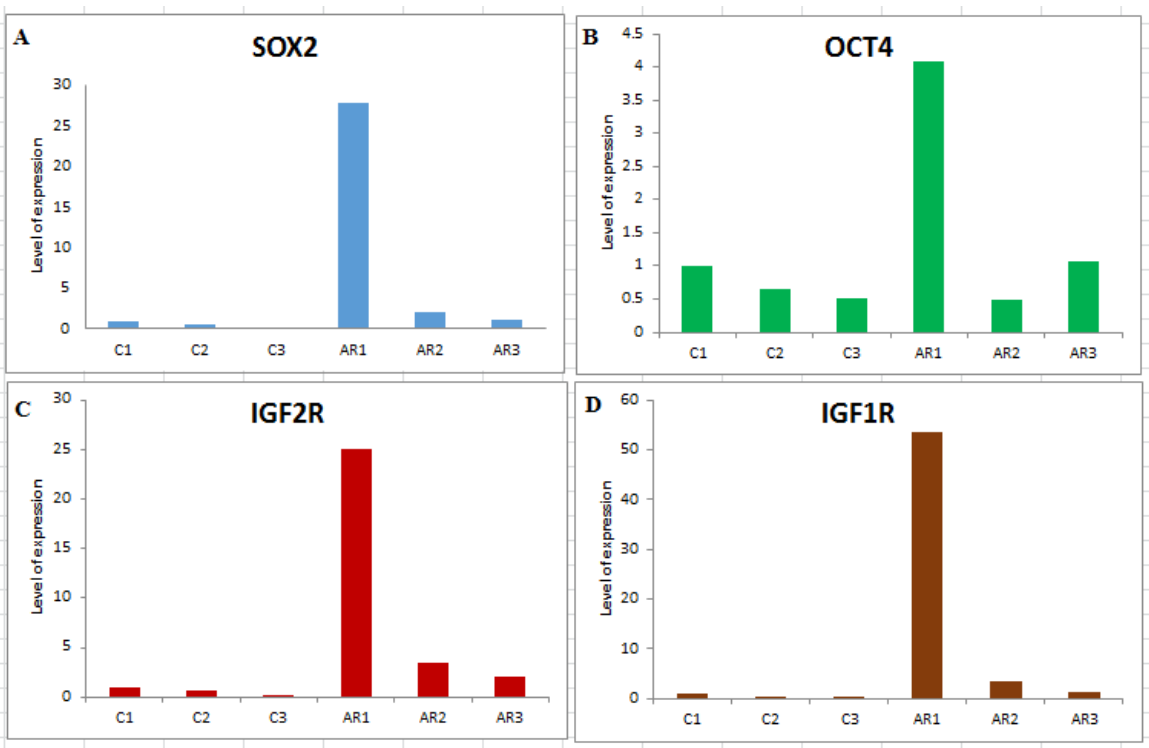

Fig 2. The effect of rosmarinic acid supplementation of maturation medium on mRNA abundance of embryos related factors: A. *SOX2*, B. *OCT4*, C. *IGF1R* and D. *IGF2R*

The information of the OCT4 gene, which is also called POU5F1, ensures the expression of a transcription factor that binds the octamer sequence "ATTTGCAT" from regions which acts as activator or silencer of some genes. The expression of these genes is very important in early embryonic development because it ensures the pluripotency of the cells in the internal mass of the embryo, so it has a role in differentiating cell lines (Kehler et al., 2004). Proper expression of the OCT4 gene is also important for the functioning of other genes such as GATA6 and FGF4 (Frum et al. 2013). Studies on relationships between NANOG, OCT4 and SOX2 gene expression and changes in two histones together with transcriptional activation (H3K4m3 and H4K16ac), but also with transcriptional repression (H3K9m2 and H3K27me3), during reprogramming of donor cells needed to obtain of somatic cell nuclear transfer (SCNT) embryos have shown that the low percentage of embryos obtained by SCNT is due to histone changes, together with the abnormal expression of the OCT4 gene (Hall, 2013)

Like the evolution of the OCT4 gene, the level of SOX2 gene expression is highest in the AR1 group. The SOX2 gene is another transcription factor that contributes to the induction of cellular pluripotency, being closely related to the OCT4 gene, ensuring the latter adequate functionality (Sakurai, 2016). The proper functioning of the two genes, SOX2 and OCT4, also has an influence on the promotion of transcription of fibroblast growth factor 4 (FGF4), which is expressed in the embryonic cell mass of the blastocyst (Hall, 2013). In group AR1 the value of this transcription factor was 27.66, and in groups AR2 and AR3 it was 2.01 and 1.13, respectively, values compared to group C, where the reference value is 1. Regarding C2 and C3 groups, the values expressed for SOX2 were below 1 (Figure 2). The evolution of IGF1R and IGF2R gene expression is similar to SOX2 and OCT4 genes. The IGF1 and IGF2 genes are part of the insulin-like growth factor (IGF) family, being essential for fetal growth and development, but also for placental development, being very strong mitogens that act as regulators of cell survival, proliferation and differentiation (Farmer, W.T.Q, 2015). The good results from AR1 group based on these four genes expression, genes important in differentiating cell lines, maintaining the pluripotency of embryonic germ cells (OCT4, SOX2), regulating cell survival, proliferation and differentiation (IGF1, IGF2) sustained the positive effect of rosmarinic acid added in IVM on bovine embryos development competence.

Conclusions

1. the morphological quality of the oocyte has a very important role in ensuring *in vitro* fertilization
2. the use of rosmarinic acid in the *in vitro* maturation environment of oocytes together with the quality of oocytes (group AR1), determined the best results regarding the expression of OCT4, SOX2, IGF1R, IGF2R genes
3. the intensified expression of the OCT4 gene in group AR1 compared to all other groups and the highlighting of increased values in the other groups as well - AR2 and AR3, highlights the positive effect of rosmarinic acid on oocytes
4. the evolution of IGFR1, IGFR2 and SOX2 gene expression, similar to that of OCT4, highlights the positive effect of rosmarinic acid on oocytes

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