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EFFECTS OF ANTIOXIDANT TREATMENT ON CELL DIFFERENTIATION IN RABBIT EMBRYOS

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Abstract

The antioxidant coenzyme Q10 can influence the expression of genes involved in apoptosis and energy metabolism of oocytes and quercetin can improve oocyte maturation and early embryonic development. In this study, the gene expression of *GATA6* and *NANOG* in rabbit embryos was assessed using the qRT-PCR reaction. The groups were: group A- control group (no treatment added), group B (hormonal treatment of superovulation, which included the administration of PMSG and hCG), group C (administration of quercitin) and group D (administration of Coenzyme Q10). Our results show that the expression of the two genes was different depending on both the stage of embryonic development and the treatment administered. The highest values of gene expression for GATA6 and NANOG were obtained in groups 2, 4, 7, 8 and 9, corresponding to morula and blastocyst stages. In addition to the fact that NANOG and GATA6 are factors that are involved in early embryonic development, we believe that the administration of extrapituitary gonadotropins and antioxidants contributed to the increase in gene expression.

Key words: rabbit embryos, gene expression, antioxidants, qR-PCR

The female rabbit has certain anatomical and physiological characteristics relevant to embryology research and the application of assisted embryo reproduction techniques.

Ovulation in the rabbit is induced by mating. Coitus leads to the nervous stimulation of the vagina, thus triggering the production of gonadotropin releasing hormone (GnRH) in the hypothalamus. Thus, under the influence of this hormone, the anterior pituitary gland secretes the follicle-stimulating hormone (FSH) and the LH (luteinizing) hormone responsible for inducing ovulation.

Q10 is an endogenous antioxidant that protects against oxidative damage and regulates gene transcription. Coenzyme Q10 supplementation may also have beneficial effects on ovarian reserve and follicular development. It also has the ability to restore mitochondrial function by reducing mitochondrial membrane damage and depletion of the intracellular antioxidant ATP. Q10 can influence the expression of genes involved in apoptosis and energy metabolism of oocytes. By reducing the expression of pro-apoptotic genes and increasing the expression of anti-apoptotic genes, Q10 protects oocytes from apoptosis and contributes to maintaining their integrity.

Quercetin can improve oocyte maturation and early embryonic development. This substance was also found to have the ability to reduce apoptosis and enhance autophagy in aged oocytes, providing a protective mechanism against age-related mitochondrial oxidative stress.

The GATA family represents a family of transcription factors of fundamental importance in gene regulation and organismal development. The *GATA6* gene is expressed since embryogenesis, even from the early blastocyst stage at 3.5 days. GATA factors, including GATA6, regulate the expression of cardiac and smooth muscle genes, having an essential role in the processes of development, differentiation and gene expression. They interact directly with GATA(A/G) DNA sequences in the promoters and enhancers of target genes, thus regulating their expression.

NANOG is an essential transcription factor in early embryonic development and in the maintenance of stem cell pluripotency. Its functions are crucial in the specification of pluripotent epiblast (EPI) cells at the expense of primitive endoderm (PrE) within the inner inner cell (ICM) of the blastocyst.

Interactions between NANOG and GATA6 are essential in controlling cell specification within the ICM. At the 16-cell stage, all ICM cells express both *NANOG* and *GATA6*, forming a population of double-positive (DP) cells. These close interactions between *NANOG* and *GATA6* are crucial in regulating the process of cell

specification and cell plasticity in the early embryo.

In this study, we evaluated the expression of *GATA6* and *NANOG* genes in rabbit embryos administered antioxidants, such as coenzyme Q10 and quercitin.

In this context, the aim of this paper was to determine if the treatment of females with hormones or antioxidants influences the expression of genes involved in cell differentiation processes in early embryos.

MATERIAL AND METHOD

For this study, we used New Zealand female rabbits. The selected females were 4 months old and had a body weight between 3 and 3.3 kg at the time of the experiment. To ensure a suitable environment, all females were reared in individual cubicles, where they benefited from natural light and were exposed to an ambient temperature between 15 and 25°C. During the experiment, the rabbits had unlimited access to water and were fed with commercial food in the form of pellets.

Table 1.

The embryonic stages of the groups analyzed

Experimental group	Embryonic stage	Stimulated/Non-Stimulated
A1-A3	7 morulae 12 blastocysts	Stimulated with male
A5-A8	19 morulae 7 blastocysts	Non stimulated
B1-B4	9 blastocysts	Stimulated with male + hormones
B1-B4	24 morulae	Stimulated with male + hormones
B1-B4	27 morulae degraded	
B5-B8	19 blastocysts	Stimulated with hormones
C1-C8	43 morulae	Stimulated with Q10
C1-C8	14 blastocysts	
D1-D8	17 morulae 2 blastocysts	Stimulated with querctin

In this study, the rabbits were grouped into four groups, each group having 8 females. The description of the experimental groups is as follows: no treatment added (group A- control group), hormonal treatment of superovulation, which included the administration of PMSG and hCG (group B), administration of quercitin (group C) and administration of Coenzyme Q10 (group D).

Group B received 120 IU/female of a PMSG analogue (Folligan, MSD) intramuscularly, then 180 IU/female of HCG (Chorulon, MSD) at 48 hours, but 6 hours before mating.

In group C, quercetin was administered orally for 20 days, at a dose of 30 mg/kg (using Quercetin 500mg/Solaray).

Group D received oral CoQ10 for 20 days at a dose of 10 mg/kg. For groups C and D, oral administration was performed daily at the same time interval using a syringe.

Embryo harvesting was performed by washing the oviducts and uterine horns with a PBS solution (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM phosphate buffer / 100 mL, VWR Life Science), after performing the ovariohysterectomy.

Gene expression analyses

Protein sequences were transcribed into mRNA sequences. These mRNA sequences were subsequently transcribed into cDNA sequences. To identify the indicator genes of the cell differentiation process, the sequences of the specific primers were taken from the specialized literature. The oligonucleotides of these primers were synthesized in the Eurogentec laboratories in Belgium and screened for use in this study. The primers sequences used in this study were: *GATA6* Forward 5' TGCGGCATCTACAGCAAGAT 3' and Reverse 5' CCCGGCCCATTGTTTCCT 3'.

For the extraction and purification of total RNA from the samples, the SV Total RNA Isolation System kit (Promega, USA) was used.

To verify the quantity and quality of the extracted RNA, the spectrophotometric method was used using the Nanodrop 8000 UV-VIS spectrophotometer (Thermo Scientific). Based on the results obtained for RNA, the amount required for the reverse transcription reaction was calculated.

For cDNA synthesis, the Hight-capacity cDNA Reverse Transcription kit (Thermo Scientific, Lithuania) was used. The required solution was prepared and transferred to ice. 10 µl of RNA was added to the prepared solution and mixing was done gently. The mixture was then transferred to a thermocycler.

The synthesis and amplification program consisted of keeping the samples for 10 minutes at 25°C, then at 37°C for 120 minutes. Reverse transcriptase activity was stopped by keeping the samples for 5 minutes at 85°C.

For qPCR reactions, the GoTaq qPCR Master Mix Kit (Promega, USA) was used. This is a ready-made solution optimized for the quantitative PCR amplification reaction. The kit contains a DNA polymerase enzyme (GoTaq DNA Polymerase) and dNTPs (nucleotide triphosphate) in a buffer optimized for PCR. It also contains a dye, SYBR GREEN, and is supplemented with a passive reference dye called ROX.

Each sample was analyzed in two replicates. To have a control for each individual primer, a sample without cDNA template was analyzed.

The gene expression ratio was normalized using the expression value of the β-actin gene, which is a constitutively expressed gene. For each sample, the number of threshold cycles (Ct) was determined. The method used for relative quantification was the Δ (ΔCt) method. According to this method, the relative ratio (R) between the control and the stressed variant was calculated using the following formula: $R = 2^{(-\Delta\Delta Ct)}$.

The obtained results were statistically interpreted using the Microsoft Excel program and ANOVA (Analysis of Variance).

RESULTS AND DISCUSSIONS

GATA6 gene expression values and their interpretation

GATA6 is an essential gene in early embryogenesis, especially during the blastocyst period, being expressed in mesoderm and endoderm derived tissues (3,4).

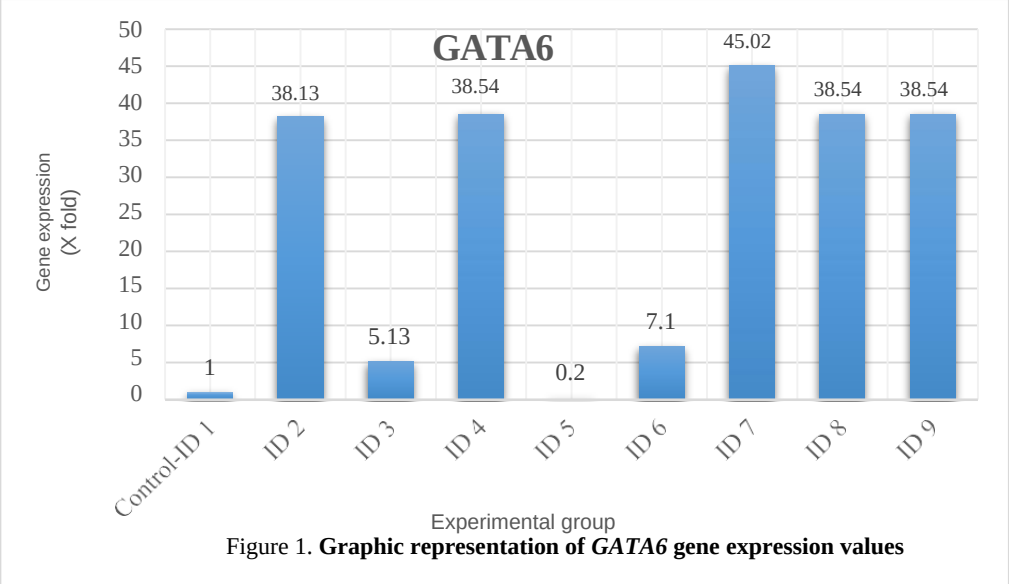

Figure 1. **Graphic representation of *GATA6* gene expression values**

In our study, the expression of this gene was identified in all 9 analyzed samples. As can be seen from Figure 1 there are differences between the expression levels. Thus, considering group 1 as a control, embryos obtained from females after stimulation with the male, which was given the value 1 and in comparison, with this unstimulated group 2 shows a gene overexpression, due to the fact that embryos were found in this group in morula and early blastocyst stage. This could be explained by the fact that GATA6 is the earliest PrE (primitive endoderm) gene to be expressed. Also, *GATA6* gene is present in all blastomeres from the eight-cell stage, alongside *NANOG*, *OCT4* and *CDX2*. (Dietrich et al., 2007)

In the case of group 3, the gene expression is higher compared to the control group, but lower than in the case of group 2, because this group consists only of blastocysts in which to measure the advancement of the embryonic stage, the expression value of this gene is lost.

Regarding group 4, the embryonic stage is late morula, when the cell differentiation process is initiated, which explains the overexpression of this gene.

Group 5 was composed of degraded morulae in which the processes of embryonic development are seriously slowed down, so that the expression of genes stimulating cellular differentiation is no longer possible, studies supporting the fact that embryos lacking gene expression die during gastrulation (Zhao et al., 2005), fact that explains such a low expression of the *GATA6* gene.

***NANOG* gene expression values and their interpretation**

The *NANOG* gene is an important gene in embryonic development and in the maintenance of stem cell pluripotency. *NANOG* is part of the family of transcription factors and is expressed in embryonic stem cells as well as other pluripotent cell types. This gene plays an essential role in maintaining pluripotent cell identity and controlling cell differentiation (Khang et al., 2017).

The data in figure 2 presented below show that in group 2 that was not stimulated, an overexpression of the *NANOG* gene can be observed due to the fact that the embryonic stage analyzed is in the optimal period of expression, more precisely in the early blastocyst stage.

Decreased *NANOG* gene expression in the early blastocyst results in embryonic death. However, Chambers et al. (2007) support the fact that embryonic stem cells in which the *NANOG* gene is weakly expressed are still capable of maintaining pluripotency, although they are prone to differentiation.

At the same time, comparing the graph data, we can see that there is an overexpression of

the gene also in group 4 (stimulated with male and hormones).

The development and maturation of ovarian follicles is dependent upon the successive actions of gonadotropines. Upon stimulation of immature antral follicles by FSH, there is an upregulation of expression levels of both aromatase and LH receptor mRNA. Subsequently, LH acts directly or indirectly through the actions of growth factors (Park et al., 2004) on the FSH stimulated follicle to facilitate steroid production, induce luteinisation and ovulation.

Activation of LH receptors also contributes to the upregulation of oviductal glycoprotein (OGP). The activation of LH receptors in the oviduct causes an increase in the synthesis of OGP that binds to the embryo and helps its growth and development. (Zheng et al., 2001)

An overexpression of the gene is also evident in groups 7 and 8 (stimulated with coenzyme Q10), but also in the case of group 9 (stimulated with quercitin), a fact that supports the theory that *NANOG* expression occurs in the early stages of the morula (Kang et.al, 2017).

NANOG is expressed in a limited number of cell types and only in cells that also express OCT4, including ESCs. NANOG is located in the center of the morula and in the ICM (inner cell mass) of the blastocyst.

An overexpression of the gene is also evident in groups 7 and 8 (stimulated with coenzyme Q10), but also in the case of group 9 (stimulated with quercitin), a fact that supports the theory that NANOG expression occurs in the early stages of the morula.

Also, the antioxidants protects the ovarian reserve, counteracts the physiological aging of the ovaries by restoring mitochondrial function and increases the rate of embryo cleavage and blastocyst formation.

In groups 3 and 6, according to the statistical analysis graphically represented in figure 2, the expression of the *NANOG* gene is weak, which reinforces the current knowledge that with the advancement of the embryonic stage the expression is suppressed in the early blastocysts, with the induction of ICM differentiation into EPI. The lowest value of NANOG expression was recorded in group 5, in which there are degraded morulae.

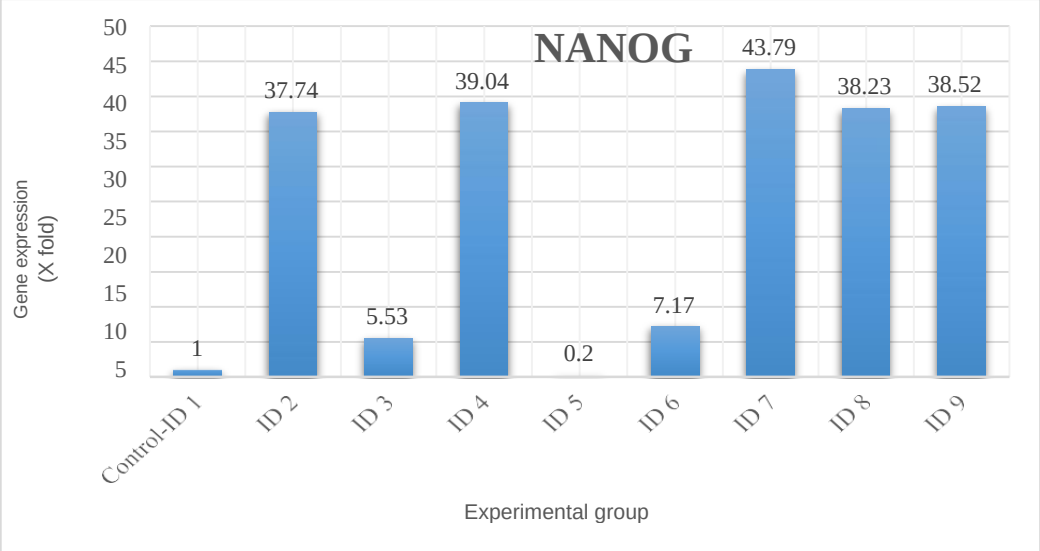

Figure 2. **Graphic representation of *GATA6* gene expression values**

CONCLUSIONS

The overexpression of GATA6 and NANOG genes was recorded in cases of groups 2, 4, 7, 8 and 9, which were in the morula and blastocyst stages. In the case of the groups to which the analogue hormones of FSH and LH and antioxidants were administered, an overexpression of the *GATA6* and *NANOG* genes was found, with the exception of group 5 in which there was a degraded morula stage.

The obtained results showed that hormonal and antioxidant treatments can influence the expression of certain genes involved in embryonic development, but these aspects are also closely correlated with the stage of development in which the embryo was captured.

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