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IN VITRO BISPHENOL A EFFECT ON TFAM AND SIRT1 GENE EXPRESSION IN PORCINE OOCYTE MITOCHONDRIA

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Abstract

Mitochondria are the main cellular organelle responsible for energy production, having an essential role in maintaining cellular homeostasis. In this study, the gene expression of *TFAM* (Transcription Factor A Mitochondrial) and *SIRT1* (Silent Information Regulator) in sow oocytes cultured in vitro was assessed using the RT-qPCR reaction. The primers were tailored after our own design. The groups were: V1 (matured oocytes, control group), V2 (matured oocytes with hormones), V3 (medium supplemented with Bisphenol A), V4 (medium supplemented with Bisphenol A and hormones). Our findings reveal a reduction in *SIRT1* activity following maturation in all experimental groups, while *TFAM* activity displayed more elevated levels, seemingly independent of the *SIRT1* gene expression. The highest values of gene expression for *TFAM* and *SIRT1* were obtained in V2 (supplemented with FSH and LH, - 0.277 and 0.010) and V4 (FSH, LH and bisphenol A - 0.272 and 0.015) without significant differences (p=0.941). Bisphenol A alone generated low values, presumably due to its endocrine disruptor action. We concluded that FSH/LH addition might rescue some of the *TFAM* expression during bisphenol treatment, but the mechanism might be independent of *SIRT1*.

Key words: sow oocyte mitochondria, *TFAM* and *SIRT1* expression, bisphenol A

Mitochondria is the maternally inherited cell organelle that uses highly efficient oxidative phosphorylation pathways to supply ATP. Except for the nucleus, mitochondria are the only cellular organelles with their own genetic information called mitochondrial DNA (mtDNA).

For transcription and translation, the availability of a sufficient number of functional mitochondria is very important, because oocyte maturation requires a large amount of ATP. Given that the mitochondria of immature oocytes do not provide sufficient energy, it is likely that the energy to support oocyte maturation is mainly carried out by cumulus cells and granulosa cells.

In the case of oocytes that have ovulated, they lose their connections with the cumulus cells that have provided them with energy so far and must activate their own mitochondria. By the time of final maturation of the oocytes, a sufficient number of mitochondria has accumulated.

In both humans and animals, the mid-cycle LH surge activates the luteinizing hormone receptor (LHR), also known as the luteinizing hormone/chorionic gonadotropin receptor (LHCGR). LHR is mainly expressed in the cells of the granulosa wall of the ovarian follicle.

FSH can indirectly influence cellular processes, including the expression of genes such as *SIRT1* and *TFAM*, which are involved in mitochondrial function and other cellular functions. While FSH is not directly involved in mitochondrial function, the energy demands of follicular growth and maturation may indirectly impact mitochondrial activity. Increased energy requirements may influence the expression and activity of *SIRT1*, which is a regulator of mitochondrial function and cellular metabolism. FSH's role in ovarian follicle development may indirectly affect *TFAM* expression, as healthy mitochondria are crucial for follicular growth.

The biological actions of LH are necessary for oocyte maturation, ovulation and corpus luteum function. In the ovarian follicle, these actions are mediated by LHR which is coupled to Gs, the G protein that activates adenylate cyclase and cAMP. This results in an increase in follicular cAMP levels that affects multiple molecules of the follicle LH signaling pathway. These pathways ultimately activate maturation-promoting factor (MPF) in the oocyte inducing oocyte maturation, resumption of meiosis, and the first meiotic division.

LH primarily functions in the context of the reproductive system and ovulation. Its indirect effects on genes like *SIRT1* and *TFAM* are mediated through hormonal changes associated

with the estral cycle, such as progesterone and estradiol fluctuations.

Supplemented FSH and LH in the IVM medium could promote the maturation rate, reduce the apoptosis rate of ovine oocytes and increase FSH concentrations in the IVM medium fluid. In addition, FSH and LH enhanced the expression levels of FSHR, LHR and GnRHR mRNA of ovine COCs. Wei et al. (2013) reported that FSHR, LHR and gonadotropin-releasing hormone receptors (GnRHR) are expressed in sheep ovaries.

The *SIRT* gene family encodes a cluster of proteins called sirtuins, which engage in an extensive array of cellular processes such as DNA repair, gene expression regulation, metabolism, and responding to cellular stress. Despite their vital functions in various cell types and processes, their specific roles within oocytes (immature eggs) remain less elucidated compared to their roles in other cell types.

SIRT1, in particular, has garnered attention in oocyte research. It is presumed to play a role in regulating various facets of oocyte development and maturation, encompassing DNA repair, mitochondrial function, and modifications to epigenetic markers. These processes are pivotal for fostering the development of a healthy oocyte capable of fertilization and supporting the growth of a viable embryo.

In contrast, *TFAM* (Transcription Factor A Mitochondrial) gene is not commonly associated with explicit functions in oocytes. Instead, TFAM is chiefly recognized for its indispensable role in governing mitochondrial DNA (mtDNA) within cells, especially in the context of mitochondrial function and the creation of new mitochondria (mitochondrial biogenesis).

Bisphenol A (BPA) is an industrial compound widely used in the manufacture of various polycarbonate plastic products and which, once in the body, interferes with the proper functioning of the endocrine system, exerting effects of the type induced by the action of estrogens, widespread with estrogen-like characteristics and is widely used. Long-term exposure to BPA can affect the normal functioning of the mammalian reproductive, immune, and neuroendocrine systems.

Following an in vivo experiment on mice, Zhang et al. (2017) found that certain concentrations of BPA can disrupt spindle formation, chromosome synapsis, and kinetochore microtubule assembly. Thus, these events can affect the release of the first polar globe, disrupting the meiotic process and ultimately affecting the reproductive capacity of mammals.

In our study, we highlighted the effect of hormones together with that of bisphenol A added to the oocyte culture medium, an effect that was later expressed by the expression levels of the two genes: *SIRT* and *TFAM*, both genes influencing mitochondrial activity.

MATERIAL AND METHOD

In vitro maturation of sow cumulus-oocyte complexes

The ovaries (n=25) were obtained from slaughterhouses. To collect the oocytes we used the follicle aspiration method. Follicular fluid was aspirated into a 5 ml syringe and then placed into sterile 50 ml tubes containing PBS (D8662, Sigma Aldrich).

Using sterile pipettes, the sediment was removed and transferred to Petri dishes containing PBS for the first washing. For the third and fourth washing, the oocytes were transferred to TCM solution and finally, to 400 µl of TCM medium without any supplement (group V1), supplemented with hormones (group V2), with bisphenol A (group V3) and with hormones and bisphenol A (group V4).

In this study, 50 COCs were obtained and classified according to their morphological aspects using a stereomicroscope (Stemi 2000-C, ZEISS) with a hot plate (33.4˚C), as follows: class I - (COC with unexpanded and compact cumulus, with full or at least 5 layers of cumulus cells, clearly visible cytoplasm, dense and homogeneous, class II – (COC with compact, thick cumulus, 2-4 layers of cumulus cells, covering all zona pellucida, dense cytoplasm, with uniform granulation).In this study, only class I and class II were used.

The TCM 199 maturation medium was prepared in our lab and consisted of 1510 mg TCM199 with Earle's salts (M2520, Sigma Altrich); 220 mg NaHCO3, 2.2 mg sodium pyruvate, 5 µl gentamicin added to 100 ml sterile water, with final pH 7.2, filtered within 0.2 µm filter (Fresenius Kabi) and kept at 40C, ready to use.

Maturation plates with TCM-199 medium were equilibrated at a temperature of 39°C and an atmosphere of 5% CO2 for 24h without the addition of hormones, these being added later on the day of using the medium. In each well of the plate, 400 µl of TCM medium were introduced, as follows: no supplement added (group V1), supplemented with 10 IU PMSG (Folligon, Intervet), 10 IU HCG (Chorulon, Intervet) (group V2), with bisphenol A (group V3) and with hormones and bisphenol A (group V4).

The maturation was carried out for 22h in the medium with hormones, then they were washed once in the PBS washing medium and inserted into eppendorf tubes, following which they were subjected to gene expression analyses.

Gene expression analyses

For isolating total RNA, we have washed the matured oocytes with PBS buffer and the mixture was subsequently centrifuge for 10 minutes at 2500G. basically it was used the SV Total RNA

Isolation System kit (Promega, Oregon, USA) with respect to producer s instructions. For synthesizing cDNA we have used High Capacity cDNA Reverse Transcription Kit (Thermo Scientific, Lithuania).

Following this step, cDNA served as a template for RT-qPCR reaction run on Stratagene Mx3000P real-time PCR equipment (Agilent) based on the protocol accompaning the qRT-PCR Brilliant III SYBR Master Mix kit (Agilent Technologies, Santa Clara, CA. USA), with beta-actin as reference gene.

The primer sequences used in this study were designed online, based on reference mRNA sequences from NCBI databases, using Primer 3 software with the pick primers function. Thus, for the *SIRT1* gene, the sequence Sus scrofa sirtuin 1 (*SIRT1*) mRNA, accession number EU030283.3 was used as matrix and for the TFAM gene was used as matrix, the sequence Sus scrofa transcription factor A, mitochondrial (TFAM), mRNA, accession number NM_001130211.1.

The primers sequences designed and used in this study were: *SIRT1* Forward 5' TGGCGGCTGAGAGGGAG 3' and Reverse 5' CCCGGCCCATTGTTTCCT 3'. The reference gene was β-actine with the sequence Forward 5' CTCGATATGAAGTGCGACG 3' and reverse 5' GTGATCTCCTTCTGCATCCTGTC 3'.

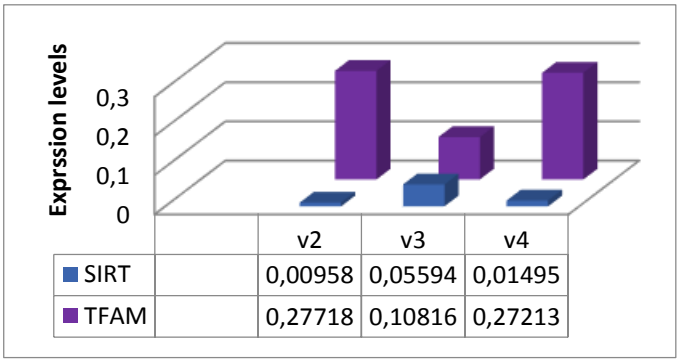
RESULTS AND DISCUSSIONS

As we know, both SIRT1 and TFAM are involved in the regulation of mitochondrial biogenesis, which is the process of generating new mitochondria. *SIRT1*, through its deacetylase activity, can regulate the activity of various transcription factors, including PGC-1α (Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-alpha), which plays a central role in mitochondrial biogenesis.

TFAM, as a transcription factor itself, is responsible for regulating the expression of mitochondrial genes, including those involved in replication and transcription of mtDNA. The coordination between *SIRT1* and *TFAM* is essential for the proper regulation of mitochondrial biogenesis.

SIRT1 and *TFAM* contribute to maintaining mitochondrial function. SIRT1's deacetylase activity can regulate the function of proteins involved in mitochondrial energy production and oxidative phosphorylation.

TFAM's role in maintaining mtDNA integrity and promoting mitochondrial gene expression ensures the production of proteins required for mitochondrial function.



		v2	v3	v4
SIRT		0,00958	0,05594	0,01495
TFAM		0,27718	0,10816	0,27213

Figure 1. **Level of gene expression for *SIRT1* and *TFAM***

For the *SIRT1* gene, hormones have hastened the maturation process because SIRT1 is low in group V2 (supplemented with hormones) and nearly absent in group V4. Based on the expression of *SIRT1* (the gene involved in the maturation process), we can conclude that hormones have partially countered the effect of bisphenol A. In group V3, the maturation process was slowed down.

The fact that they are higher in *TFAM* (groups V2 and V4) and lower (group V3) means that bisphenol A has counteracted the gene's effect.

In group V2 (where only hormones were added), the expression is increased because TFAM is involved in other processes. First, this protein maintains mtDNA copy number by regulating mtDNA replication. The mtDNA copy number correlates with Mt gene expression levels, but also with Mt respiratory activity.

TFAM may also exhibit a structural function that refers to the fact that it fully wraps mtDNA to form a nucleoid structure, like histones in the nucleosome.

Specific binding of *TFAM* to the two mtDNA promoters results in the activation of transcription by recruiting mitochondrial RNA polymerase (POLRMT) and mitochondrial transcription factor B2 (TFB2M). Nonspecific binding of *TFAM* enables the packaging of the mitochondrial genome into protein-DNA complexes for the formation of the mitochondrial nucleoid. (Hillen., 2017)

In group V4, hormones manage to maintain the gene's activity even in the case of bisphenol A treatment.

In all experimental groups, we observed a reduction in *SIRT1* activity after the maturation process, while for the *TFAM* gene the levels were higher, seemingly independent of the SIRT1 gene expression.

The highest values of gene expression for *TFAM* and *SIRT1* were obtained in group V2 (supplemented with FSH and LH, - 0.277 and

0.010) and V4 (FSH, LH and bisphenol A - 0.272 and 0.015) without significant differences (p=0.941). Bisphenol A alone generated low values, presumably due to its endocrine disruptor action.

As a metabolic and endocrine-disrupting chemical, BPA can affect oxidative homeostasis through direct and indirect mechanisms, including increasing oxidative mediators and reducing antioxidant enzymes, causing mitochondrial dysfunction, altering cell signaling pathways, and inducing apoptosis. BPA induces oxidative stress by decreasing antioxidant enzymes, such as superoxide dismutase (SOD), catalase, glutathione reductase (GR), and glutathione peroxidase (GSH-Px). (Ma Y., 2019)

Guo et al. (2017) found that bisphenol A influences oxidative stress in embryonic development in sows. Thus, the embryo obtained in vitro is of low quality. Excessive production of free radicals and exposure to oxidative stress are the main obstacles in the development of embryos in vitro. When ROS production exceeds the antioxidant capacity of embryos, oxidative stress occurs. In early embryonic development, ROS production is important and excess will induce apoptosis and metabolic disturbances.

Doshi et al. (2011) observed that administration of bisphenol A to rats during the first five days of life induced hypermethylation of the promoter regions of estrogen receptors in the testes, thus leading to disruption of fertility and spermatogenesis.

We consider that FSH/LH addition might rescue some of the *TFAM* expression during bisphenol treatment, but the mechanism might be independent of *SIRT1*.

The melting curve analysis performed in the real-time RT-qPCR and also the obtained gene expression are entitling us to consider that we have done the correct primer design.

CONCLUSIONS

SIRT1 and *TFAM* are interconnected in the regulation of mitochondrial function, biogenesis, and maintenance. *SIRT1*'s deacetylation of *TFAM* and its impact on mitochondrial gene expression, combined with *TFAM*'s role as a transcription factor for mitochondrial genes, highlight their cooperative roles in ensuring proper mitochondrial function and health.

Dysregulation of either *SIRT1* or *TFAM* can have detrimental effects on mitochondrial function and overall cellular health.

Based on our working protocol and outcome of the RT-qPCR, we conclude that we have correctly designed the primers.

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