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ORIGINAL ARTICLE

Selenium affects genes associated with immunity and apoptosis in *in vitro* follicles of ewes

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ABSTRACT. Selenium plays an important role in bodily functions. It activates immune cells such as neutrophils and immunity through T cells. However, its role in ovarian follicular gene expression has not been reported. The effects of selenomethionine (SeMet) on gene expression in *in vitro* ewe follicles and their relationship with immunity and apoptosis were studied. Preovulatory follicles were randomly cultured, and SeMet was added to the culture, followed by incubation for 24 h. Total RNA was extracted from follicles and placed on a microarray chip. Gene expression was analyzed using GenArise and classified using the DAVID v6.8 bioinformatics program based on the Gene Ontology and Kyoto Encyclopedia for Genes and Genomes (KEGG). Gene pathways were designed using Cytoscape v3.7.2. There were 2,538 differentially expressed genes (DEGs): 1,228 upregulated and 1,310 downregulated. The upregulated genes were classified into 32, 18, and 12 nodes related to biological processes, cellular components, and molecular functions, respectively. KEGG assigned these genes to 41 metabolic and signaling pathways. 94 genes were involved in eight pathways associated with immune processes. The expression levels of *CD8*, *NFAT2*, and *CD48* were quantified using RT-qPCR. Selenium activated gene expression in the *in vitro* preovulatory follicles of ewes, some of which were related to the immune system. The relationship among *CD8*, *NFAT2*, and *CD48* genes suggests a possible immune pathway related to follicular apoptosis regulation.

Keywords: DNA microarray, preovulatory follicles, organic selenium, apoptosis, follicular immunity.

INTRODUCTION

Designing improved methods for managing production and reproduction is important in animal farms. Therefore, extending the knowledge of how the immune system regulates ovarian events to improve the follicular state is important, because ovarian follicles contain developing oocytes. Consequently, oocytes should be maintained in homeostasis (health) to ensure fertilization and future development of the embryo and fetus, thereby contributing to the enhanced reproductive success of farmed animals.

Selenium is an important trace element in animal physiology. Its main function is to act as an antioxidant by interacting with selenoproteins. It also functions in general immune regulation by promoting T helper cell differentiation. Additionally, it increases the proliferation and development of preovulatory follicles. Selenium increases steroidogenesis in goat granulosa cells. According to Yang *et al.* (2017), Selenium reduced apoptosis (programmed cell death) in rat ovaries and increases embryonic development *in vitro*. Similarly, it also activates gene expression and improves the formation and quality of bovine yolk blastocysts.

In footrot animals, selenium increases the mRNA levels of proteins involved in leukocyte migration. The mRNA expression of selenoproteins and proteins related to lipid metabolism also increased in lambs fed diets containing selenium. Different lipid forms arise during lipid metabolism, including cholesterol, which is a constitutive molecule in the cell membranes and the base of steroid molecules in ovarian follicles.

Several studies have been conducted on ovarian tissues. Ovarian follicle development proceeds through several stages of maturation until differentiation or apoptosis (programmed cell death), which produces high quantities of reactive oxygen species, some of which are linked to immune processes and the maintenance of follicular health. The current study was based on the hypothesis that selenium modifies gene expression in ovarian tissues, some of which may be linked to the immune system. Therefore, this study aimed to investigate the effects of selenomethionine (SeMet) on the follicular tissue of ewes, detect changes in gene expression, and select immunity-related genes with a high probability of being present in the follicles.

MATERIALS AND METHODS

Ovaries of adult ewes of mixed breeds (mainly wool-based) were collected from a local slaughterhouse following Mexican Official Norm NOM-051-ZOO-1995. The ovaries were immersed in cold saline solution (0.9% sodium chloride; 4 °C) with 100 mg L^{-1} gentamicin, kept on ice, and transported to the laboratory.

Subsequently, the adipose tissue and ligaments surrounding the ovaries were removed and the ovaries were repeatedly washed with cold saline solution (habitual handling). Follicles were extracted from the rest of the ovar-

ian tissue under a stereoscopic microscope (Carl Zeiss de México S.A. de C. V., CDMX, Mexico) and follicular diameter was measured using a caliper rule. Preovulatory follicles (diameter ≥ 6 mm) were dissected from ovaries under sterile conditions using a scalpel and dissecting forceps. Before *in vitro* culture, the follicles were immersed in a 2:1 mixture of culture medium (MEM; Sigma-Aldrich, St. Louis, MO, S.A de RL. de CV., Toluca, Estado de México) and saline solution with 100 mg L-1 gentamicin.

In vitro culture

The experimental design for the culture included two treatments: a) without SeMet (control) and b) with SeMet (18 replicates per treatment).

Thirty-six preovulatory follicles were selected and placed in Eppendorf tubes containing 1 mL culture medium. The control group consisted of 18 preovulatory follicles in culture medium containing 100 IU mL^{-1} equine chorionic gonadotropin (eCG; VIRBAC XICO S.A. de C.V.). The treatment consisted of 18 follicles (six tubes with three follicles each) treated with 10 ng mL^{-1} SeMet (Sigma-Aldrich Química, S.A de RL. de CV. Toluca, Estado de México) in addition to other components of the control. The follicles were incubated for 24 h; incubations were carried out at 37°C in 95% humidified air with 5% CO^2. After 24 h, the follicles were removed from the culture medium, placed in clean Eppendorf tubes, frozen in liquid nitrogen, and stored at -80°C until total RNA extraction.

Total RNA extraction

Total RNA was extracted from follicles using TRIzol Reagent and Invitrogen (Thermo Fisher Scientific Inc. MA, USA) according to the manufacturer's instructions. Total RNA was quantitatively and qualitatively evaluated (based on absorbance) using a NanoDrop spectrophotometer (ND-2000; Thermo Scientific, MA, USA), and band separation was performed on 1.5% agarose gel (Green & Sambrook, 2012). Total RNA samples were used for cDNA synthesis using SuperScript IV Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific Inc., MA, USA), and microarray hybridization (Vallée *et al.*, 2006) was performed using the cDNA microarray M22K _11_06. Alexa 555 and Alexa 647 were used to label samples developed in the DNA Microarray Unit of the National Autonomous University of Mexico.

Evaluation of genes related to immunity and apoptosis in *in vitro* SeMet-treated follicles

The three differentially expressed genes (DEGs) that were overexpressed in *in vitro* SeMet-treated follicles were selected and validated by RT-qPCR. Furthermore, differences in expression among the three genes were analyzed using primers (Table 1) based on the ewe transcripts (the primers were designed using the CDS of ewe genes reported in GenBank and Primer3 software) instead of the expression determined using mouse-based microarrays. RT-qPCR was performed at the Genomic Services Unit (LANGEBIO-CINVESTAV, Gto, Mexico) using three technical replicates. The ACTB (beta-actin) gene was used as a housekeeping gene. The $2^{-\Delta\Delta CT}$ quantification method was used for qPCR.

Quantification and classification of data

To quantify and analyze gene expression, the fluorescence data emitted by the microarray were normalized us-

Table 1.
Primers used for qPCR of the three upregulated genes involved in immunity and apoptotic process in *in vitro* SeMet-treated follicles of ewes.

Target gene	Primer sequence (5´-3´)	Sense	Product size (bp)	GenBank reference
ACTB	CATCGGCAATGAGCGGTTCC	Forward	146	NM_001009784
	CCGTGTTGGCGTAGAGGT	Reverse		
CD8A	GGAGTGAACTGAACCCTGGA	Forward	225	XM_027966754
	TGACCCAGGAGCATGTTTGA	Reverse		
NFATc2	CCACTTTTCTCCAACAGCCC	Forward	193	XM_027977331
	GAACCCACCCACTGAAACAC	Reverse		
CD48	GCTTGGCATCCTTCTCATGG	Forward	250	XM_012183742
	GCCCTTCTCCGAGTCTTTCT	Reverse		

ing the GenArise microarray analysis tool, and genes with z-scores between 1.5 and 2 were considered DEGs.

The DEGs were further classified based on term annotation using the Gene Ontology (GO) database, which considers $p \leq 0.01$ and $p < 0.05$, and the Kyoto Encyclopedia for Genes and Genomes (KEGG) database of metabolic pathways and signaling ($p < 0.05$) using DAVID Bioinformatics Resources v6.8. The interactions and pathways (gene networks) of genes with immune activity were studied using Cytoscape v3.7.2.

RESULTS

After comparing fluorescence emission between follicles exposed to selenium and those not exposed to selenium using mouse microarray data, 2,538 DEGs were identified. Of these, 1,228 and 1,310 genes were upregulated and downregulated, respectively.

GO and KEGG classification of DEGs in *in vitro* SeMet-treated preovulatory follicles

A total of 1,411 genes were analyzed using the DAVID program for GO analysis and categorized into three functional nodes ($p < 0.01$): 32 nodes related to biological processes (BP), 18 related to cellular components (CC), and 12 related to molecular functions (MF). According to GO analysis, most genes were ranked in the BP category. The upregulated genes under BP were related to the transcription, transport, and development of multicellular organisms, whereas those under CC included genes encoding proteins related to the cytoplasm, nucleus, cytosol, and membrane. (Figure 1).

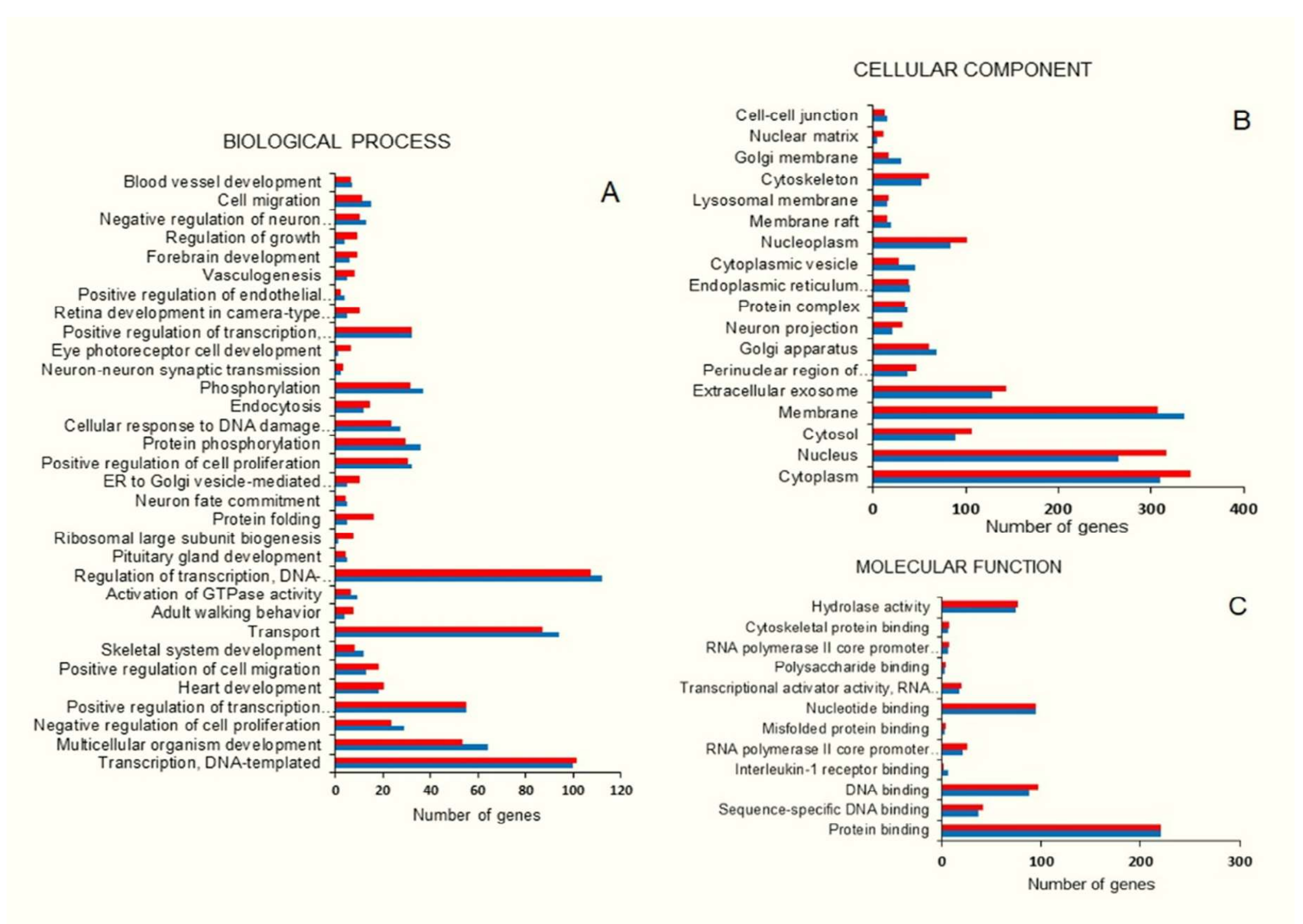

Figure 1.
Gene Ontology (GO)-based number and ranking of differentially expressed genes in *in vitro* SeMet-treated preovulatory follicles of ewes ($p \leq 0.01$). Red bars = upregulated genes; blue bars = downregulated genes. A: Genes involved in biological processes; B: Genes involved in cellular components; C: Genes involved in molecular functions.

One hundred ninety-five DEGs were associated with immune processes, and GO analysis classified them into 115 functional genes in BP, 42 in CC, and 38 in MF ($p < 0.05$). Most of the upregulated genes were related to positive (40) and negative (59) regulation of apoptosis (Table 2; $p < 0.01$).

DEGs related to metabolic and signaling pathways were classified into 41 pathways using KEGG ($p \leq 0.05$). The pathways with the highest number of upregulated genes were related to cancer, infectious diseases, and activation and signaling (e.g., mitogen-activated protein kinase

Table 2.
Genes associated with biological processes, specifically the immune system, in *in vitro* SeMet-treated preovulatory ovarian follicles of ewes.

Term	Genes
Term: GO:0006897 ~endocytosis. Fold enrichment: 1.8, p = 0.0048	**Up-regulated** (14): *CD209B, RAB1A, ARHGAP27, AP2M1, APP, ARR3, EPS15, PIK3CB, PSTPIP1, PACSIN1, PACSIN3, SORT1, SNX1, SNX4.* **Down-regulated** (12): *ATP9A, CD209B, GAPVD1, HRAS, RAB34, ARHGAP27, WIPF2, ARC, AP2M1, APP, ARR3, A4.*
Term: GO:2000353 ~positive regulation of endothelial cells apoptotic process. Fold enrichment: 4.7, p = 0.0066	**Up-regulated** (2): *FOXO3, AGER.* **Down-regulated** (4): *AKR1C18, COL18A1, PRKCI, RGCC.*
Term: GO:0043524 ~negative regulation of neuron apoptotic process. Fold enrichment: 1.80, p =0.0066	**Up-regulated** (10): *ISL1, JAK2, KRAS, CACNA1A, MTNR1B, MT1, NGF, NRBP2, SOD1, UBE2V2.* **Down-regulated** (13): *BTG2, HRAS, CORO1A, EN1, FOXB1, FZD9, HSPD1, HIPK2, LGMN, MDK, NPM1, PRKCI, SIX4.*
Term: GO:0043065 ~positive regulation of apoptotic process. Fold enrichment: 1.50, p = 0.0108	**Up-regulated** (18): *JAK2, SOX4, B4GALT1, WT1, AIFM1, AGER, CASP1, CTNNB1, CCAR1, DAPK3, EEF1E1, FOXO3, MSX2, NGF, NET1, PTEN, ZAK, TOP2A.* **Down-regulated** (21): *CLIP3, RBCK1, APBB2, CTNNBL1, CLU, DHODH, HSPD1, HMGA2, ING5, IP6K2, IL24, MUC2, LPAR1, NTRK3, KCNMA1, SFRP1, SAV1, STK4, TEX261, TFAP4, TGFB1.*
Term: GO:0010667 ~negative regulation of cardiac muscle cells apoptotic process. Fold enrichment: 3.38, p = 0.0145	**Up-regulated** (5): *PCMT1, QK, JAK2, HSF1, NFE2L2.* **Down-regulated** (2): *GHRH, NPM1.*
Term: GO:0006909 ~phagocytosis. Fold enrichment: 2.41, p = 0.0205	**Up-regulated** (4): *ANXA3, MEGF10, TULP1, TUSC2.* **Down-regulated** (6): *GATA2, CORO1A, EIF2AK1, HCK, ABL2, VAV1.*
Term: GO:0043066 ~negative regulation of apoptotic process. Fold enrichment: 1.31, p = 0.0286	**Up-regulated** (32): *ARF4, ARAF, BCL11B, CD38, CD74, HHIP, JAK2, ARHGAP10, SMARCA4, TRIAP1, WT1, AIPL1, AVEN, CRYAB, CBS, TEK, EPCAM, FXN, GAS1, MAEA, MSX2, PROP1, PRDX5, PTEN, PLAC8, PLK1, RHBDD1, SLC40A1, SPHK1, SOD1, TEX11, ZFP830.* **Down-regulated** (27): *OGG1, BTG2, LIMS1, PTK2, STIL, ALB, AKR1B3, APBB2, ARNT2, BFAR, CLU, DPEP1, GNAQ, HSPD1, HELLS, HCK, HMGA2, IL24, LTK, NPM1, PAX4, KCNJ1, PRKAA2, SFRP1, SIX4, SPHK2, UCP2.*
Term: GO:0038096 ~gamma receptor signaling pathway involved in phagocytosis. Fold enrichment: 9.41, p = 0.0341	**Up-regulated**: 0 **Up-regulated** (3): *WAS, WASL, CDC42.*
Term: GO:0042110 ~T-cells activation. Fold enrichment: 2.58, p = 0.0495	**Up-regulated** (2): *CD48, CD8A.* **Down-regulated** (5): *WAS, HSPD1, ITGAV, TGFB1, VAV1.*
Term: GO:0030097 ~hemopoiesis. Fold enrichment: 1.90, p = 0.0395	**Up-regulated** (6): *CD34, TIPARP, ADD2, CTNNB1, TEK, GFI1.* **Down-regulated** (7): *GATA2, BRCA2, CUL4A, KIRREL3, PICALM, RUNX1, SFRP1.*

(MAPK) and Ca^{2+} signaling). Eight gene nodes were related to immunity: natural killer cell-mediated cytotoxicity, Wnt signaling, B-cell receptor signaling, T-cell receptor signaling, transendothelial migration of leukocytes, MAPK signaling, inflammatory mediator regulation of transient receptor potential (TRP) channels, and phagocytosis mediated by crystallizable fragment (Fc) gamma R (Figure 2).

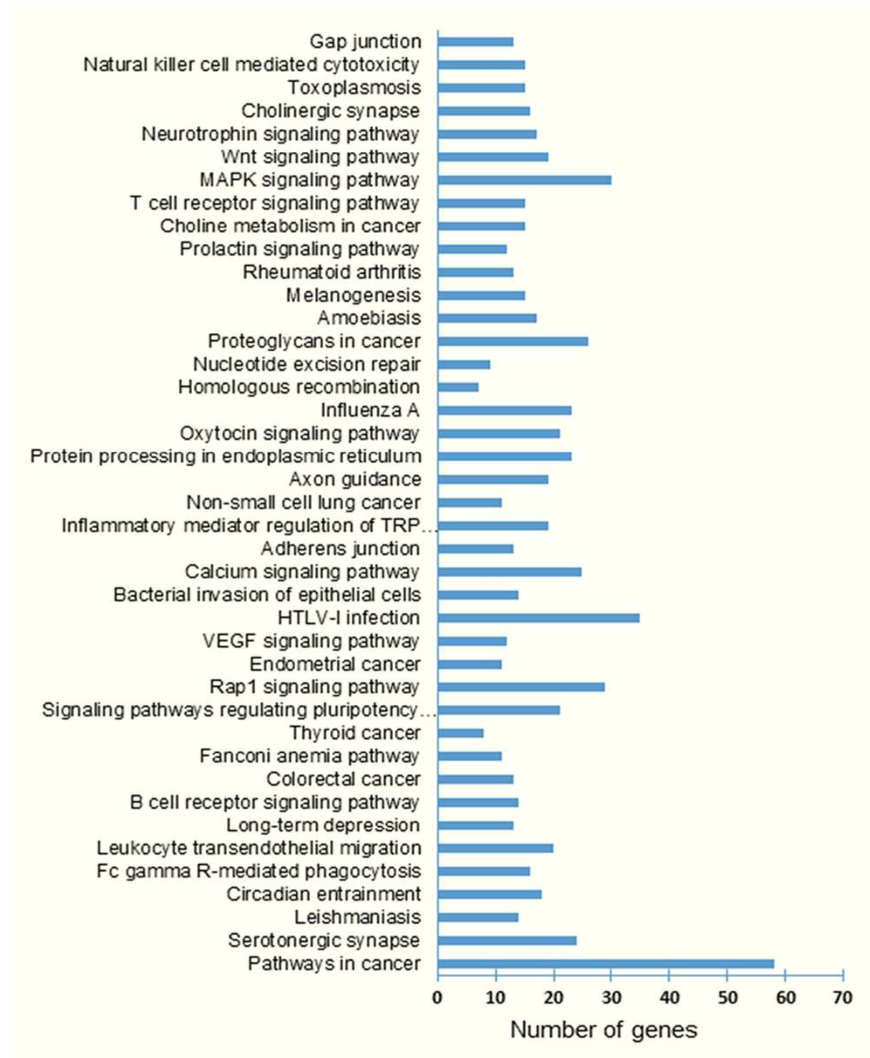

Figure 2.
Kyoto Encyclopedia for Genes and Genomes (KEGG)-based classification of differentially expressed genes (DEGs) related to metabolic and signaling pathways in *in vitro* SeMet-treated preovulatory follicles of ewes.

Immune gene network in *in vitro* SeMet-treated preovulatory follicles of ewes

The gene expression network involved in the immune system of *in vitro* SeMet-treated preovulatory follicles of ewes was constructed using 100 upregulated and downregulated genes.

Using GO terms, eight gene nodes were found to be related to cellular and humoral immunity: T-cell receptor signaling, B-cell receptor signaling, natural killer cell-mediated cytotoxicity, and leukocyte transendothelial migration. The upregulated genes included *CACNA1*, *VAV2*, *PIK3CD*, and *WNT8A*. *CD48* is linked to the natural killer cell pathway and mediates cytotoxicity, which, in turn, is linked to the T-cell receptor signaling pathway mediated by *NFATC2* to communicate with *CD8A*. The pathways used by *CD48*, *NFATC2*, and *CD8A* are active immune pathways involved in cell apoptosis (Figure 3; lines with stars). *NFATC2* was not only associated with *CD48* and *CD8A* through the natural killer cell-mediated cytotoxicity pathway and T cell receptor signaling pathway, but was also related to the *WNT* signal-

ing pathway and B cell receptor signaling pathway (Figure 3; lines with squares); the latter involved humoral immunity. The *CD8A*, *NFATC2*, and *CD48* genes were also located in these pathways and subsequently validated by RT-qPCR.

RT-qPCR validation of the three genes involved in immunity and apoptosis in *in vitro* SeMet-treated preovulatory follicles of ewes

Changes in *CD8A*, *CD48*, and *NFATC2* gene expression in *in vitro* SeMet-treated preovulatory follicles of ewes observed using a mouse gene-based microarray were further confirmed using RT-qPCR, and gene expression was compared between the two techniques (Figure 4). The gene expression signals obtained using microarrays were higher than those obtained using RT-qPCR.

DISCUSSION

In this study, 2,538 DEGs were identified, suggesting that *in vitro* selenium (10 ng mL^{-1}) treatment of preovulatory follicles affects the transcriptome. These results are in accordance with those observed in ewes supplemented with a high concentration of organic selenium (0.40 mg), wherein 1,186 differentially expressed transcripts, includ-

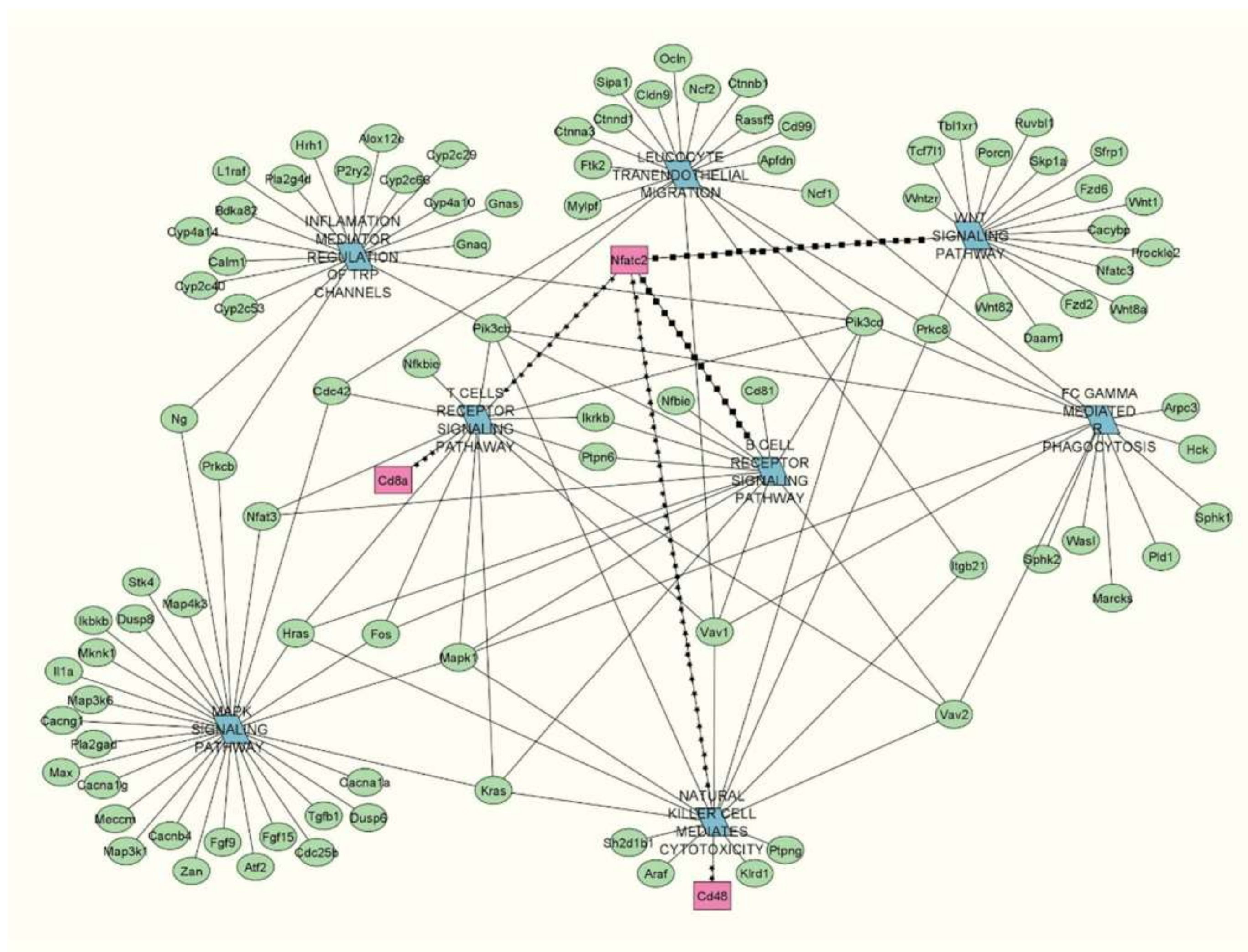

Figure 3.
Network of genes involved in the immune process of *in vitro* SeMet-treated preovulatory follicles of ewes. Circles represent 105 genes: blue polygon, principal family nodes; pink rectangles and dotted lines; genes tested by RT-qPCR and involved in the apoptotic process by two family nodes related to immunity.

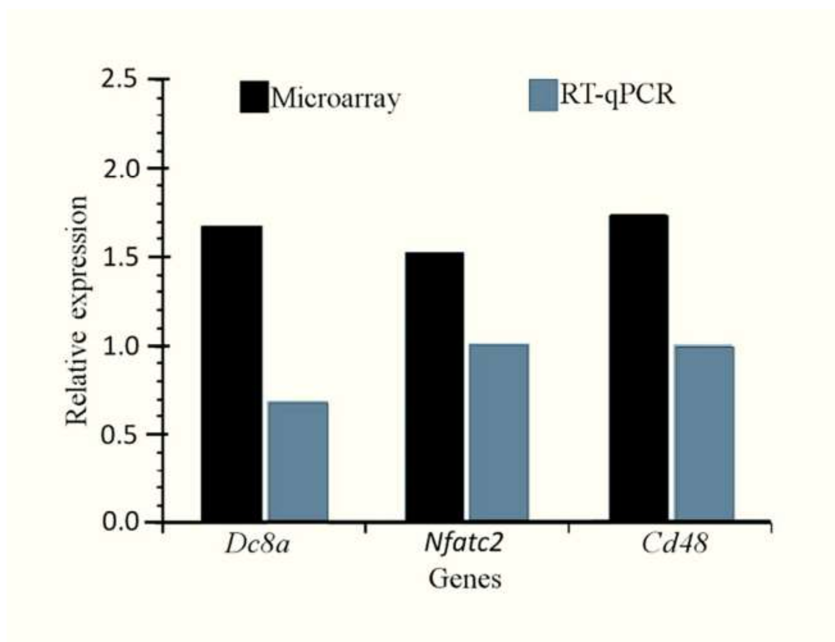

Figure 4.
The three immunity-related genes expressed in *in vitro* SeMet-treated preovulatory follicles of ewes and validated using RT-qPCR.

ing those of genes related to immunity, were observed. However, the study by Elgendy *et al.* (2016) was conducted on blood cells and not on ovarian follicles, as in the present study. Notably, mouse microarray results were consistent with the genes expressed in ovine follicles. Furthermore, Elgendy *et al.* (2016) reported that the number of transcripts detected using a mouse microarray was higher than that detected using an ovine microarray.

According to GO, most DEGs were classified under the BP category, specifically those related to transcription processes, which can be attributed to the activation of selenoprotein transcription and elevation of biological activities involving selenium.

In this study, functional analysis of these genes identified 41 pathways, including those related to the immune system. These results are similar to those reported by Song *et al.* (2013) for pig leukocytes treated with selenium (0.54 mg kg^{-1} of diet), wherein 28 upregulated and 24 downregulated genes were observed. The first factor is related to immunity. Similar results have been reported by Elgendy *et al.* (2016). Collectively, these results highlight the importance of selenium in the genetic regulation of immune processes in various tissues.

Functional clusters, particularly those related to cell activity and death (apoptosis), were identified in 195 genes. These results can be attributed to the follicular development process in which both events (i.e., cell differentiation and death) occur in the follicles.

Construction of a gene network with 94 genes highlighted the nodes and pathways related to immune processes. Among these nodes, there are gene interactions for apoptosis process regulation, namely, a signaling node of the T cell receptor that, together with interleukins, acts in many immunological conditions, and a node of cytotoxicity mediated by natural killer cells that are related to multiple functions in the immune process and during apoptosis. This study verifies the direct relationship between these cell nodes.

These results indicate that the participation of these gene nodes, together with leukocyte transendothelial migration, is required to regulate preovulatory follicle activity and apoptosis. Previously, it has been observed that selenium causes autophagy and apoptosis in cancer cells. Furthermore, it plays an alternative role in apoptosis owing to its antioxidant capacity and free radical scavenging activity. Therefore, at the follicular level, while some cells are in the process of apoptosis, others overcome the event and move towards differentiation, which can also be verified by the sub-regulation of genes involved in follicular cell proliferation observed in this study. Considering the differences in follicular cell populations, the effects of Se on gene expression in different follicular cell populations should be studied further.

Three genes involved in immunity, *CD8A*, *NFATC2*, and *CD48*, were evaluated by RT-qPCR. These genes were upregulated in preovulatory follicles in response to SeMet.

CD8 and *CD4* appear to be involved in signaling damage during follicular apoptosis. The *CD8A* and *CD4* genes are essential because their protein products act as receptors for the major histocompatibility complex (MHC), which facilitates the presentation of antigens to T cells and activates specific immune responses. CD4(+) T cells are essential for the formation of protective memory CD8(+) T cells following infection or immunization. Therefore, CD8 is important for maintaining immunity throughout the lifetime, which can act during repeated attacks by microorganisms or against cyclic physiological events, such as apoptosis, during which it is necessary to eliminate toxic products from the cell.

NFATC2 encodes a transcription factor that regulates the genes and proteins involved in immune responses. For example, *NFATC1* and *NFATC2* deficient T cells cause a scarcity of Th cytokine production, hyperactivation of B cells, and *NFAT* controls the exhaustion of CD8+ T cells. Therefore, in the absence of *NFAT*, T cells exhibit decreased immune protection. The relationship between *NFATC2* and *CD8* was observed in our study, and *CD8*, *NFATC2*, and *CD48* were found to be directly linked. Another important effect of *NFAT* is cytokine regulation, which plays an important role in regulating apoptosis and follicular atresia. Additionally, NFATC2 a member of the *NFAT* family, is a transcription factor that activates the expression of cytokine and T cell genes and is involved in the induction and apoptosis of T lymphocytes.

Similar to *CD8* and *NFATC2*, *CD48* is linked to the natural killer cell-mediated cytotoxicity pathway. *CD48* activates innate lymphoid cell progenitors in humans. This receptor is present in different cell types, including natural killer cells and innate lymphocytes. *CD48* also participates in immune-cell adhesion. Similarly, *CD48* on T cells can promote T cell receptor signaling and activation and anchorage between GPI or Lck. *CD48* allows them to contact cholesterol-rich lipid rafts, facilitating intracellular calcium flux in a cholesterol-dependent mechanism. Both follicle and luteal tissues have large amounts of lipids (cholesterol) for steroidogenesis, which may explain why the expression of this gene was observed in this study. The results of this study are related to the observations of Basini & Tamanini (2000), who reported an increase in steroidogenic activity of granulosa cells following selenium administration.

Selenium acts as an immunostimulator by activating T cell proliferation and natural killer cell differentiation, and by stimulating other innate immune cells. To our knowledge, this is the first study to determine the effects of Se on gene expression in preovulatory follicles *in vitro*. Furthermore, the pathway through which *CD8A*, *NFATC2*, and *CD48* were quantitatively (RT-qPCR) validated is related to the immune response triggered by cytotoxicity. Based on the pathway formed by *CD8A*, *NFATC2*, and *CD48*, we suggest that these genes could be part of the immune control during apoptosis. Another reason may be that the differentiation process of follicular cells is similar to that observed in T cells and natural killer cells. The results of this study highlight the potential importance of selenium in enhancing immunity in the preovulatory follicles.

The *CD8A*, *NFATC2*, and *CD48* genes were validated using RT-qPCR. Although the gene expression levels differed between the two techniques, they were complementary, implying that one technique was better than the other owing to the precision of each technique, sequence, selection of primers, primer design, and other experimental conditions. Regardless of the signals detected, the genes identified using mouse microarrays were also detected using RT-qPCR based on ewe genes. Independent of the technique used, the expression of *CD8A*, *NFATC2*, and *CD48* in *in vitro* SeMet-treated preovulatory follicles of ewes was verified. Remarkably, a microarray based on mouse gene libraries was helpful in the absence of an ovine-origin microarray. Verification of the expression of *CD8A*, *NFATC2*, and *CD48* could have been carried out using other techniques in addition to qPCR, which may be a limitation of our study. Finally, this study provides useful information regarding the reproductive system of ewes. Selenium administration can improve reproduction in ewes by increasing the quality of the follicular immune system and the possibility of mature oocytes.

CONCLUSIONS

SeMet activates several genes, including those related to the immune system, in the preovulatory follicles of ewes *in vitro*, some of which are likely involved in follicular cell apoptosis. If follicular apoptosis is diminished, free radical levels and follicular toxicity are decreased. This cascade of events can increase follicular development and number of lambs per reproductive season.

Acknowledgments

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Conflict of Interest

María Monserrat L.V. was supported by grants from the National Council for Science and Technology (CONACyT). The authors declare that they have no conflicts of interest.

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