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BIOLOGICAL RELATIONSHIP BETWEEN INSULIN-LIKE GROWTH FACTORS (IGF1 AND IGF2) GENES AND STEROIDOGENESIS IN OVINE OVARIAN ANTRAL FOLLICLES

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1.ABSTRACT

Reproductive inefficiency in sheep is a critical source of reduction in small ruminant profitability worldwide. The importance of insulin-like growth factors1 and 2 belonging to the insulin-like growth factors system were identified as pivotal key roles in ovarian follicular development and steroidogenesis in different species. This study aimed to detect the IGF-1 and IGF-2 genes and Oestrogen levels in the ovine small and large antral follicles collected from an abattoir using conventional PCR and ELISA, respectively, as well as to investigate the relationships between IGFs genes and steroidogenesis using bioinformatics approaches. Our results showed that IGF-1, IGF-2, and CYP19A1 were significantly present in the local sheep ovary's small and large antral follicles. Furthermore, the oestrogen levels were significantly different between these two ovarian follicle types. The string database suggested that IGFs and CYP19 have strong relationships. Functional annotation (GO terms) revealed that IGFs and CYP19 genes were mainly associated with responses to hormone and insulin growth factor bindings. In addition, KEGG pathways mapped to ovarian steroidogenesis and Ras signaling pathways. Overall, these findings are consistent with the critical functions of IGF-1 and IGF-2 in regulating steroidogenesis, growth, and viability of follicular cells in the sheep ovary. Therefore, evaluation of IGFs genes can be useful for the understanding of reproduction performance in ewes.

KEYWORDS: *IGF, ovine, ovarian follicles, PCR , Bioinformatics.*

2.INTRODUCTION

Ovine is an economically important animal in Iraq as it provides about 60%, 25%, and 15% of meat, dairy, and wool products, respectively. Sheep can be raised in intensive and extensive production systems and cold to hot environments (Dhahir et al., 2022 and FAO. Food and Agriculture., 2003 and Ghoneim et al., 1974). For the sheep livestock industry, appropriate ewe folliculogenesis is pivotal to the decline in the infertility rate and produces healthy offspring(Al-Thuwaini, 2021). Follicles are the functional unit of the ovary within which the female germ cell, the oocyte, develops and matures before being released during ovulation(Hernandez-Medrano et al., 2012 and Monniaux, 2016). Folliculogenesis is a highly organised process that involves the growth and regression of ovarian follicles within the cortex compartment. Follicles can be classified as pre-antral (gonadotropin-independent) and antral phases. Pre-antral follicles include primordial, primary, and secondary follicles, while antral follicles are also referred to as tertiary follicles, this typical phase is mediated by autocrine and paracrine signaling (Webb et al., 2007). The process of Folliculogenesis in sheep and cattle requires about 180 days for a follicle to grow from its primordial state (40 um) to ovulatory (sheep ≥ 5 mm) size (Cahill & Mauléon, 1980; Juengel et al., 2018). In cyclic ewes, 3-4 waves of follicle development occur during the estrous cycle, ovarian antral follicles emerge and grow from a pool of 14-15 small follicles (2 or 3 mm in diameter) in a wave-like pattern, reaching diameters of ≥5mm before regression or ovulation(Driancourt, 1991). The intraovarian regulatory factors have stimulatory effects on the development and function of ovarian follicles, these factors act by regulating the expression of several protein-coding genes involved in cellular proliferation and hormone production within the

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ruminant reproductive organs(Hernandez-Medrano et al., 2012; Webb et al., 2007). Mammalian ovaries express a large set of growth factors such as insulin-like growth factors (IGFs: IGF-1 and IGF-2). IGF-1,is a70 amino-acid single chain peptide that is expressed under the control of growth hormone, whereas, IGF-2 is a67 amino-acid single chain peptide, but its production is not associated with growth hormone. They both revealed significant amino acid sequence homology and share a similar ternary structure(LeRoith et al., 2021). Expression of the IGFsis postulated to be conserved across several mammalian species including mares, humans, bovines, sheep and pig (Garcia-Guerra et al., 2018; Ginther et al., 2003; Hunter et al., 2004; Mihm & Evans, 2008). The main purposes of the present study were (1) to investigate the presence of IGF-1 and -2 genes in ovarian antral follicles, (2) to analyse the follicular oestradiol level, and 3) to evaluate the functional annotation by using in silico-based analysis to verify whether there is an association between IGFs genes and steroidogenesis.

3. MATERIALS AND METHODS

3.1. Ovarian collection

Ovaries from 50 ewes were collected from an abattoir in Duhok city, and transported to the college of Veterinary Medicine, the university of Duhok in PBS on ice. In the Duhok research center laboratory, the ovaries were rinsed with 70% Ethyl alcohol then follicles were dissected out and their diameters measured using Vernier a Calliper. Follicles were classified into healthy and atretic according to published criteria and only healthy follicles were used in this research.

3.2. Follicular fluid isolation

Small (< 3 mm diameter) and large antral follicles (>5 mm) were collected as well as follicular fluid aspirated by syringe, placed in an Eppendorf tube then centrifuged at 500g for 5 mins to obtain the follicular fluid-free cells (Gutiérrez et al., 1997) .

3.3. DNA isolation

After removing follicular fluid, DNA was isolated from ovarian antral follicles using the Add Prep Genomic DNA extraction kit (Addbioinc, Korea) based on the manufacturer´s instructions. The concentration of DNA was confirmed by a Nanodrop spectrophotometer.

3.4. Conventional PCR

The genes were amplified using primers and a PCR kit following manufacturer᾽s instructions. The primers were designed using Primer3 and blasted against the nucleotide database of the BLASTN (http://www.ncbi.nlm.nih.gov/BLAST) website to ensure identity among reported BLAST sequences for the target genes . In a total volume of 20 µL, a PCR mixture was performed. Each reaction contained 10 µL 2× Add Start Taq Master (Addbio, Korea), 0.3 µm of each forward and reverse ovine-specific primer for IGF1, IGF2, CYP19, and alpha-tubulin (Table 1), and 2 µL DNA. PCR cycling conditions were as follows; 5 min at 95ºC followed by 35 cycles of the 30s at 95ºC, 30s at 58ºC, and 30s at 72ºC followed by 5min at 72ºC (Ye et al., 2012).

Table (1): PCR primers sequence were used in the present study.

Gene	Primer Sequence 5'-3'	Amplicon Size bp	Reference
IGF-1	FW primer ATTACAAAGCTGCCTGCCCCTT RV primer CACATCTGCTAATACACCTTAC CCG	265	(Darwish et al., 2017)
IGF-2	FW primer CAAGTTCTTCCAATCTGACACCTG RV primer TAGAGATGTTGTTCTGATCCCCTC	423	This study
CYP19	FW primer CAGACTGTTGTTGGTGAAAGAGAC RV primer CTCAAGTCTGTGCATTCTTCCAAG	207	This study
Alpha-Tubulin	FW primer AAATACATGGCCTGCTGCCT RV primer GCACCCATCCACAAACTGGA	110	This study

3.5. Gel Electrophoresis of Amplicons

The genes were detected using a 1.2% (w/v) agarose gel prepared from 1X (TAE) buffer and Prime Safe Dye. A DNA marker of 1 kb was used and gel was visualized under the UV transilluminator light.0

3.6. Oestradiol Quantification

The oestrogen concentration in the follicular fluid was determined using the Sheep Estrogen ELISA kit (BT LAB, Shanghai Korain, China) as directed by the manufacturer. After collection, follicular fluid specimens were immediately centrifuged at 6500 rpm at 4 C for 10 minutes and the fluid was quantified. The results were calculated using a logit-log of calibration curve of known standard ranges (0.2-60ng/L). The intra and inter-assay coefficients of variance were 5 and 6.5%, respectively.

3.7. Protein-protein interactions and gene ontology analysis

Protein-protein interaction (PPI) analysis of identified genes was performed using STRING (https://string-db.org/) based on ovine species (Ovis aeries) . The interactions between IGFs and steroidogenic genes were mapped into the PPIs network. Furthermore, String enrichment analysis was utilised to detect Gene Ontology (GO) term and Kyoto Encyclopedia of Gene and Genome (KEGG) pathways. STRING enable an analysis of genes from several organisms including ovine and it can recognize PPI types based on direct physical as well as indirect functional associations. The defined GO terms and KEGG pathways with ($P<0.01$) were considered significantly enriched in the detected genes (Szklarczyk et al., 2015)..

3.8. Statistical analyses

Results from oestrogen experiments were analysed using T-tests in GraphPad statistical software v8.2. Data are presented as mean ± SEM, and found to be statistically significant at ($P \leq 0.05$).

4.RESULTS

4.1. Morphological classification of the ovarian antral Follicles

Follicle size was measured using vernier Caliper, follicles were categorized into populations of small and large antral follicles according to their diameter (Figure 1, A). Healthy follicles were found to have a well-vascularized wall and transparent amber follicular fluid without debris observed under dissecting microscope (Figure 1, B).

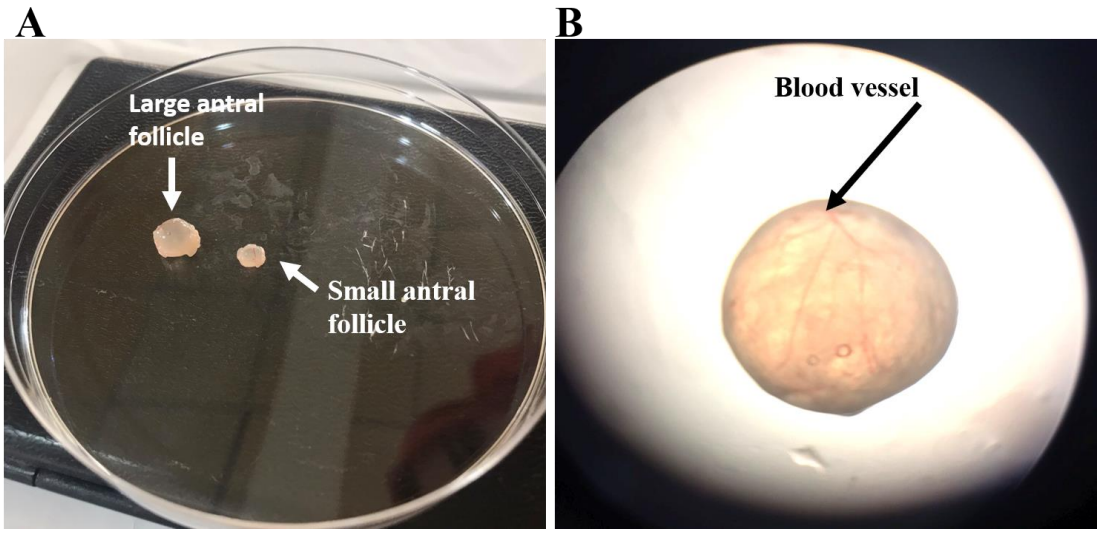

Fig. (1. A):Image of the sheep ovarian antral follicles (small and large follicles) after separation from ovaries. B). Image of the healthy antral follicle showing the blood vessel under a dissecting microscope.

4.2. Detection of IGF genes in sheep ovarian antral follicles

The BLAST results for the IGF-1, IGF-2, CYP19, and reference gene, alpha-tubulin primers revealed a 100% homology of the nucleotide sequences to Ovis Aries genes, mRNAs, and transcript variants. Afterward, ovine antral follicles were examined using PCR and gel electrophoresis to detect the presence of these genes. IGF-1 and -2 were found to be abundant in both small and large antral follicles (Figures 2A, B). Moreover, the steroidogenic gene (Cyp19) was observed in both types of sheep antral follicles. However, the large antral

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follicle showed a strong band of CYP19 (Figure 2. C). Furthermore, the internal control, alpha-tubulin, demonstrated a clear band in both antral follicles and the negative control (No template) showed no signal (Figure.2).

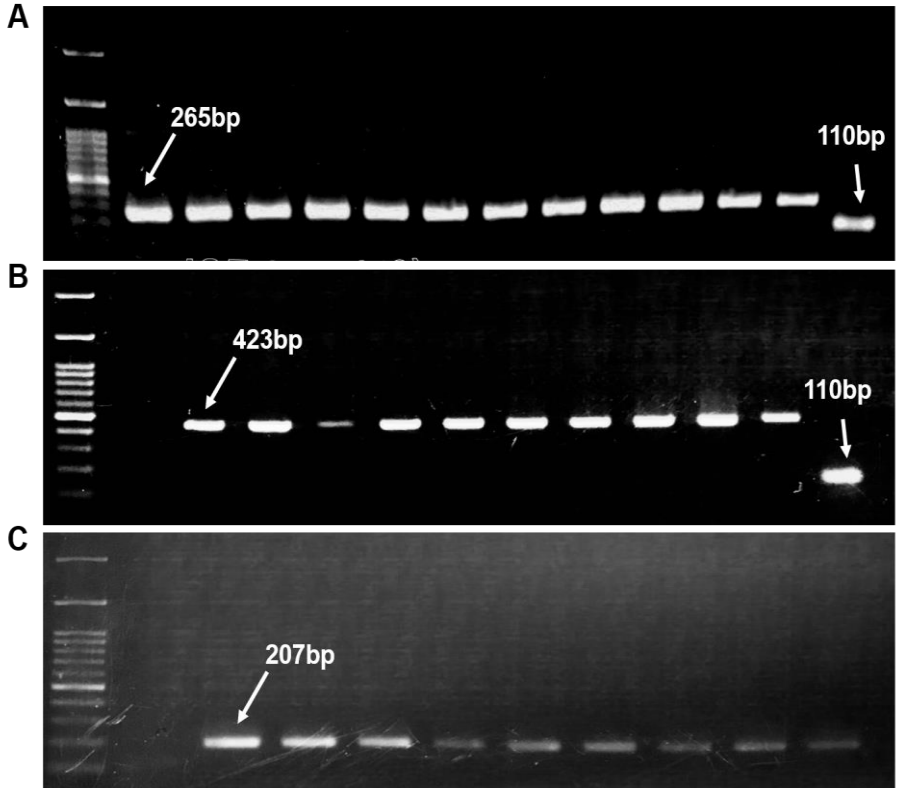

Fig. (2): Analysis of PCR amplified genes on the 1.2% agarose gel electrophoresis. Lane 1 is a 100bp DNA marker, Lane 2 is negative control (No template) and the last lane (110) is positive control (alpha-tubulin). A. Lanes (265 bp) are the amplified fragment for the IGF-1 gene. B. lanes (423 bp) are the amplified fragment for the IGF-2 gene and the last line is the amplified gene for the positive control (110). C. Lines (207bp) are amplified genes for CYP19A1.

4.3. Follicular fluid Estrogen concentration analysis

The concentration of estrogen in the follicular fluid was used to determine the difference between the main reproductive hormone and the viability between the two antral follicle types of the sheep. Generally, it was indicated that the content of estrogen in large antral follicles was higher than that in the small antral follicle (Figure 3).

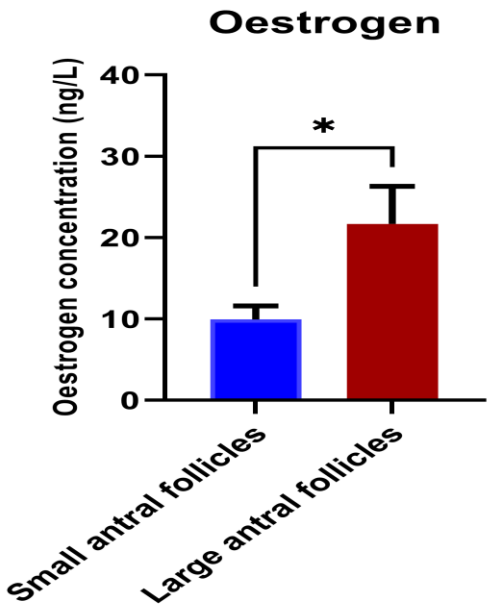

Fig. (3): The concentration of the Oestrogen in the small and large antral follicular fluid in sheep (n=35) using ELISA. The statistical analysis was performed using GraphPad Prism v8.2 and P value <0.05 was represented significant (*).

4.4.PPI network and gene enrichment analysis of the genes

Protein-protein interactions involving the IGF1, IGF2, and CYP19 genes were analyzed using STRING. As shown in Figure 4, a total of 84 edges and 23 nodes were obtained. The network revealed that there were significant relationships between IGFs genes and CYP19 in sheep.

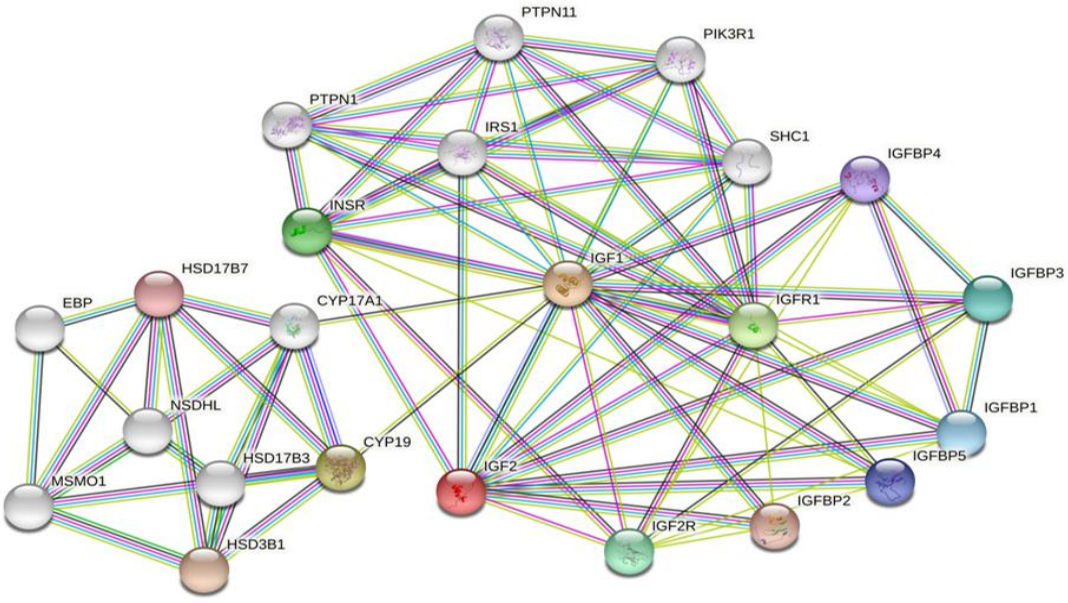

Fig. (4): Protein-protein interaction network showing interactions among (IGF-1, IGF-2, and CYP19) genes. The nodes and edges were constructed using the STRING tool. Each node represents a gene, while edges represent protein-protein associations.

GO enrichment analysis revealed that these genes were potentially present in20 biological processes (BP), 7 cellular components (CC), and 17 molecular functions (MF)(Figure 5. A-C). The detected genes were directly linked to GO terms including a response to peptide hormone

(GO:0043434, P<6.56E-09), Endomembrane system (GO:0012505, P<1.36E-05), and Insulin-like growth factor binding (GO:0005520, P<2.18E-14), in BP, CC and MF categories, respectively.

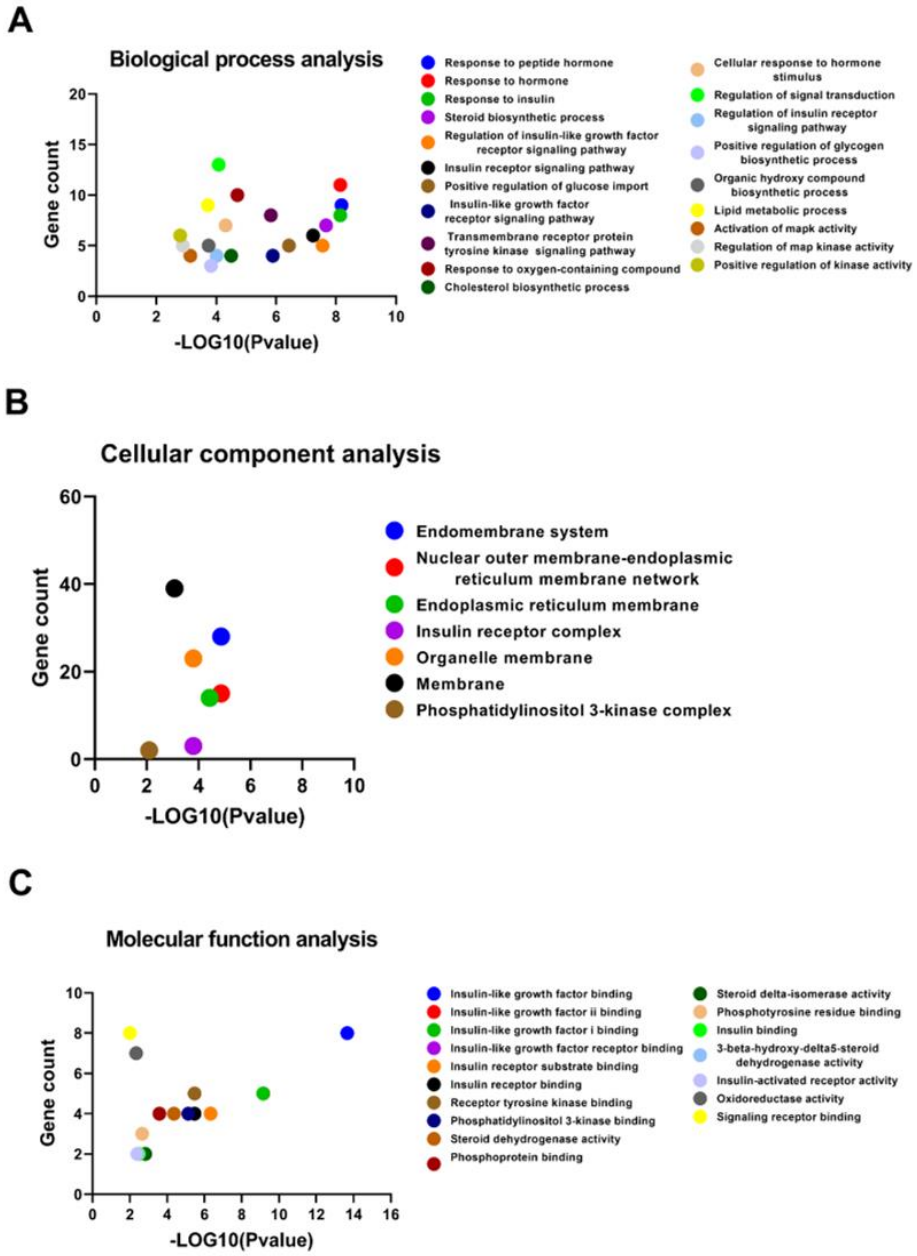

Fig. (5): Functional annotation terms enriched for detected genes (IGF1, IGF2, and CYP19) usingString enrichment software. The bubble represents a GO term, each color represents a functional group, and the term enrichment significance, is $p \leq 0.01$. Terms are connected based on shared genes. A. Biological processes (BP), B. Cellular components (CC), and C. molecular function (MF). The bubble plots were generated by GraphPad prism v8.2.

KEGG pathway enrichment showed the top 20 significantly enriched pathways for the identified genes, the most significant of which was the ovarian steroidogenesis (oas04913, P<1.00E-08) and Ras signaling pathway (oas04014, P<3.24E-06) (Figure 6 A). The insulin signaling pathway, the GnRH signaling pathway, the steroid hormone biosynthesis pathway, and the TGF- signaling pathway in granulosa cells are all involved in the mediation of ovarian steroidogenesis (Figure 6 B).

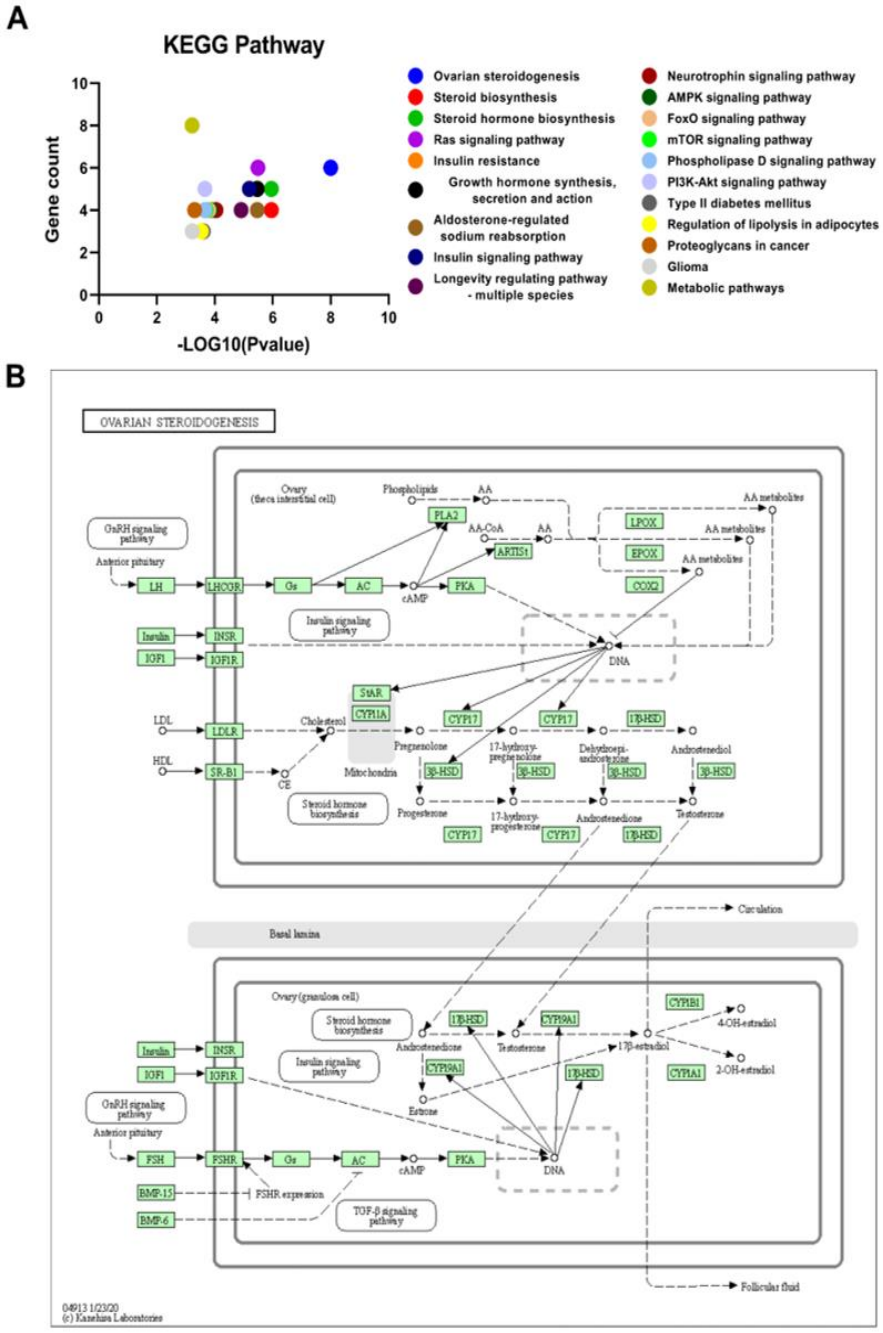

Fig. (6): A. Functional KEGG pathways enriched (P≤0.01) for the identified genes (IGF-1, IGF-2, and CYP19) using String enrichment software. Pathways are connected based on shared genes. B. Ovarian steroidogenesis pathway displayed as an interaction network using the KEGG tool. The bubble plot of the KEGG pathway was generated by GraphPad prism v8.2.

5. DISCUSSION

The current study sought to characterize the presence of IGF-1 and -2 in ovine ovarian antral follicles and to identify their functional roles using bioinformatic analysis. Previous studies have utilized IGFs to improve the ovulation rates and litter size in different species including sheep (for a review see (Juengel et al., 2021)). Our study reported that IGF-1 and-2 were markedly observed in the ovine small and large antral follicles. In agreement with this finding, several studies have reported that IGF-1 expression was dramatically abundant in the steroidogenic cells of the sheep antral follicles(Leeuwenberg et al., 1995).Nevertheless, the mRNA level of IGF-1 was relatively reduced in all follicle types throughout the oestrus

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cyclewhileIGF-2level was remarkedly seen in the healthy antral follicles in sheep and their expression decreased as the follicles become atretic(Hastie & Haresign, 2006). Our study demonstrated thatCYP19A1 was detected in both types of antral follicles, whereas, oestrogen level was significantly enriched in large antral follicles in sheep ovary. In sheep, the IGFs system has stimulatory effects on both cellular proliferation and oestradiol production (Monget et al., 2002; Scaramuzzi et al., 1999; Skinner et al., 2010). Activation of IGF-1 promotes steroidogenesis through inducing the production of essential enzymes, such as P450 cholesterol desmolase (CYP11A1) and aromatase (a key step in the biosynthesis of oestrogen, CYP19A1(Scaramuzzi et al., 2011; Webb et al., 2007).

Our PPI analyses identified that IGF-1is strongly associated with steroidogenic genes and that IGF-2 enhances steroidogenesis via IGF-1. Three receptors mediate IGF-2 signaling, the IGF1R, the insulin receptor (INSR), and the IGF1R–IR-A hybrid receptor(Talia et al., 2021). Gene enrichment analysis showed the top biological process was a response to the peptide hormone process which exhibit a relevant role in the regulation of follicular development and fertility (Ipsa et al., 2019). In addition, the detected genes were highly engaged in the molecular functions (GO terms) related to Insulin-like growth factor bindings which have a central role in the induction and inhibition of several growth factor signaling pathways that are potentially responsible for the regulation of proliferation, maturation, and apoptosis of ovarian follicular cells(Mazerbourg & Monget, 2018). Interestingly, the identified genes in the topKEGG terms were significantly involved in ovarian steroidogenesis and Ras signaling pathways. The genes inthe ovarian steroidogenesis pathway are the key roles in metabolic hormone production in the ovarian cells.Consistent with these KEGG findings, several in vitro studies have shown the biological and molecular functions of the IGFs in different species including sheep (Monte et al., 2019; Singh et al., 2015; Spicer & Aad, 2007; Yu et al., 2003). Moreover, thefunctional roles of these genes in the Ras signaling pathway were identified as participating in cellular proliferation and survival, in turn regulates the ovulation and luteinization of the antral follicles(Fan et al., 2012; Fan & Richards, 2010). These results can provide insight into certain cellular mechanisms that may be involved in the effects of IGF-1 and -2 in regulating cellular proliferation, survival, and maturation of female reproductive cells.

6. CONCLUSION

The present study revealed that IGF-1, IGF-2, and CYP19A1 were notably found in small and large antral follicles in the local ovine ovary. We also provided functional annotation evidence suggesting the involvement of IGFs which can signal through their IGF receptors in steroid regulation and mitotic pathways in the ovary. Overall, given the importance of IGF signaling in regulating steroidogenesis and follicular development, future investigations are needed to focus on the in vitro and in vivo effects of preantral exposure to IGFs to clarify their significant impacts on ovine reproductive performance.

7. ACKNOWLEDGMENT

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8. CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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