Dynamic expression pattern of genes related to implantation and vascularization in pregnant dromedary camels

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1. Abstract

Pregnancy is a complex process that requires critical events such as successful implantation of embryo into the receptive endometrium as well as placental vascularization. In the current study we investigated the expression pattern of genes related to implantation and placental formation as insulin like growth factor (IGF1), vascular endothelial growth factor A (VEGFA), FOS and JUN protooncogenes in pregnant compared to non-pregnant she-camels. For this, 30 genital tracts of she camels (15 non-pregnant and 15 pregnant) as well as blood samples were collected from slaughterhouses. Uterine tissues of she camel after slaughter were divided into two portions; the first portion was used for RNA extraction and genes expression, while the second portion was immediately fixed in 10% neutral buffer formalin for histological examination. Serum progesterone level was measured between the two groups. The results revealed that, the IGF1, VEGFA, c-FOS and c-JUN mRNAs were up-regulated ($P<0.001$) in the pregnant group compared to non-pregnant ones. Also, progesterone was higher ($P<0.001$) in pregnant group than those obtained in non-pregnant. Histological examination of the uterine tissue of pregnant she-camel showed uterine mucosa with more glands, less fibrous stroma, and increased number of blood vessels compared to non-pregnant. In conclusion, the tight regulation of genes (IGF1, VEGFA, FOS and JUN) related to endometrial receptivity and placental formation is essential for embryonic implantation in pregnant she-camel.

Keywords: *Camel, Pregnancy, Uterine tissue, Gene expression, Serum progesterone*

2. Introduction

Dromedary camels are one of the most important domestic animals in arid and semi-arid regions because they can produce high-quality food at a low cost and harsh environments. Camel is extremely resistant to high temperatures, solar radiation, and water shortage **[1].** The reproduction of female dromedary camels is characterized by seasonal activity and induced ovulation **[2].** Camel reproductive efficiency is generally thought to be low in natural conditions. This is most likely due to a relatively short breeding season, a longer pre-pubertal period, a long gestation period (13 months), a prolonged (8–10 months) period of lactation-related anestrus, a long inter-calving interval, and the deficiency in reproductive techniques such as: embryo transfer and artificial insemination **[3].**

One of the most common complaints in the camel reproduction is foetal and perinatal loss, which is one of the most important

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factors contributing to the reduced reproductive efficiency **[4].** The most common type of reproductive loss in South American Camelids (SACs) is early embryonic death, which is estimated to affect 10–15% of all pregnancies in the first 60 days of pregnancy **[5].** It was indicated that the percentage of embryonic death is reaching up to 60–80 percent in the first 90 days of gestation in extreme cases **[5].** Moreover**,** the estimation of the camel foetus age provides useful information in the diagnosis of gestation during clinical examination **[6]** and to study the aborted feti cases as well as those collected at abattoirs **[7].**

The process of implantation and successful pregnancy exhibit a great diversity based on anatomo-histology of the uterus as well as endocrine and molecular regulation between the uterine and the embryonic tissues **[8].** The conceptus (embryo/fetus as well as associated extra embryonic membranes) and the endometrium interact together during pregnancy **[9,10].** Progesterone (P4) and placental hormones, which are required for pregnancy establishment and maintenance, have an effect on conceptus–endometrial interfaces during the peri-implantation period. Additionally, the expression of endometrial genes localized in the endometrium is well established to be regulated by the ovarian steroid progesterone via the P4 receptor **[11,12].** So we focused on the molecular mechanisms of some genes that related to the establishment and progression of pregnancy as the insulin-like growth factor (IGF), vascular endothelial growth factor A (VEGFA), c-FOS and c-JUN proto-oncogenes.

During pregnancy, the IGF family plays a critical role in embryonic development **[13].** It was found that IGF1 stimulates embryonic development by reducing apoptosis and increasing cell proliferation **[14,15].**

The VEGFA, is a potent regulator of vascular functions such as angiogenesis, vasculogenesis, vascular permeability, and lymphangiogenesis **[16,17].** Furthermore, it is indicator of uterine receptivity for implantation in humans and bovine **[9].** Its release can be stimulated by IGF1 **[18]**. The activities of c-Jun and c-Fos proto-oncogenes are involved in cellular proliferation, differentiation, and invasion processes **[19,20].** The c-Fos/c-Jun are widely expressed and activated in the human placenta, implying their role in the regulation of placentation and foetal development **[21].**

Most embryonic losses are a consequence of loss of communication between embryo and uterus that leads to implantation and placentation failure **[22]**. The mechanisms leading to embryo implantation and survival in she camels is far from being well explained, Therefore, the present study aimed to investigate the expression profile of IGF1, VEGFA, c-FOS and c-JUN transcripts in pregnant *vs.* non-pregnant she-camels.

3.Materials and Methods

Ethical approval

The research protocol was discussed and accepted by the Council of Theriogenology Department, Faculty of Veterinary Medicine, Cairo University, approval number (Vet CU 12/10/2021/368).

3.1. Collection of genital tracts and blood samples

During the breeding season (November, 2020 to March, 2021), 30 genital tracts (15 non-pregnant and 15 pregnant) of mature female dromedary camels with unknown history of reproduction were obtained from slaughterhouses in Cairo-Egypt. At the time of slaughter, all camels were examined by qualified veterinarians and determined to be clinically healthy. The reproductive tracts and whole blood were collected immediately after slaughter and transported on chilled saline (0.9% NaCL) to the laboratory. The genital tracts were washed three times in the laboratory with warm sterile saline,then washed once quickly in

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ethanol 70% then rewashed three times again with warm sterile saline. The size of ovarian follicles and the presence of small CL were used to define the stage of oestrus in non-gravid uterus (conceptus-free). If the follicles were smaller than 1 cm in diameter, the female had most likely not ovulated yet **[23,24].** The stage of pregnancy was roughly estimated for gravid uterus by measuring the crown-vertebral rump length (CVRL) of the foetuses using the formula: age in days = CVRL + 23.99/0.366 **[25].** The CVRL was 10.9±1.63 cm correspond to 94.5 ± 4.42 days of pregnancy. Following that, the uterine tissues were divided into two parts; the first was snap frozen at –80˚C for RNA isolation and gene expression, and the second was immediately fixed in 10% neutral buffer formalin for histological examination.

3.2. RNA isolation and cDNA synthesis

Total RNA was isolated from both pregnant and non-pregnant uterine tissues using the miRNeasy Mini kit (Qiagen) according to the manufacturer's protocol. To remove potential contaminations of genomic DNA, the extracted RNA was subjected to on-column DNA digestion with RNase free DNase (Qiagen). Nano-drop 2000/c was used to measure total RNA concentration and degradation level (Thermo Fisher Scientifc, Wilmington, USA). The cDNA for gene expression analysis was produced from isolated total RNA using the GScript first strand synthesis kit (Gene direx, Taiwan) according to the manufacturer's protocol. Concisely, 20 µL total reaction volume was prepared: 5 µL of total RNA samples mixed with 4 µL 5X 1st strand buffer, 1 µLOligo$(dT)_{20}$, 1 µLDTT , 1 µLdNTP mix, 2µLGScript RTase and 6 µL RNase free water was added to the RNA mixture in a PCR strip and run in a thermocycler (BioRad, USA) programmed at 55˚C, 60 minutes; 70˚C, 15 min and hold at 4˚C. Immediately the synthesized cDNA was stored at −20˚C.

3.3 .Quantitative real-time PCR analysis

Gene specific primers; IGF1, VEGFA, c-fos and c-Jun is presented in (Table1). The specificity of each primer amplicon was assessed by sequencing the PCR products. Quantitative real-time PCR of mRNAs will be performed in a StratageneMx3005P Real-Time PCR System (Agilent Technologies), using SYBR Green/ROX Mix (ThermoScientific), with the following program: 95˚C for 10 min, 40 cycles at 95˚C for 15 s, 60˚C for 30 s and 72˚C for 30 s. Melting curve was estimated at the end of the run to observe the specificity of the amplification. The data was analyzed by the comparative threshold cycle (ΔΔCt) method and normalization was achieved using geometric mean of housekeeping genes (GAPDH, and β-ACTIN). NormFinder was used to select the most stable reference gene for gene expression **[26].**

3.4. Assay of the progesterone concentrations

Serum samples were separated in the lab and stored at -20˚C for further analysis. Progesterone concentrations were determined using a commercial ELISA kit (progesterone Imbian-ELISA Kit) according to the manufacturer's assay procedure. The optical density (OD) value was measured at 450 nm, using ELISA microplate reader (BioTek ELx800, Vermont, USA), sensitivity was not exceed 0.5 nmol/l.

3.5. Histological examination

Tissue samples were taken and fixed in 10% neutral buffered formalin. Following fixation, tissues were processed in various grades of alcohols and xylenes before being embedded in paraffin. For light microscopy, 5 m sections were cut and routinely stained with hematoxylin and eosin (H&E) **[27].** Tissue slides were

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examined with an Olympus BX43 light microscope equipped with a DP-27 digital camera (Olympus, Japan).

3.6. Data Analysis

Unpaired t-test was used to analyze gene expression data and P4 levels in serum. The data was graphed and presented as mean ±SEM, with *P*-values <0.05 considered statistically significant. GraphPad Prism 9.0 was used for data analysis and plotting (Graphpad Software, Inc., SanDiego, CA, USA).

4. Results

4.1. The gene expression pattern related to implantation, vascularization and placental formation

The mRNA expression of IGF1, VEGFA, c-FOS and c-JUN was significantly (*P*<0.001) up-regulated in the pregnant compared to those observed in non-pregnant she-camels (Fig. 1).

4.2. The serum level of progesterone in pregnant and non-pregnant she camel

Progesterone concentration was significantly increased (*P*<0.001) in the pregnant group compared to those recorded in the non-pregnant ones (Fig. 2).

4.3.Histological examination of the uterine tissue of the pregnant and non-pregnant she camel

The non-pregnant she-camel uterine tissue revealed that, the uterine glands was surrounded with proliferating fibrous tissue. While the uterine tissue of pregnant she-camel revealed that, the uterine mucosa was surrounded with more glands, less fibrous stroma, and increased number of blood vessels (Fig. 3).

5. Discussion

Endometrial receptivity is main concept in implantation biology **[28].** Major causes of early pregnancy loss include insufficient angiogenesis and steroidogenesis at the feto-maternal interface **[29].** A result, the current study reveals the pattern of expression of genes associated with implantation and placental formation. Our data revealed that the IGF-1 was significantly upregulated in the pregnant group compared to non-pregnant. The result is in agreement with **[30]**, who focused that the pregnant cows that have higher levels of IGF1 mRNA in reproductive tissues. During early pregnancy, the expression of IGF1 and its receptor increased in cats **[31].** In mares, IGF-I was found to be embryonic and endometrial origin during early pregnancy **[32].** Early pregnant sows have higher levels of IGF1 mRNA than non-pregnant sows **[33, 34].** However, there was a significant decrease in IGF1 at gestational day 20 in the endometrium of healthy attachment site gilts compared to virgin ones, but according to immunohistochemistry, IGF-1 was expressed in all endothelium and epithelium **[35].** The IGF1 was found to be expressed in early pregnant bitches **[36].** However, there was no difference in the expression of IGF1 mRNA between the peri-implantation group and the non-pregnant bitches **[37].** The levels of uterine IGF-I mRNA expression in ewes were reduced in early pregnancy than at estrus **[38].**Also, IGF-I was found in high concentrations in the early developing placentas of equines and pigs using immunohistochemistry **[39,40].** Furthermore, IGF1 mRNA levels were suppressed in small gestational age neonates compared to appropriately grown neonates, representing their role in placentation **[41].** So we indicated that IGF1 play role in growth regulation and placental formation, recorded here in.

In the present study the expression of VEGFA gene was higher in the pregnant

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group than that in non-pregnant she camels. This is similarly with the expression pattern of VEGF in canine that was upregulated in early pregnancy, which was found to be significant in the pre-implantation period **[42].** In addition the VEGFA increased in utero placental compartment following implantation and during midgestation, denoting the role of VEGFA in establishing the uterine edema and vascularization required for embryo attachment and implantation as it was found in capillaries and epithelial uterine components **[43].** In contrast, VEGFA mRNA in alpacas, bovine, and heifers was highly expressed in the endometrium of non-pregnant animals than early pregnant females **[9,44,45].** This is may attributed to species difference. At day 15, mRNA expression of all VEGFA isoforms was higher in the caruncular endometrial tissue of heifers than in the intercaruncular endometrial tissue. It is possible that the implantation process will begin at the caruncular implantation sites **[45].** In pig, the VEGF transcript showed constant expression throughout the cycle, with a significant increase on days 22–25 of gestation **[18].** It also was found at the feto-maternal interface of pregnant pig uteri **[46]**. In the decidual cells of early pregnancy in humans there was intense immunostaining of VEGF **[47],** and it was also noticed during implantation in mice uterus **[48].** This might indicate the role of VEGFA in vascularization and placental formation.

FOS and JUN genes bind together to form the transcription factor activating protein 1 (AP1) complex **[49].** The AP-1 family is a main regulator of cellular invasion **[50].**

In the present study, pregnant she-camel c-fos and c-jun expression was significantly higher than non-pregnant. The proto-oncogenes c-fos and c-jun were expressed in preimplantation embryos of some species, including the pig, mouse, sheep, and bovine **[51, 52, 53, 54].** The peak expression of c-jun was shown to be in early gestation, accompanying with the regulation of gene expression related to cell proliferation in the human placenta **[55].** In contrary to the suppression of c-fos and c-jun mRNA expression in the human endometrium during pregnancy, this concurs with the disappearance of estrogen receptors **[56].** Previous research revealed that FOS suppression resulted in reduction of cell invasion **[57].** Data from immunostaining revealed a reduction in the number of FOS positive extra-villous trophoblasts in early-onset Preeclampsia placentas **[58]**. This may reveal their role in implantation and cell proliferation process.

In our result, the serum progesterone level was higher in the pregnant she camels group than non-pregnant one. This coincides with the pervious result reported during early pregnancy in dromedary camels **[60],** alpcas **[61],** cow **[62],** goat [**63]** indicating its importance in pregnancy maintenance.

In the current study the histological examination revealed that the uterine tissue of the non-pregnant she-camel showed uterine glands with proliferating fibrous tissue surrounding in contrast to the uterine tissue of pregnant she-camel that showed uterine mucosa with more glands, less fibrous stroma, and increased number of blood vessels. As the luteal progesterone effect and histotroph production for embryo survival during early pregnancy **[64].**

Progesterone was showed to regulate IGF-1 expression in the endometrium **[65]**. IGFs primarily act on endothelial and luteal cells to promote angiogenesis via VEGF stimulation **[58, 66, 67]**. The VEGFA is a key angiogenic factor in human endometrium **[68]** which is also regulated by progesterone to allow uterine angiogenesis and vascular remodeling **[69].** In addition The progesterone receptor (PR) isoforms, PR-A and PR-B, cooperate with other transcription factors, such as FOS, JUN to regulate the expression of many target genes that work together to regulate the uterine epithelial proliferation, stromal

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differentiation, angiogenesis, and local immune response, allowing the uterus to be receptive for embryo implantation **[70].**

6. Conclusion

It could be concluded that the high level of serum progesterone concentrations in the pregnant she-camel could regulate the expression profiles of IGF1, VEGFA, c-Fos and c-Jun genes, thereby angiogenesis and vascular remodeling occur for successful implantation and pregnancy maintenance. Further studies are needed to investigate the molecular mechanisms that regulate the proper feto-maternal interaction in she-camels.

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Table 1: List of primers used for qRT-PCR analysis

Gene name	Accession No.	Sequence 5′ to 3′	Annealing ˚C
IGF1	XM_010990020.1	F: GAGACAGGGGCTTTTATTTC R: GACTTCGTTTTCTTGTTGGTAG	55
VEGFA	XM_010979532.2	F: GTTTACCCTCCTCCTTTTTC R: CTCTTTCTTCTCTCTGCTGATT	54
JUN	XM_031465225.1	F:TGAACTGCACAGCCAGAACA R:GGGTTGAAGTTGCTGAGGTT	54.7
FOS	XM_010976475.1	F:GTCGTGAAGACTATGACAGGA R: GCGGACTTCTCATCTTCTAAT	55
GAPDH	XM_010990867.1	F:GTCTATTACCATCTTCCAGGAG R:AATCTTGAGGGACTTGTCATAC	223
B-ACTIN	XM_010997926.1	F:CAGATCATGTTCGAGACCTT R:GTGAGGATCTTCATGAGGTAGT	221

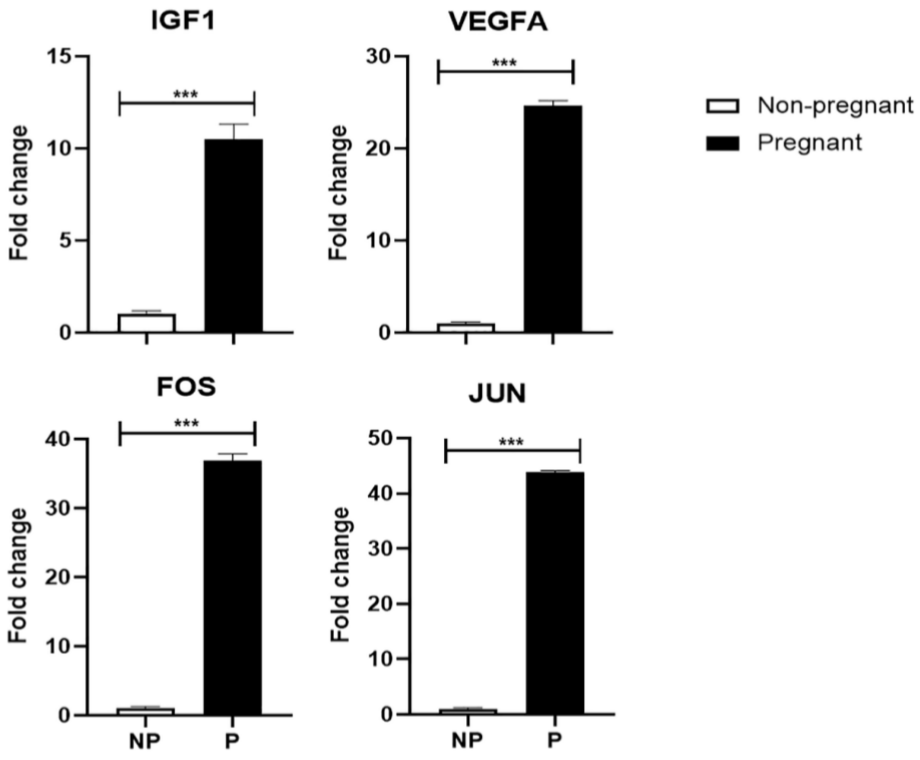

Fig. 1: The relative abundance of IGF1, VEGFA, FOS and JUN genes in pregnant and non-pregnant she-camel. ***; Statistical significant at $P< 0.001$

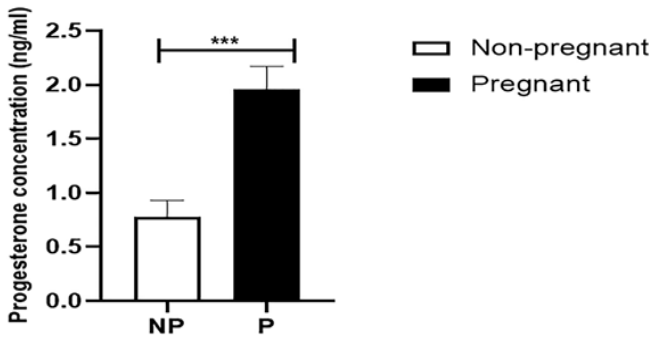

Fig. 2: Progesterone concentrations between the pregnant and non-pregnant she-camels. ***; Statistical significant at $P< 0.001$

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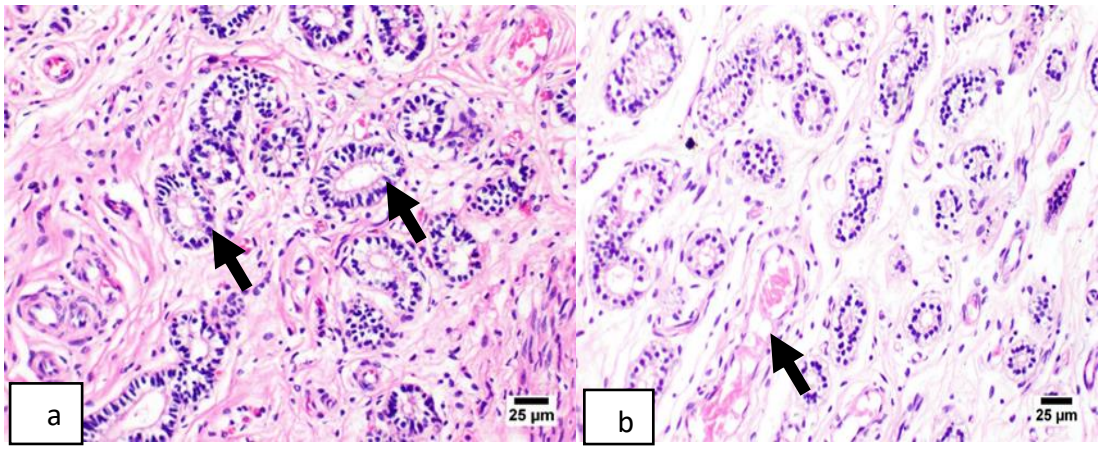

Fig. 3: Histological examination of the uterine tissues of the pregnant and non-pregnant she-camels. (a) The uterine tissue of the non-pregnant she-camel showed uterine glands (arrows) with proliferating fibrous tissue surrounding (H&E). (b) The uterine tissue of pregnant she-camel showed uterine mucosa with more glands, less fibrous stroma, and increased number of blood vessels (arrow) (H&E).

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