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Fetal Fluid Extracellular Vesicles Isolation and Gene Expression Profile in the Endometrial Tissues and Fetal Membranes at Mid-Stage Pregnant Dromedary Camels

Amira Mohamed[1], Karima Gh. M. Mahmoud [2*], Hossam R. El-Sherbiny[1], Sayed Taha Ismail[1] and Sally Ibrahim [2]

[1] *Department of Theriogenology, Faculty of Veterinary Medicine, Cairo University, Egypt.*

[2] *Department of Animal Reproduction and Artificial Insemination, Veterinary Research Institute, National Research Centre, Dokki, 12622 Giza, Egypt.*

Abstract

EXtracellular vesicles (EVs) facilitate communication between the embryo and uterus. However, there is a lack of literature on their role in camel pregnancy. Our objectives are to (1) isolate EVs from fetal fluids and (2) analyze the gene expression profile of endometrial tissues and fetal membranes in mid-stage pregnant camels. We obtained 25 camels' genital tracts at 4-9 months of gestation from slaughterhouses. The pregnancy stage was estimated by measuring the fetus's crown-vertebral-rump length. EVs were isolated from fetal fluids using ultracentrifugation. mRNA from endometrial and fetal membrane tissues was extracted for transcription analysis of the genes VEGFA, FOS, IGF1, PGF, PDGFA, JUN, and PTEN. EVs were characterized in fetal fluids using transmission and scanning electron microscopy as well as dynamic light scattering. The mRNA expression of JUN and PTEN was significantly ($P<0.01$) lower compared to other genes in the endometrial tissue and fetal membranes of pregnant camels at mid-pregnancy. However, the VEGFA, FOS, IGF1, PGF, and PDGFA had significantly higher expression levels in both endometrial tissue and fetal membranes compared to JUN and PTEN. Overall, a similar expression pattern was observed in both endometrial tissue and fetal membranes of mid-pregnancy camels. In conclusion, EVs was isolated from fetal fluids for the first time in pregnant camels. There is a strong interaction between endometrial tissue and fetal membranes, as evidenced by the similar expression of all candidate genes. Further investigation is required to explore the molecular function and the role of extracellular vesicles in camel pregnancy.

Keywords: Extracellular vesicles, Camels, Gene expression, Pregnancy.

Introduction

Camels, primarily inhabiting dry and semi-dry regions, are split into two species: the single-humped (Dromedary) camel and the double-humped (Bactrian) camel, making up 94% and 6% of the population, respectively [1]. The worldwide camel count totaled 39,295,752 individuals, with a large portion residing in African nations such as Somalia, Chad, Sudan, and Egypt [2]. According to FAO [2] statistics, Egypt's camel population reached 119,885, partly due to increased imports from African countries [3]. Camels contribute to the global economy by providing 3,114,525 tons of milk and 602,645 tons of meat [4]. Additionally, rural economies can prosper by improving their adaptability to harsh climates [5-7]. Female camels face reproductive challenges such as delayed first delivery, extended gestation periods, calving intervals [8], and pregnancy losses [9-10]. A successful implantation and pregnancy rely on a well- coordinated interaction among the ovary, endometrium and the developing conceptus during

*Corresponding author: Karima Gh. M. Mahmoud, E-mail: karimamahmoud@yahoo.com Tel.: 00201006316384
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the peri-implantation phase [11] and normal molecular function for pregnancy maintenance in female camels [12].

Extracellular vesicles, which are small, nanosized structures encased in a double-layer phospholipid membrane, serve as carriers of molecular messages, transmitting cargo from the originating cells to the recipient cells [13], and include bioactive components like lipids [14], proteins [15], mRNAs, and non-coding RNAs such as microRNAs (miRNAs) [16] in various species [17-18]. Numerous EV types have been identified, mainly categorized by size or biogenesis [19]. Ectosomes (microvesicles/microparticles) form when the plasma membrane buds outward, while exosomes originate from fusion of the plasma membrane with multivesicular bodies come from endosomes [20].

Nearly every cell type releases these EVs into the surrounding environment, found in body fluids as milk [21], blood, urine, bronchoalveolar lavage fluid, saliva, and bile [22-25] as well as the bovine oviduct [26-28]. EVs secreted by the chorioallantoic membrane and endometrium contain proteins essential for the formation of the endometrial blood vessels and angiogenesis, contributing during the initial pregnancy stages [29]. In vitro, bovine fetal fluid EVs can encourage cell growth and prevent cell death in ovarian cortical stromal cells by stimulating the production of progesterone and androstenedione [30]. Uterine EVs from the conceptus and uterine epithelium boost conceptus development, successful implantation, and subsequent placentation [31-33]. Moreover, EVs from bovine embryos have been demonstrated to enhance the survival and growth of cloned embryos and improve implantation rates [34]. EVs from uterine flushing of pregnant or non-pregnant ewes have increased trophoectoderm cell growth [35].

There has been no research on the isolation and characterization of EVs in camelids, specifically from fetal fluid. As of now, the function of extracellular vesicles in pregnant female camels (Camelus dromedaries) isn't thoroughly comprehended. Thus, our objective is to (1) isolate and characterize EVs from fetal fluid and (2) examine the gene expression profile of endometrial tissue and fetal membranes at mid-stage pregnancy in female camels.

Material and Methods

Samples collection

A total of 25 reproductive tracts from clinically healthy dromedary camels at mid-stage pregnancy (4-9 months), as determined by veterinary examination, were collected from slaughterhouses and transferred to the laboratory in chilled saline solution (NaCl 0.9%; 5°C). The pregnancy stage was approximately assessed by measuring crown-vertebral-rump length (CVRL) of the fetus, using formula: age in days = CVRL + 23.99/0.366 [36]. In the lab, the collected uteri were washed twice (3 times each) with warm physiological saline, with one rapid washing in ethanol (70%) in between. Endometrial and fetal membrane tissues were stored at -80°C for analysis of gene expression, and fetal fluids were collected for EV isolation.

Isolation of extracellular vesicles

EVs were extracted from fetal fluids through sequential centrifugation, as described by Thery et al. [37] and Gurunathan et al. [38], with slight modifications. Fetal fluid was centrifuged at 2000 xg for 20 minutes at 4°C to eliminate cellular debris and blood. Subsequently, the supernatant was moved to new tubes and centrifuged at 25,000 xg using a fixed-angle rotor for 30 minutes at 4°C, yielding a microvesicle pellet. The microvesicle pellet was resuspended in 1x PBS and stored at −80°C. Following this, the supernatant was ultracentrifuged at 120,000 xg, using a fixed-angle rotor, for 190 minutes at 4°C to acquire an exosome pellet that was resuspended in 1x PBS and stored at −80°C.

Characterization of extracellular vesicles by transmission electron microscopy (TEM)

EVs were examined using TEM following the procedure of Thery et al. [37] with modifications. Briefly, about 5 µl of each sample (three independent replicates/isolation techniques) were deposited onto a parafilm sheet and subsequently onto a carbon-coated 400-mesh copper grid directly from the sample. The grid was immersed in drop of 2% phosphotungstic acid and stained for 30–60 seconds. After drying, images were acquired through a high-resolution electron microscope.

Characterization of extracellular vesicles by scanning electron microscopy (SEM)

Following Wu et al. [39], EV samples (three separate replicates/isolation techniques) were placed on a SEM stage using carbon paste, followed by the application of a gold-palladium alloy coating of 2–5 nm was applied via sputtering prior to imaging with a scanning electron microscope, at beam energies of 25 kV.

Characterization of extracellular vesicles by dynamic light scattering (DLS)

The size and arrangement of particles within EVs extracted from fetal fluids of pregnant camels were measured using DLS. A separate pool of EV samples from fetal fluid (five separate replicates/isolation techniques) were suspended in 5 ml of 1x PBS, and the analysis was performed with NICOMP 380 Z LS instrument utilizing the 632 nm line of a HeNe laser at a 90° angle and Zeta potential at an external angle of 18.9°.

RNA isolation and synthesis of cDNA

Total RNA was extracted from endometrial and fetal membrane tissues of pregnant camels using the miRNeasy Mini kit from Qiagen due to the manufacturer's instructions. Possible genomic DNA contamination was eliminated by on-column DNA digestion with an RNase-free DNase set on-column (Qiagen). The concentration of total RNA and its degradation levels were assessed using a NanoDrop 2000/c (Thermo Fisher Scientific, Wilmington, USA). The total RNA was used to synthesize cDNA with SuperScript II (Invitrogen). A total reaction of 20 µL was prepared including: 5 µL of total RNA combined with 4 µL 5X 1st strand buffer, 1 µL Oligo(dT), 1 µL dNTP mix, 1 µL DTT , 2 µL GScript RTase, and 6 µL RNase-free water, all added to the RNA mix in a PCR strip and subsequently run in a thermocycler (BioRad, USA) set at 55°C for 60 minutes, followed by 70°C for 15 minutes, with a hold temperature set at 4°C. The resulting cDNA was preserved at -20°C.

Quantitative real-time PCR analysis

Primers specific to the genes are shown in table (1). The uniqueness of every primer amplicon was verified by sequencing the PCR products. The real-time PCR of mRNAs was conducted in a StratageneMx3005P Real-Time PCR, utilizing Simply Green qPCR Master Mix, ROX (GeneDireX, Inc), with the following program: 95°C for 10 minutes, 1 cycle; 95°C for 15 seconds, and 60°C for 60 seconds. A melting curve was estimated at the end to assess amplification specificity. Data analysis was executed using the comparative threshold cycle (ΔΔCt) method, normalizing with geometric mean of GAPDH and β-ACTIN housekeeping genes. NormFinder was employed to identify the most stable reference gene for gene expression analysis.

Data Analysis

The data were prepared in means ± SEM and examined with the Kruskal-Wallis one-way ANOVA test followed by Dunn's multiple comparisons test by the GraphPad Prism version 5.0 Software. The results were considered significant at $P < 0.05$.

Results

Morphology and size distribution of EVs

The morphology and size of EVs in the fetal fluids of dromedary camels were verified through electron microscopy (Fig. 1). The TEM images displayed a cup-like shape, which is a typical characteristic of EVs. Additionally, the TEM and SEM images indicated that EVs from fetal fluids are variable in shape. The size of the vesicles was between 81.1-119 nm. The DLS results demonstrated that EVs in the fetal fluids of dromedary camels showed a distinct asymmetrical size distribution (Fig. 2).

The gene expression pattern in the endometrial tissue and fetal membranes

The pattern of gene expression in the endometrial tissue and fetal membranes revealed that the mRNA levels of JUN and PTEN were significantly reduced ($P<0.01$) in comparison to other genes in both the endometrial tissue and fetal membranes of pregnant female camels at the mid stage of pregnancy (Fig. 3). However, VEGFA, FOS, IGF1, PGF, and PDGFA showed higher expression levels in both the endometrial tissue and fetal membranes relative to JUN and PTEN. Overall, a similar expression pattern was observed in both endometrial tissue and fetal membranes of pregnant female camels at the mid stage.

Discussion

Although some research has been carried out to identify the transcripts present in endometrial tissue during early pregnancy in camels [20, 40], the exact mechanisms through which these factors operate during embryo maintenance have yet to be defined. EVs are potentially involved in aiding the conceptus implantation into the uterine epithelium during the peri-implantation timeframe [41]. The distinctive EV population within the uterine cavity during pregnancy may be vital for successful implantation and progression of pregnancy [42]. Therefore, our study aimed to isolate EVs from fetal fluid and investigate the gene expression profiles of endometrial tissue and fetal membranes in mid-stage pregnant female camels. This includes genes related to remodeling of uterine extracellular matrix; FOS, and JUN, vascularization of the uterine tissue and formation of the placenta; VEGFA, PGF, and PDGFA, as well as embryonic growth and development; IGF1 and PTEN.

As far as we know, this is the initial research to extract EVs from fetal fluids in dromedary camels. In this study, the adapted serial ultracentrifugation technique was effective for isolating EVs from fetal fluids, accommodating the large volume of the sample [43]. The typical volume of amniotic fluid in camels was roughly 1 liter, consistently less than the allantois fluid volume, which approximates 9 liters. However, the volumes of amniotic and allantoic fluids increased as the pregnancy progressed [44]. We observed that a sequential increase of centrifugal force was optimal for isolating EVs from fetal fluids, yielding a heterogeneous EV population. Our findings align with earlier research by Barranco et al. [45]. Additionally, TEM, SEM images and DLS, showed that the size of EVs had classical spherical shapes [43,46]. Research on mammals has indicated that EVs influence various reproductive processes [47], including conceptus recognition during implantation [35], maintenance of pregnancy, and parturition [48]. Furthermore, EVs play roles in

pathological conditions like pregnancy loss, polycystic ovarian syndrome, endometriosis [49].

In this research, we investigated the expression patterns of genes associated with uterine extracellular matrix remodeling, specifically JUN and FOS, which exhibited a significant decrease ($P<0.01$) compared to other studied genes in both endometrial tissue and fetal membranes of pregnant female camels at mid-stage pregnancy. In pigs and sheep, FOS and JUN expressions levels increase during luteolysis, whereas they decrease during the onset of pregnancy [50,51].

In the current study, VEGFA, FOS, IGF1, PGF, and PDGFA were significantly higher in both endometrial tissue and fetal membranes compared to JUN and PTEN in pregnant camels. Our findings corroborate the heightened VEGF gene expression throughout the uterine body and uterine horns of pregnant camels [12]. Overall, VEGFA, PDGFA and PGF, hold significance in vascularization and placental development in pregnant female camels. Growth factors like IGF1 promote embryonic development by minimizing apoptosis and augmenting cell proliferation [52]. Additionally, Adel et al. [12] explored the necessity of IGF1 and PTEN genes for growth and development during the early stage of camel pregnancy.

Our results showed a consistent gene expression pattern across both endometrial tissue and fetal membranes of pregnant female camels at mid-stage, elucidating the communication between embryos and maternal uterine tissues. Moreover, EVs from camel fetal fluids potentially modify gene expression in endometrial tissues, suggesting significant involvement of EVs in safeguarding pregnancy. However, in the dialogue between embryo and mother, EVs notably impact gene expression and the embryo-endometrial interface, which is vital for a successful implantation [53].

Conclusion

EVs were isolated from fetal fluids for the first time in pregnant female camels. There is a tightly interaction between endometrial tissue and fetal membranes concerning the similar expression of all candidate genes. Additional research is necessary to unravel the molecular mechanisms underlying the role of EVs in camel pregnancy.

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This study received no fund from any place.

Declaration of Conflict of Interest

There is no conflict in this work.

Ethical of approval

It unnecessary to carry out the approval as the samples was obtained from slaughterhouses.

TABLE 1. List of primers utilized for qRT-PCR assessment.

Gene name	Accession No.	Sequence 5′ to 3′		Annealing °C
IGF1	**XM_010990020.1**	**F**	GAGACAGGGGCTTTTATTTC	**55**
		R	GACTTCGTTTTCTTGTTGGTAG	
VEGFA	**XM_010979532.2**	**F**	GTTTACCCTCCTCCTTTTTC	**54**
		R	CTCTTTCTTCTCTCTGCTGATT	
PDGFA	**XM_031471204.1**	**F**	GGACGGTCATTTACGAGATA	**60**
		R	CACCTGGACCTCTTTTAACTT	
FOS	**XM_010976475.1**	**F**	GTCGTGAAGACTATGACAGGA	**55**
		R	GCGGACTTCTCATCTTCTAAT	
PGF	**XM_031454061.1**	**F**	GTCTATTACCATCTTCCAGGAG	**60**
		R	R:AATCTTGAGGGACTTGTCATAC	
PTEN	**XM_010979934.1**	**F**	GGAGTAACTATTCCCAGTCAGA	**55**
		R	TTTAGCTGGCAGACTACAAAC	
JUN	**XM_010976475.1**	**F**	TGAACTGCACAGCCAGAACA	**54.7**
		R	GGGTTGAAGTTGCTGAGGTT	

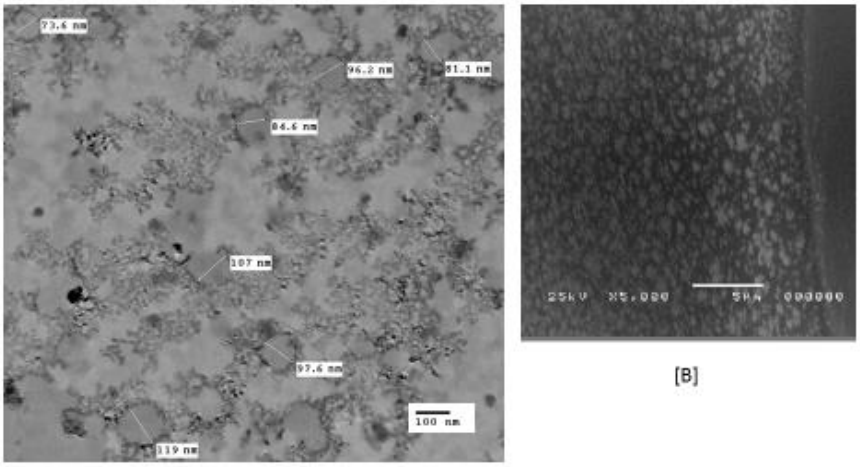

Fig. 1. Transmission electron microscopic (A) and scanning electron microscopic (B) visualization of EVs isolated from fetal fluids of dromedary camels by serial ultracentrifugation.

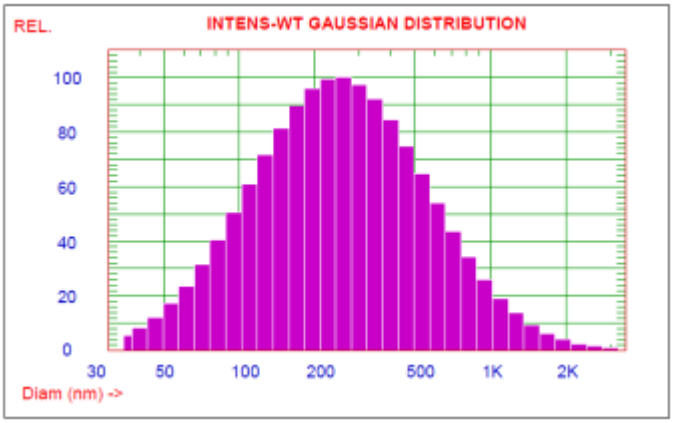

Fig. 2. Dynamic Light Scattering analysis and particle size distribution of EVs extracted from fetal fluids of dromedary camels by serial ultracentrifugation.

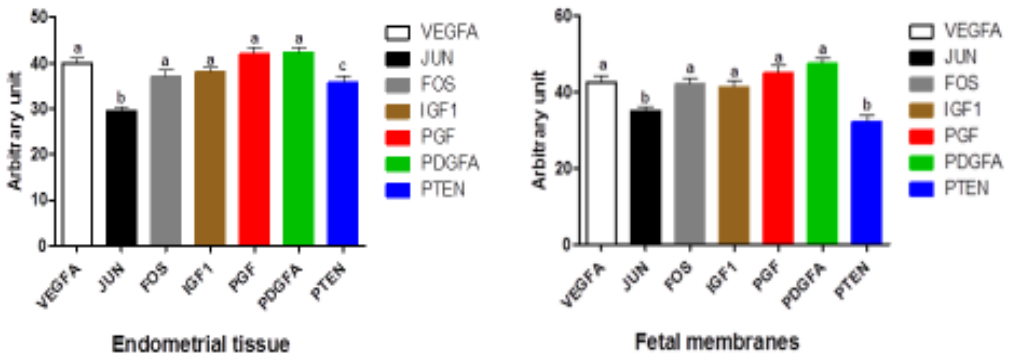

Fig. 3. Gene expression profiles in both endometrial tissue and fetal membranes collected from slaughtered pregnant she-camels at mid stage: VEGFA (vascular endothelial growth factor A), JUN (Jun proto-oncogene, AP-1 transcription factor subunit), AP-1 transcription factor subunit), FOS (Fos proto-oncogene, IGF1 (insulin like growth factor 1), PGF (placental growth factor), PDGFA (platelet derived growth factor subunit A) and PTEN (phosphatase and tensin homolog), P<0.01.

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عزل الحويصلات خارج الخلية في السائل الجنيني ونمط التعبير الجيني في أنسجة بطانة الرحم والأغشية الجنينية فى الإبل العشار في المرحلة المتوسطة من الحمل

أميرة محمد [1] ، كريمة غنيمي محمد محمود[2] ، حسام الشربيني[1]، سيد طه إسماعيل[1] وسالي إبراهيم [2]

[1] قسم التوليد والتناسل والتلقيح الاصطناعي، كلية الطب البيطري، جامعةالقاهرة، الجيزة، مصر.
[2] قسم التكاثر في الحيوان والتلقيح الاصطناعي، معهد البحوث البيطرية، المركز القومي للبحوث، الدقي، مصر.

الملخص

تُسهّل الحويصلات خارج الخلية (EVs) التواصل بين الجنين والرحم. ومع ذلك، هناك نقص في الدراسات حول دورها اثناء الحمل الإبل. لذلك تهدف الدراسة الى (1) عزل الحويصلات خارج الخلية من سوائل الجنين، و(2) تحليل نمط التعبير الجيني لأنسجة بطانة الرحم والأغشية الجنينية فى الإبل العشار في المرحلة المتوسطة من الحمل. تم تجميع عينات من الجهاز التناسلي لـ 25 جملًا في الفترة من 4 إلى 9 أشهر من الحمل من المجازر. تم تقييم مرحلة الحمل بقياس طول crown-vertebral-rump للجنين. عُزلت الحويصلات خارج الخلية من سوائل الجنين باستخدام الطرد المركزي الفائق. تم عزل الحمض النووي الريبوزي الرسول (mRNA) من أنسجة بطانة الرحم والأغشية الجنينية لتحليل التعبير الجينىVEGFA ، وFOS، وIGF1، وPGF، وPDGFA، وJUN، و .PTENتم تمييز الحويصلات خارج الخلوية (EVs) في سوائل الجنين باستخدام المجهر الإلكتروني النافذ والماسح، بالإضافة إلى التشتت الضوئي الديناميكي. كان التعبير الجيني لجينات JUN و PTENفي mRNA أقل بشكل ملحوظ ($P<0.01$) مقارنةً بالجينات الأخرى في أنسجة بطانة الرحم والأغشية الجنينية للإبل الحوامل في منتصف الحمل. ومع ذلك، كان لدى VEGFA و FOSو IGF1و PGFو PDGFAمستويات تعبير أعلى بشكل ملحوظ في كل من أنسجة بطانة الرحم والأغشية الجنينية مقارنةً بجينات JUN و .PTENوبشكل عام، لوحظ نمط تعبير مماثل في كل من أنسجة بطانة الرحم والأغشية الجنينية للإبل في منتصف الحمل.

و الخلاصة انه تم عزل الحويصلات خارج الخلوية من سوائل الجنين لأول مرة في الإبل العشار. و هناك ارتباط قوي بين أنسجة بطانة الرحم والأغشية الجنينية، كما تبين من التعبير المتشابه لجميع الجينات تحت الدراسة. و يوجد احتياج إلى مزيد من البحث لاستكشاف الوظيفة الجزيئية ودور الحويصلات خارج الخلوية اثناء الحمل في الإبل.

الكلمات الدالة: . الحويصلات خارج الخلية، الجمال، التعبير الجيني، الحمل