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RESEARCH ARTICLE

Scanning and Transmission Electron Microscopic Changes in the Ovarian Vasculature of Female Dromedaries in Relation to Breeding Season and Hormonal Profile

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ABSTRACT

The primary goal of this study was to elucidate variations in the structure of ovarian vasculature through light and electron microscopy during breeding (BS) and non-breeding season (NBS) in female dromedaries. Tissues samples of ovarian artery (OA) and ovarian vein (OV) from ten adult female dromedaries during BS and NBS were collected after the slaughter of animals. Histological analysis was done to calculate different characteristics of OA and OV through Hematoxylin & Eosin, Masson's trichrome and Weigert's elastin staining. Ultrastructural changes were evaluated by transmission (TEM) and scanning electron microscopy (SEM). Blood was collected to determine the serum levels of estrogen and progesterone. Statistical analysis revealed that the diameter of OA was recorded significantly ($P<0.05$) larger and fiber content (collagen, elastic) was found significantly ($P<0.05$) lower in the OA during BS than that of NBS. The area of intra-mural venules (IMV) in unit area observed in OA was significantly ($P<0.05$) higher during BS compared to NBS. Ultra-structurally, IMVs were simple endothelial tubes surrounded by loose collagenous tissue. The endotheliocytes contained small cytoplasmic vacuoles which showed significantly ($P<0.05$) larger diameter in OA during BS compared to NBS. The diameter of OA and serum estrogen level showed a strong positive correlation ($r=0.68$) during BS in contrast to the serum progesterone level which was found inversely correlated ($r=-0.34$). It is conceivable from the data that the structural organization of OA and OV behaves differently according to the breeding season in the female dromedaries. These changes are directly related to the changes in the ovarian hormonal transport, locally and systemically. To produce paracrine effects, IMV in the OA and cytoplasmic vacuoles in the endothelial cell may be purposed as a site of the transport.

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INTRODUCTION

Dromedaries are a good source of milk, meat and transport, especially in deserts, and can be a good replacer of ruminants (Skidmore, 2011). The reproductive performance of dromedaries is regarded as very low under natural environmental conditions, which can be related to lack of selective breeding, long gestation period, and calving interval, improper housing, and management (Akram *et al.*, 2008; Ali *et al.*, 2009). Dromedaries are seasonal breeders and induced ovulators, however, the seasonality of breeding patterns may be revoked by adequate green feeding (Padalino *et al.*, 2015) and photoperiodism. Dromedaries that are well fed and well-watered may show polyestrous sexual activity throughout the year (Gauly and Bourke, 1997; Monaco *et al*., 2015). Extrinsic environmental factors, changes in nutrition with mineral supplementation and photoperiod, are suggested to affect not only reproductive efficiency but also breeding season (Ali *et al*., 2017). It is widely assumed that seasonality affects the secretion and transfer of the sex hormones locally as well as systemically. The entire reproductive vasculature of females is involved in the local transfer of these hormones (Krzymowski and Stefanczyk-Krzymowska, 2012). Local transport of these hormones maintains reproductive thermoregulation in

different animals (Stefaficzyk-Krzymowska *et al.,* 1998; Skipor *et al*., 2010). In dromedaries, the reproductive vasculature is like other ruminants except for the middle uterine artery which is absent. Gross morphology and microvasculature in the female dromedaries are well documented (Ali *et al.,* 2011, 2017). However, ultrastructural variation in the reproductive vasculature of female dromedaries in consideration of breeding season yet needs to be investigated. The primary objective of this study was to elucidate the ultrastructural changes in the ovarian artery and ovarian vein during breeding and non-breeding seasons with differential staining, transmission and scanning electron microscope. Furthermore, a relationship between the structures of ovarian vessels with ovarian hormones is ascertained.

MATERIALS AND METHODS

A total of 20 healthy adult female dromedaries were selected from those slaughtered during breeding (November to April) and non-breeding season (May to October) in the local abattoir in Faisalabad- Pakistan. Age was determined by dentition as described by Rabagliati (1924). Multiparous animals of more than five years of age were selected for this study. Rectal temperature, respiration rate and heartbeat were recorded before the slaughter of selected animals to verify the health status of each animal.

Collection of blood: Ten ml of blood was collected from the jugular vein in a sterile vacutainer from each animal before slaughter. These vacutainers were subjected to centrifuge at 500 g for 10 minutes for serum separation. Serum was decanted in labeled cuvettes and stored in the refrigerator at -20℃ until hormone analysis.

Collection of tissues: The entire female reproductive system of dromedaries was collected immediately following slaughtered, washed and photographed. The ovarian artery (OA) and ovarian vein (OV) close to the ovary were located carefully and collected. Tissue samples of OA and OV were taken before entering the arterio-venous complex. The debris from the tissues was removed with normal saline. These samples were shifted into the neutral buffered formalin for histological studies. Tissue samples for electron microscopy were fixed in 5% glutaraldehyde solution prepared in in pipes buffer having pH 7.2.

Tissues processing technique: Following fixation, samples were subjected to dehydration through ascending grades of alcohol followed by clearing and embedding in paraffin. Tissues sections of 5 µm thickness were cut by rotary microtome (Microm HM 315). The sections were stained by hematoxylin and eosin, Masson's trichrome, and Weigert's elastic stain to mark collagen and elastic fibers (Suvarna *et al.,* 2019). These slides were examined and photographed under the camera fitted microscope (Optica B-150).

Transmission electron microscopy (TEM): Sugar crystal sized samples of OA and OV were collected and fixed in 5% glutaraldehyde fixative prepared in pipes buffer having pH 7.2 and placed in the thermal box at 4℃ for 2-4 hours. Tissues were then transferred to a post fixative 1% solution of osmium tetroxide (RT-19152, EMS-UAR) for at least 18 hours at room temperature. After post-fixation, the samples were dehydrated in ascending grades of ethyl alcohol. Embedding was done in spur resins and some sections of about 1mm thickness were stained with toluidine blue for identification of the study area (Hayat, 1986). Ultra-thin sections of 120 nm thickness were cut with RMC-Microtome® (MT-7000 Ultra) using a diamond knife. These sections were immersed in uranyl acetate (5%) (RT-22400**,** EMS-UAR) for staining for 30 minutes followed by washing twice with distilled water and transferred to lead citrate for at least 10 minutes in NaOH chamber. These prepared sections were examined under JEOL® TEM (JEM1010, 100kV, USA) at 6000, 8000 and 10000X.

Scanning electron microscopy (SEM): For scanning electron microscopy, glutaraldehyde (5%) (G6257**,** Sigma Aldrich) fixed samples were used followed by post-fixation in 1% solution of osmium tetroxide (RT-19152, EMS-UAR) solution for at least 18 hours at normal temperature. Samples were then subjected to ascending grades of alcohol for dehydration. These dehydrated samples were then transferred to the 1–2 ml of hexamethyldisilazane (CAS No 999-97-3, Sigma Aldrich) for 10 minutes for critical point drying. Mounting of samples was done on the aluminum stubs with double-sided tape for gold coating on the samples (Bozzola, 2007). These samples were examined under an International Scientific Instrument scanning electron microscope (S-2380N, Hitachi) operated at 9 kv.

Image analysis: The hematoxylin and eosin-stained sections were analyzed by Image J® software (Jawa 1.50i, National Institute of Health Science, USA) for the measurement of internal diameter and wall thickness (µm) of vessels. Masson's trichrome stained slides were analyzed to quantify collagen fibers and elastic fibers contents (%) were determined by Weigert's elastic stained sections. Electron micrographs were also analyzed to determine the diameter of cytoplasmic vacuoles in OA and OV (µm).

Hormonal Analysis: Serum estrogen (pg/ml) and progesterone (ng/ml) levels were determined by Radioimmunoassay (RIA) using commercially available kits (Randox Laboratories, UK).

Statistical Analysis: The means (±SEM) and ranges of studied parameters were worked out using the computer software package Microsoft Excel (Microsoft Office 2016). The data were analyzed by one-way analysis of variance (ANOVA). The complete randomized design was applied to the data obtained and for the comparison of means Tukey's honest significant test was applied in Minitab-15® (https://www.minitab.com/en-us/). The coefficient of correlation "r" was also calculated to determine the relationship between OA diameter and hormones. Significance among different mean parameters was calculated at $P<0.05$.

RESULTS

Histological examination: Different parameters of the ovarian artery (OA) and ovarian vein (OV) were determined in histological sections stained with different dyes during breeding (BS) and non-breeding seasons (NBS).The recorded diameter of OA (225.7±10.1 µm) was found significantly (P<0.05) higher in the breeding season (BS) than that of the non-breeding season (207.3±13.8) (Table 1). Wall thickness of OV measured in BS (214.2±13.2 µm) and NBS (209.6 ±17.4 µm) given in the Table 1. No alteration in the wall thickness of OA was found in breeding and non-breeding seasons, however, ovarian arterial (OA) wall thickness was significantly (P<0.05) thicker than that of OV (Table 1).

Collagen (23.1%) and elastic fibers (29.9%) content were seen significantly (P<0.05) low in OA during BS as compared to NBS (29.9 and 36.9 %, respectively) (Table 1 & Fig. 1). The collagen and the elastic fiber content of OV, however, remained unaltered in both seasons.

The intramural venules (IMV) in unit area (μm^2) in the tunics of the OA was significantly (P<0.05) higher in the BS than that of NBS. The intramural venules were not present in the ovarian vein (Table 1 & Fig. 1).

Transmission electron microscopic examination: In the ultrastructural micrograph of TEM, the endothelium formed of the squamous shaped endothelial cells was visible. The surface of the endothelial cells was smooth, and no cytoplasmic projections were seen. These cells are mainly occupied by the nucleus and have a variable thickness. Cytoplasmic vacuoles (CV) in the endothelial cells were visible and recorded more in the breeding season than in the non-breeding season (Fig. 2-A). Small venules were seen in the sub-endothelial layers of arteries called intramural venules (IMV). The IMVs were mainly endothelial tubes surrounded by loose collagenous contents (Fig. 2-C&D). Contrary to the ovarian arteries, the IMVs were absent in the OV. The fiber content was observed more during NBS in the OA. Elastic and collagen fibers were prominent in the sub-endothelium (Table 1; Fig. 2-E&F) of both vessels (OA, OV). In the series of different sections, vacuole like structure appeared in the cytoplasm of the entire endotheliocytes. These vacuoles, observed in different sections, were close to the luminal surface of the cells (Table 1; Fig. 2). These cytoplasmic vacuoles were observed more in BS in OA than in the NBS and OV. Diameter (0.93±0.14 µm) of CV was significantly (P<0.05) higher in endothelial cells of OA during BS as compared to NBS. In veins, endothelial cells also showed CV but the size and diameter remained unaltered by the season. In vein's ultra-micrographs, the sub-endothelium contained fibrous substance embedded in the ground substances. Smooth muscle cells were also visible in the tunica intima of the vessels. Longitudinal section of smooth muscle fibers was arranged in the form of lamellae (Fig. 2-B).

Scanning electron microscopic examination: In SEM, OA showed developed tunics, and IMV was seen almost in all layers. During breeding season, the diameter and area of IMV per unit area were significantly (P<0.05) higher than the non-breeding season, as explained earlier in the light microscopy (Fig. 3-A, B & C). In a comparison of OA and OV, the well-developed t. intima, and t. media were observed in OA along with IMV while these features were absent in the scanned EM graph of veins (Fig. 3-D & E).

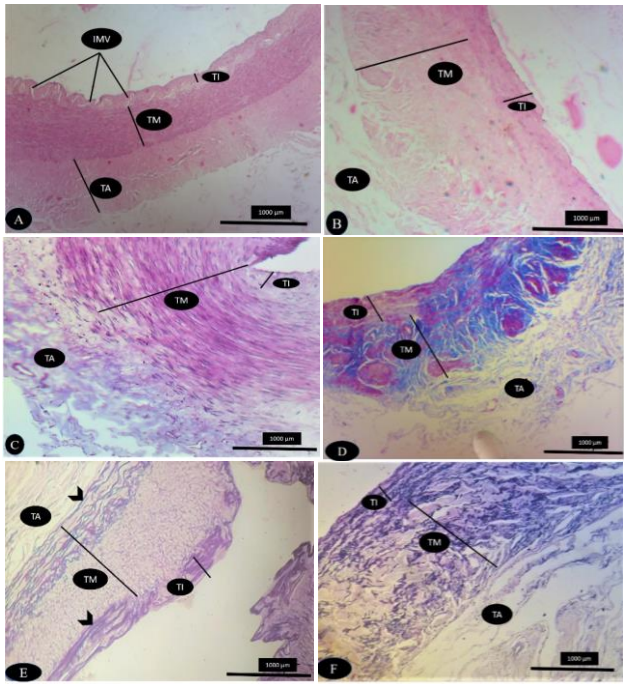

Fig. 1: Histological pictures of ovarian artery (OA) and ovarian vein (OV): A well-developed tunics can be seen OA (A) as compared to the OV (B) and small venules called Intra-mural venules (IMV) can be seen in OA (H & E, 40 X). In Fig. 1-C & D (Masson's Trichrome, 100 X), blue color represented the collagen fibers. Collagen fibers were seen significantly more in the OV (D) as compared to OA (C). Elastic fibers (black thread like structures) can also be seen in the Fig. 1-E&F (Weigert's Elastin, 100 X). Significantly more elastic fibers were seen in the OV (F) than the OA (E). TI: Tunica intima, TM: Tunica media, TA: Tunica adventitia, IMV: Intra-mural venules.

Hormonal Analysis: Serum estrogen and serum progesterone levels trend during the breeding and non-breeding season are shown in Fig. 4. The level of estrogen was found significantly (P<0.05) higher in BS (58.65±9.2 pg/ml) as compared to NBS (32.7±3.6 pg/ml) (Table 1). The highest estrogen level was determined in January and February months (61.4 and 69.5 pg/ml, respectively) while the lowest in June and July (20.2 and 17.9 pg/ml, respectively) (Table 1). The serum progesterone level remained non-affected by the season of breeding activity. There was a non-significant (P>0.05) change in the progesterone measured during BS (0.17±0.06 ng/ml) and NBS (0.160±0.04 ng/ml) (Table 1). The coefficient of correlation 'r' was also ascertained to determine the relationship between hormonal pattern and ovarian artery diameter. The value of 'r' was found positive in BS (0.48) and NBS (0.68) for estrogen and diameter of OA, indicating a directly proportional relationship between these two parameters. In the case of serum progesterone level, the value of the coefficient of correlation "r" calculated in BS was -0.08 and in NBS was -0.34. These negative "r" values represent the inverse relationship between the diameter of OA and serum progesterone level (Fig. 4).

Table 1: Data analysis of different microscopic characteristics of ovarian artery (OA) and ovarian vein (OV) during breeding season (November to April) and non-breeding season (May to October)

Parameters	Ovarian Artery		Ovarian Vein	
	BS	NBS	BS	NBS
Diameter (µm)	225.7±10.1 [a]	207.3±13.8 [b]	214.2±13.2 [b]	209.6±17.4 [b]
Wall Thickness (µm)	162.24±21.2 [a]	159.45±17.2 [a]	142.21±11.2 [b]	138.35±10.2 [b]
Smooth Muscles fibers (%)	25.43±2.7 [b]	19.1±2.2 [c]	29.7±5.6 [a]	30.6±7.5 [a]
Collagen %	23.1±2.1 [c]	29.9±2.1 [b]	37.7±4.2 [a]	33.3±2.1 [a]
Elastic %	29.9±1.6 [c]	36.9±3.2 [b]	33.4±3.5 [a]	31.21±4.3 [a]
IMV Area (μm^2)	45.4±9.1 [a]	36.8±8.2 [b]		
Cytoplasmic Vacuoles (µm)	0.93±0.14 [a]	0.82±0.15 [b]	0.73±0.12 [bc]	0.72±0.3 [bc]

Means having different alphabets in superscript are statistically different at P<0.05. BS: Breeding season, NBS: Non-breeding season, IMV: Intra-mural venules.

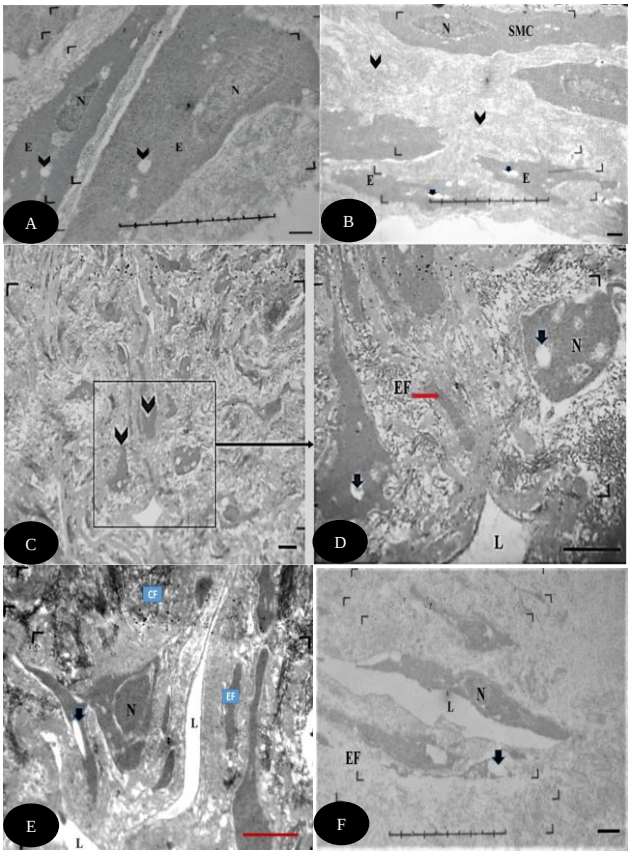

Fig. 2: Transmission electron microscopy of ovarian artery (OA) and ovarian vein (OV) in breeding and non-breeding season. A: Ovarian artery in breeding season showing squamous type of endothelial cells and more number of cytoplasmic vacuoles shown by arrow heads (8000 X, Scale bar: 1µm). B: Endothelial cells of different shapes are present and showing the cytoplasmic vacuoles in OV. The sub endothelium contained fibrous substance embedded in the ground substances (arrow head). Smooth muscles cells are also visible in the tunica intima of the vessels. Longitudinal section of smooth muscle fibers, arranged in the form of lamellae (6000X, Scale bar: 1µm). C & D: Intra-mural venules (IMV) having lumen in the ovarian artery along with cells showing nucleus and cytoplasmic vacuoles. IMV are lined with fibers (C: 3000 X, D: 6000 X, Scale bar: 1µm). E: IMV having lumen in the OA along with cells showing nucleus and cytoplasmic vacuoles. IMV are lined with fibers and collagen fibers. (10000X; Scale bar: 1µm). F: IMV having lumen in the OA along with cells showing nucleus and less number of cytoplasmic vacuoles in non-breeding season. IMV are lined with elastic fiber (8000X, Scale bar: 1µm): N; Nucleus, L; Lumen, CF; Collagen fibers, EF; Elastic Fibers; E; Endothelial cells.

DISCUSSION

Dromedaries are seasonal breeders and manifest reproductive activity in a specific period of the year. The reproductive systems of dromedaries both in males and females show significant gross morphological and histological changes during breeding (BS) and non-breeding season (BS). The male and female gonadal hormonal patterns also alter by the breeding season which is directly related to the anatomy and physiology of the reproductive system. Transfer of these hormones, local and systemic, depends upon the vasculature of the reproductive system. Morphological and histological characteristics of ovarian arteries and veins in females of several species (ewe, cow, pig, monkey, human) are well documented. The ultrastructural changes in the ovarian artery (OA) and ovarian vein (OV) during BS and NBS in female dromedaries are studied for the first time. The diameter of OA was significantly (P<0.05) larger in BS than in the NBS. The diameter of OV and thickness of both vessels, OA and OV, remained unaltered by the BS. No literature is available to compare these results. Ovarian hormones have effects on blood circulation involving the mechanisms which control blood flow. Estrogens have direct effects on arterial walls. It has been further reported that elevated levels of estrogen during breeding season affect receptors on the myocytes of the ovarian artery and cause relaxation of vascular smooth muscles (Eid *et al.*, 2007; Kim *et al.*, 2018).Consequently, increase in ovarian weight might be due to larger diameter of the OA that augments blood supply to the ovary. Hence this can also be considered as a rationale for the larger diameter of OA in breeding season.

The fibrous contents of OA like elastic and collagen fibers were seen low in the breeding season (Fig. 1- C to F). The smooth muscle arrangement was seen circumferentially around the vessels. Same arrangement was reported in the muscular artery of porcine (Silver *et al.*, 2003). Vascular properties depend upon the coupling of fiber contents (collagen and elastic) with the cellular compartment within the extracellular matrix. The biomechanical properties of connective tissue are related to the smooth muscles, elastic, and collagen fiber contents of the vessels (Silver *et al*., 2003; Zócalo *et al.*, 2013). No study is available that describe these changes associated with the different breeding season in female dromedaries. However, the least fiber contents of OA in the breeding season may give clues towards the easy diffusion of different hormones to produce paracrine effects by local transfer. Mensah-Brown *et al.* (2004) reported decreased collagen fibers in the stroma of connective tissue of the anal canal of rats by estrogen and progesterone therapy. The higher serum levels of estrogen during BS might have an inverse effect on collagenous fibers which consequently reduced collagen in the walls of artery. In Ultrastructural studies, intramural venules (IMV) observed in OA, showed thinner endothelial cells neighboring by collagen

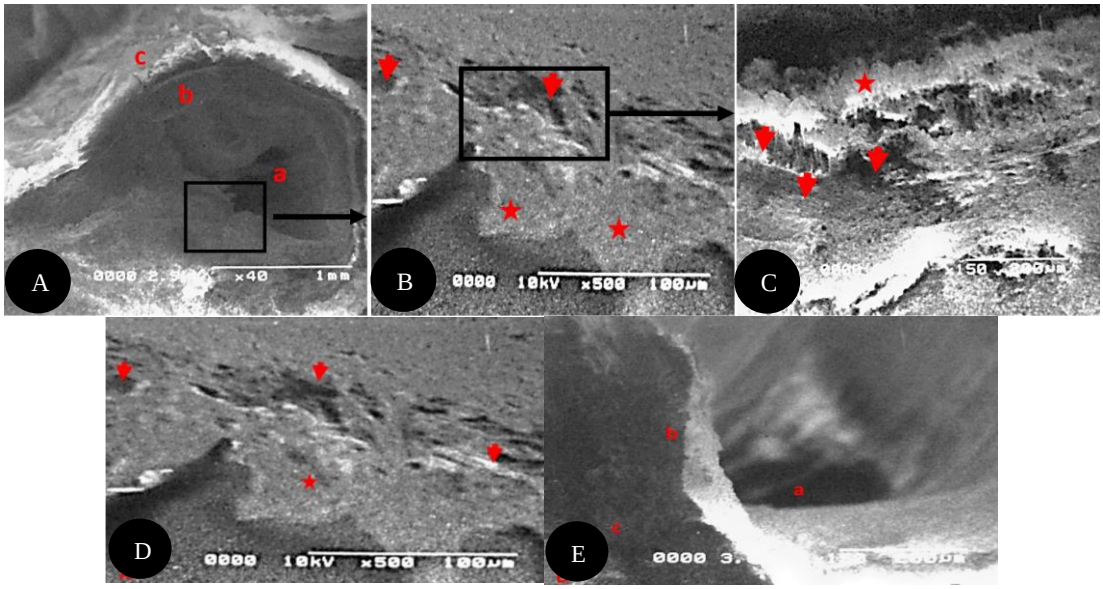

Fig. 3: Scanning Electron Micrograph of perfused fixed transverse section of the ovarian artery and vein. Ovarian artery micrograph (A) represents lumen (a), tunica media (b) and tunica adventitia (c). At higher magnification (B & C), the walls of the ovarian artery showed some kind of small vessels like structure called intramural venules (small red arrows) and the endothelial cells (red stars). Small red arrows represented the intramural venules area, which was observed more in the breeding season (D). No such type of IMV structure was seen in the OV (E).

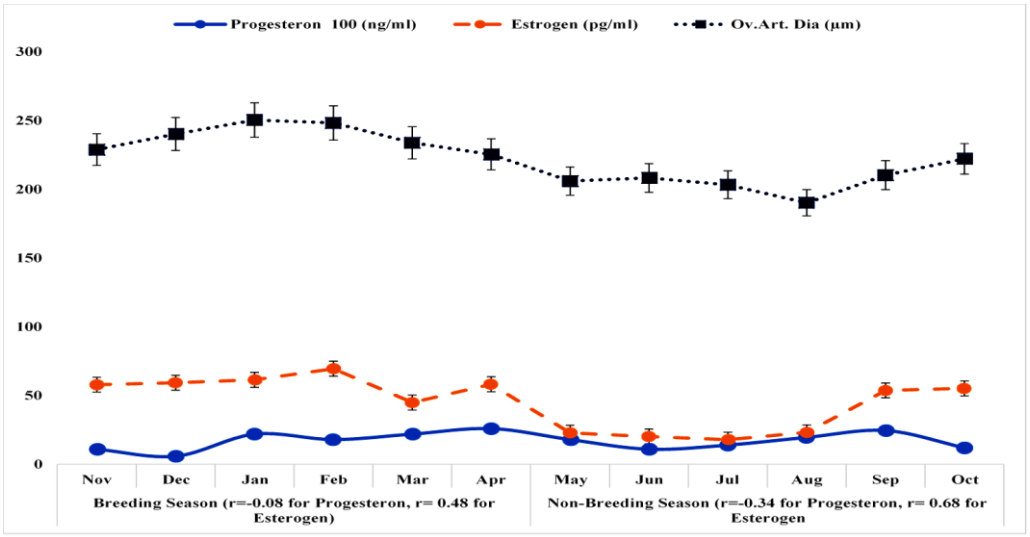

Fig. 4: Changes in the estrogen and progesterone along with their relationship with ovarian artery diameter during the different months of season.

or elastic fibers (Fig. 2 & 3). In bulls, a similar structure was described by Polguj *et al*. (2011) in the spermatic artery. The assumed function of IMV in a male is the regulation of the testicular blood supply and reduction of the arterial blood pressure in the testicular artery before the blood reaches the gonad (Pradidarcheep *et al.*, 1998; Skowroński, 2003). There are no contemporary studies available in the literature that describe the structure and role of IMV in OA. Keeping in view the function of IMV in males, it could be assumed that it might have a role in the transfer of hormones and regulation of the ovarian blood supply. This hypothesis can be supported by more IMV area recorded in BS and the absence of IMV in OV. However, more detailed studies are required to explore the anatomy, histology, and physiology of OA and OV at the molecular level.

In electron micrographs of endothelial cells, intracytoplasmic vacuoles were seen close to the lumen (Fig. 2). Chen *et al*. (1998) and Stan (2013) reported some type of cytoplasmic pocket-like structure in the endothelial cells. However, Claesson-Welsh (2015) described that these vacuoles as a prominent feature in tumor cells. These vacuoles are reported to form a trans-endothelial pathway for material transport and endocytosis (Musalam and Eid, 2010). The vesicular path is the most important one present in epithelia, to which endothelium belongs. Through this process, large molecules are transported across the endothelium cells in membrane-bounded vesicular carriers (Stan, 2006). Hormones, including human chorionic gonadotropin and leptin, are reported to transport through transcytosis (Kolka and Bergman, 2012) and the intra-cellular gaps called para-cellular transport. Pi*et al*. (2018) and Yazdani *et al*. (2019) described the ways by which transport across the blood-brain barrier takes place and trans-endothelial pathway was one of them. These vacuoles formed during transcytosis are derived from the caveolae, a specialized region on the endothelial plasma membrane (Claesson-

Welsh, 2015). Keeping in view these hormonal levels, there is a dearth of literature highlighting the underlying mechanism of estrogen and progesterone local transport across the endothelial cells. The cytoplasmic vacuoles in the endothelial cells of the IMV may have some role in the formation of trans-endothelial channels that might have helped in the local and retrograde transfer of hormones.

Conclusions: The structural organization of OA and OV behaves differently according to the breeding season in the female dromedaries. These changes are directly related to the ovarian hormonal transport, locally and systemically. To produce paracrine effects, IMV in the OA and cytoplasmic vacuoles in the endothelial cell may be purposed as a site of the transport.

Authors contribution: This manuscript is from PhD thesis of MU. ASQ conceived and designed the study. MU conducted the experiment. All authors were involved in data interpretation, write up and final approval of the manuscript. All authors declare no conflict of interest.

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