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Original article

ANATOMICAL AND HISTOLOGICAL STUDIES ON THE CERVICAL CANAL OF RAHMANI SHEEP

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Summary

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Macroscopic and microscopic findings of Rahmani sheep cervical canal were evaluated for designing a trans-cervical catheter. The study was carried out on 160 cervices divided into four groups: (Group A; young follicular, Group B; old follicular, Group C; young luteal and Group D; old luteal). All morphometric parameters of cervical canals of each group including length, average length and the external os diameter and shape were measured and tabulated. Epoxy casts were made to demonstrate the anatomic variations of each group. Data collected were statistically analysed using ANOVA. One hundred and twenty tissue sections from each group were sectioned and stained with Harris haematoxylin and eosin, Masson's trichrome stain for demonstration of collagen fibres, Alcian blue pH 2.5 for demonstration of acidic mucins and PAS technique for demonstration of neutral mucins. In conclusion, the current study is the first report on the morphometric structure of the cervical canal of native Egyptian Rahmani ewes.

Key words: catheter guide, cervical canal, epoxy cast, morphometric parameters, Rahmani ewe

INTRODUCTION

The major Egyptian breeds of sheep are Ossimi, Rahmani and Barki. Rahmani ewes are small to medium sized sheep with coarse wool and fatty tail. All Egyptian breeds are raised mainly for wool, milk and lamb production (Marai *et al.,* 2009).

Artificial insemination using frozen-thawed spermatozoa enhances the production of genetically superior lambs by maximising the utilisation of superior rams. However, the conception rates in sheep following insemination using frozen-thawed spermatozoa are still unsatisfactory (Kershaw *et al.,* 2005). Transcervical artificial insemination in sheep is still limited with unsatisfactory pregnancy rates due to presence of the cervical rings that act as insulating barrier during artificial insemination (More, 1984) and the narrow cervical lumen especially at the level of the first three cervical folds (Halbert *et al.,* 1990). Unfortunately, those factors hinder the insertion of the insemi-

nating pipette into the lumen of the cervical canal. On the other hand, laparoscopic insemination in ewes increases the pregnancy rates using frozen-thawed spermatozoa but its application requires highly qualified operator and expensive tools (Ax *et al.,* 2000). Ovine cervical canal is a long tubular organ with highly tortuous lumen and has 4 to 7 cervical rings that are a barrier to any contaminants (Fukui *et al.,*1978). Age and breed variations are the two commonest factors affecting the ewe cervix morphometry, the shape of cervical folds varies from a ring in young ewes while becoming a flap shape in the older ewes (Kaabi *et al.,* 2006). The average length of the ewe cervical canal found by More (1984) was 6.7 cm with presence of six annular folds forming crypts that can hold spermatozoa, while Eppleston *et al.* (1994) found that the average length of the ewe's cervical canal was 4.87 cm in Merino breed with 3.97 average numbers of cervical folds. The cervical folds appear as funnel-shaped arrangement that project caudally into the lumen of the cervical canal; the second cervical fold is eccentric while the other folds appear concentric (Naqvi *et al.,* 2005). This anatomical configuration hinders the passage of the inseminating pipette during insemination. Histologically, the cervical canal of sheep is lined by non-secretory columnar epithelium with mucous secreting goblet cells; the cervical stroma is composed of collagen bundles running in different directions with many fibroblasts, blood capillaries and few smooth muscle cells. The thick muscular coat of smooth muscles was demonstrated and covered externally by serosa (Dobson, 1988).

The aim of the current study was to record the morphometric parameters as well as the histological criteria including the histochemical mucins types and the submucosal collagen fibres area of the cervical canal of Rahmani breed ewes during both follicular and luteal phases of the estrous cycle in either young or old animals to obtain sufficient data for manufacturing a suitable inseminating pipette and facilitate the proper manipulation of the cervix.

MATERIALS AND METHODS

Cervices collection

One hundred and sixty non-pregnant ewe cervices (Rahmani breed) were collected from the local abattoir between September 2016 to October 2017. Cervices were divided according to the age (young; less than 18 month-old; old; 18–48 months of age) and the stage of estrous cycle (follicular and luteal; depending on the ovarian structures) into four groups: Group A (young follicular, n=40), Group B (old follicular, n=40), Group C (young luteal, n=40) and Group D (old luteal, n=40).

Monitoring the shapes of the external cervical os

All uteri were transported to the laboratory 2–3 hours post-slaughter, cervices were excised and separated cranially at the level of the uterine body and caudally at the level of the posterior vagina, and the external os was exposed by making a longitudinal incision through the anterior vagina and the cervix (Fig. 1). The shape of the external os was observed and classified into four types (Halbert *et al.,* 1990). The identified four types were: flab, duckbill, rosette and papilla.

Cervical canal measurements

All morphometric parameters including 1) average length of cervical canal taken at the level of the external opening to its

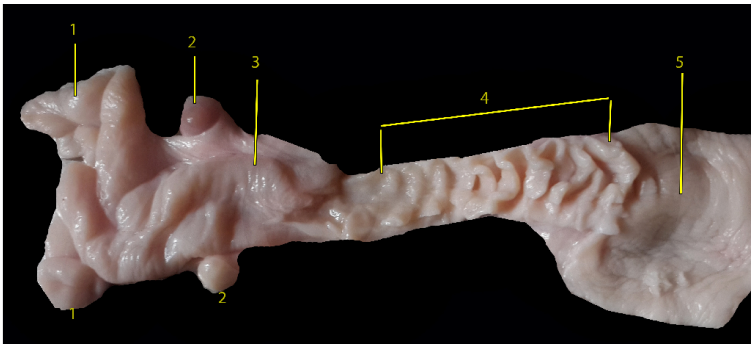

Fig. 1. A photograph showing longitudinal incision in the cervical canal of ewe: 1. uterine horns, 2. ovaries, 3. uterine body, 4. cervical canal folds, 5. vagina.

union with the body of the uterus; 2) average length of cervical canal at different levels; 3) average diameter of cervical canal and 4) the external os diameter in each group were measured using Vernier caliper. The average number of cervical folds was estimated and recorded.

Epoxy injection technique

Uterine specimens were flushed by injection of warm sterile normal saline from the body of the uterus toward the vagina to remove any mucus or debris followed by injection of E151N20 epoxy resin (Schummer, 1951). Specimens were left for 3–4 days in the refrigerator for hardening, then dissected carefully to declare the epoxy casts of the cervical canal.

Tissue architecture

Collected cervical tissue samples were fixed in 10% neutral buffered formalin for 72 h. Samples were trimmed and processed by ascending series of alcohols, cleared in xylene, infiltrated with Paralast synthetic wax and blocked out into Paraplast tissue embedding media. Sections of 3–5 µm were cut by rotatory microtome. The sections were stained with Harris haematoxylin and eosin as a general staining method, Masson's trichrome stain for demonstration of collagen fibres, Alcian blue pH 2.5 for demonstration of acidic mucins and PAS technique for demonstration of neutral mucins (Bancroft & Stevens, 2010). Ten random non-overlapping fields from each tissue section of different stains in different groups with a total 120 tissue sections from each group per stain were analysed using Leica application suite for slide analysis equipped with full HD microscopic camera (Leica Biosystems, Germany).

Statistical analysis

Data concerning average length of cervical canal, average length of cervical canal at different levels, external os diameter and average diameter of cervical canal at different levels were analysed by one-way analysis of variance (ANOVA) using SPSS 10.0 Means ± SEM were calculated. Average number of cervical folds and different shapes of external os were analysed using the chi-square test.

RESULTS

Shapes of the external os and number of cervical folds

Different shapes of the external os were monitored according to age and stage of the estrous cycle (Fig. 2). The flap shape

was predominant in Group A (34/40; 85.0%), duckbill shape was frequent in group B (31/40; 77.5%), a shape of papilla was common in group C (28/40; 70.0%) and the rosette shape was prevailing in Group D (29/40; 72.5%). The average number of cervical folds was 6 in 38/40 or 95.0% of Group A, 36/40 (90.0%) of Group B and 35/40 (87.5%) of Group D, while 7 fold were found out in 37/40 (92.5%) of Group C.

Average external os diameter and cervical canal length

The external os diameter was significantly ($P<0.05$) higher in group B (1.2±3.3 cm) compared to 0.67±2.1, 0.40±1.2 and 0.73±0.9 cm respectively for Group A, C and D (Table 1).

The average diameter of the cervical canal was significantly higher in group B (1.80±0.42 cm) than in other groups (0.71±2.7, 0.78±3.1 and 1.10±3.7 cm for Groups A, C and D, respectively) at the level of 2nd cervical fold. There was no significant difference ($P>0.05$) in the average diameter of cervical canal at the level of 4th fold between Groups B and D (1.30±3.3 and 1.20±2.8 cm, respectively) but both were significantly higher than diameters recorded in Groups A and C (0.61±1.9 and 0.57±1.7 cm, respectively).

At the level of the sixth fold, the average diameter of both Groups B and D

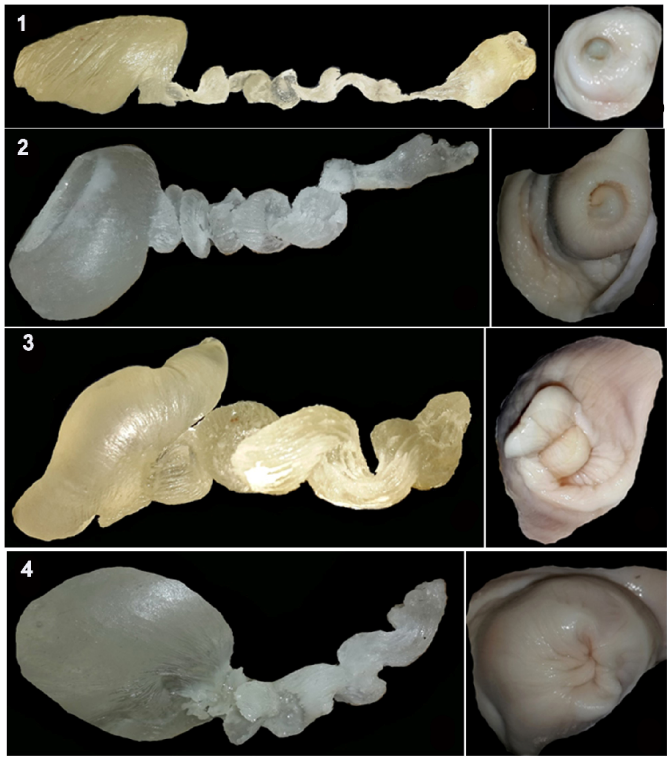

Fig. 2. Epoxy casts of the cervical canal of different groups (left) and external os appearance (right): 1. Flap (Group A; young follicular); 2. Papilla (Group B; young luteal); 3. Duckbill (Group C; old follicular); 4. Rosette (Group D; old luteal).

Table 1. Cervical canal diameter in Rahmani ewes (cm) at different levels. Data are presented as mean ± SEM, n=40

Groups	Group A (young follicular)	Group B (old follicular)	Group C (young luteal)	Group D (old luteal)
External os diameter	0.67±0.15	1.2±0.26	0.40±0.14	0.73±0.35
Cervical canal diameter at the 2nd cervical fold level	0.71±0.13[b]	0.78±0.14[b]	1.80±0.42[a]	1.10±0.45[b]
Cervical canal diameter at the 4th cervical fold level	0.61±0.20[b]	0.57±0.11[b]	1.30±0.36[a]	1.20±0.47[a]
Cervical canal diameter at the 6th cervical fold level	0.47±0.11[b]	0.42±0.18[b]	1.20±0.53[a]	0.90±0.22[a]

Different superscripts within the same row are significantly different at P<0.05.

Table 2. Average length of cervical canal at different levels. Data are presented as mean ± SEM, n=40

Groups	Group A (young follicular)	Group B (old follicular)	Group C (young luteal)	Group D (old luteal)
Cervical canal length between external os and 1st fold	0.4±0.15[b]	0.9±0.18[a]	0.3±0.17[b]	0.5±0.22[b]
Cervical canal length between 1st fold and 2nd fold	0.7±.13[b]	1.2±0.21[a]	0.5±0.13[b]	0.7±0.41[b]
Cervical canal length between 2nd fold and 3rd fold	0.6±0.14[b]	1.7±0.17[a]	0.4±0.11[b]	0.5±0.23[b]
Cervical canal length between 3rd fold and 4th fold	0.7±0.17[b]	1.4±0.15[a]	0.3±0.18[b]	0.5±0.16[b]
Cervical canal length between 4th fold and 5th fold	0.6±0.17[b]	1.5±0.18[a]	0.4±0.15[b]	0.6±0.19[b]
Cervical canal length between 5th fold and 6th fold	0.8±0.17[b]	1.3±0.12[a]	0.4±0.17[b]	0.4±0.21[b]
Cervical canal length	5.2±1.8	4.5±1.3	8.4±2.1	4.8±1.4

Different superscripts within the same row are significantly different at P<0.05.

(1.20±4.8 and 0.90±8.7 cm) was significantly higher than that recorded in Groups A and C (0.47±1.5 and 0.42±1.2 cm) (Table 1).

The average length of cervical canal was significantly (P<0.05) higher in Group B (8.4±3.2 cm) than in all other groups (4.8±1.32, 4.6±2.73 and 5.2±1.77 cm for Group A, C and D, respectively) as shown in Table 2.

The average length of the cervical canal between external os and 1st fold, 1st and 2nd folds, 2nd and 3rd folds, 3rd and 4th folds, 4th and 5th folds and 5th to 6th folds

were 0.9±1.8, 1.2±3.1, 1.7±3.7, 1.4±1.5, 1.5±0.8 and 1.3±2.2 cm respectively for Group B. Interestingly, all average lengths of Group B were significantly higher than those of Groups A, C, D at the same levels of the cervical canal ($P<0.05$; Table 2).

Epoxy casts of the cervical canal

Epoxy cast for each group was dissected carefully to be a clear model during the future manufacture of the inseminating catheter (Fig. 2).

Histological findings

Histologically, cervical mucosal epithelium of surveyed ewes was composed of simple columnar secretory cells and occasionally was ciliated non-secretory or stratified columnar epithelium. Regarding mucins dynamics in terms of area percentage of expression and reactivity in the secretory epithelial cells, the highest percentage of reactivity of Alcian blue stain PH 2.5 was demonstrated in the middle cervical region of both young follicular (Group A) and old luteal ewes (Group D) attaining up to 28.5% and 26.5% respectively from the secretory epithelium (Fig. 3). However, the least reactive regions were the cranial cervical part of both young and old follicular ewes (Group A and B; Fig. 3).

In terms of reactivity of epithelial mucins to PAS technique, the highest reactivity was limited to the caudal region of the cervical tube of both old follicular (Group B) and old luteal (Group D) ewes occupying up to 22% of the secretory epithelial area. However, PAS reactivity was nearly absent in other regions of different groups (Fig. 4). Masson's trichrome staining of different cervical regions of all groups demonstrated enormous amount of collagen fibres in submucosal layer of all

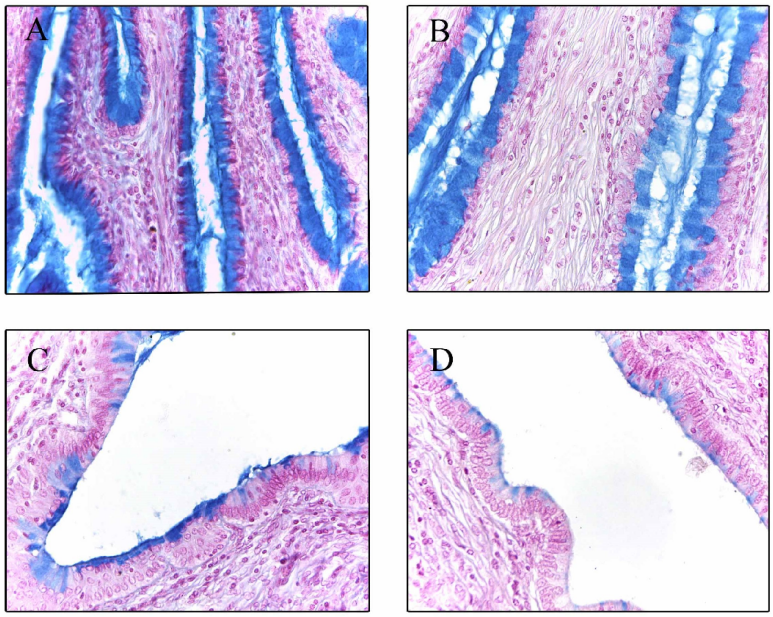

Fig. 3. Expression levels of acidic mucins by cervical epithelium in caudal part of the cervix of the old luteal ewes (A) as well as in follicular stages (B) and in cranial segments of the cervix of old follicular ewes (C) as well as in ewes in follicular stages (D). Alcian blue pH=2.5, 400×

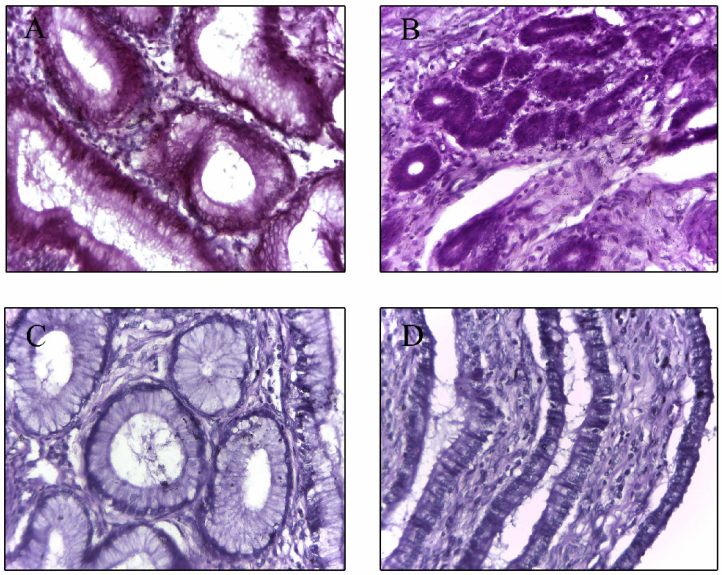

Fig. 4. Expression levels of neutral mucins by cervical epithelium of caudal cervical part of old luteal (A) multiparous ewes in follicular stages (B). Negative reactivity was observed in the caudal part of hefiers follicular (C) and hefiers luteal ewes (D) as well as all other segments of different groups. PAS stain, 400×.

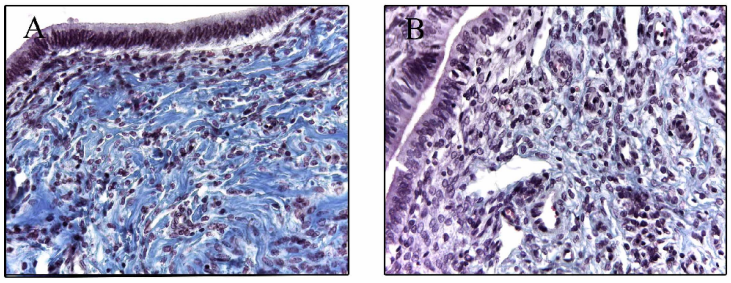

Fig. 5. Collagen fibres optical densities in middle cervical segment of multiparous follicular ewes (A) as well as in cranial cervical segment of multiparous luteal ewes (B).

regions of different groups. Moreover, the middle cervical region demonstrated the highest area percentage of collagen fibres as well as optical density values in different surveyed ewes of each group (Fig. 5).

The examined tissue sections of the middle cervical region of young luteal (Group C) and old luteal ewes (Group D) recorded the highest mean value of collagen fibres percentage among all examined groups. However, the highest optical density values of demonstrated collagen fibres were recorded in Group B, D and C respectively (Table 3).

DISCUSSION

The current study is considered the first report on the anatomical and the mor-

Table 3. Percentage of reacted mucin area using alcian blue, submucosal collagen and optical density (OD) of collagen fibres using MTC stain in different areas of the cervical canal of ewes. Data are presented as mean ± SEM, n=40

Groups		Group A (young follicular)	Group B (old follicular)	Group C (young luteal)	Group D (old luteal)
Mean alcian blue reactivity area, %	cranial	0.30±0.12^{b}	1.5±0.28^{b}	8.6±4.1^{ab}	11.5±2.3^{a}
	middle	28.4±1.3^{a}	15.0±2.3^{b}	9.2±4.3^{c}	26.5±2.4^{a}
	caudal	18.7±1.5^{a}	17.0±2.5^{a}	11.8±4.6^{b}	19.7±2.6^{a}
Mean % of submucosal collagen	cranial	56.4±2.2^{a}	55.0±2.3^{a}	59.1±2.7^{a}	53.2±2.6^{a}
	middle	62.2±3.1^{a}	62.4±3.2^{a}	69.4±3.5^{a}	66.2±2.9^{a}
	caudal	59.2±4.3^{a}	58.7±3.9^{a}	56.5±2.8^{a}	56.2±4.5^{a}
Mean OD of collagen fibres	cranial	0.51±0.09^{a}	0.48±0.05^{a}	0.55±0.04^{a}	0.45±0.03^{a}
	middle	0.61±0.05^{a}	0.73±0.6^{a}	0.69±0.07^{a}	0.70±0.08^{a}
	caudal	0.57±0.12^{a}	0.68±0.14^{a}	0.65±0.18^{a}	0.56±0.14^{a}

Values with different superscripts in the same row were significantly higher at $P<0.05$.

phometric structure of the cervical canal of native Egyptian ewes.

The results showed that the shape of the external cervical os differed according to the age and the stage of estrus cycle. On the other hand, Cruz *et al.,* (2014) found that the duckbill shape was the most predominant in Santa Ines ewes, and rosette and flap (Kershaw *et al.* (2006) or spiral shape (Naqvi *et al.,* 1998) were the predominant shapes in other breeds – this difference is attributed to the breed variation. The average number of cervical folds recorded in the present study was 6 in Groups A, B and D while it was 7 in Group C. Cruz *et al.* (2014) reported that the number of cervical folds ranged from 3–7 per cervix (mean 4.4±1.06) depending on the age, where ewes at age < 6 months or 6–12 months had narrower cervices with large number of folds. In line with our results, Gultiken *et al.* (2009) found no significant difference ($P>0.05$) between number of the cervical folds in lambs (5.16±0.93) and ewes (4.73±0.7).

Our results showed that the average length of cervical canal was significantly higher in old follicular ewes (Group B) than in other groups. Similarly, Habibibzad *et al.* (2015) found that the length of cervical canal varied from 3.93±0.25 cm in young lambs <6 months of age to 7.58±0.17 cm in ewes older than 4 years in Sanjabi breed. Naqvi *et al.* (2005) added that the length of the cervix was 5.36 cm in mature ewes while it was 3.87 cm in young ewes. These variations in results of the length of the cervical canal can be attributed to breed size variation, age and the stage of estrous cycle. Also, our study demonstrated that the external os diameter was significantly higher in Group B. Cruz *et al.* (2014) reported different diameters of the external os depending on the different shapes of external os and age of ewes. Interestingly, our results demonstrated a significant variation in both diameter and length of cervical canal at different levels of cervical folds.

The variation in length and diameter of cervical canal at different levels was one of the main obstacles during artificial insemination as it differs according to age and breed (Naqvi *et al.,* 2005). The length of cervical canal especially at the level of

the external os to the first cervical fold and that from first to second cervical fold was greater than that reported either in Karayaka ewes (Gultiken *et al.,* 2009) and tropical breeds (Naqvi *et al.,* 2005). For the first time, cervical canal measurements were recorded in our study using epoxy injection not by making silicone molds as done by Naqvi *et al.* (2005).

Several authors have discussed the mucins dynamics in cervical region of Merino strain and other breeds (Becher *et al.,* 2004). It was reported qualitatively that sialomucins were found in the basal part of cervical folds unlike sulfomucins which were limited to the tips of mucosal folds (Pluta *et al.,* 2011). The current study quantified the area percentage of total sialomucins as well as sulfomucins in examined tissue sections for each region. It is evident that the middle and caudal cervical regions of cervical canal play a critical role in mucins production levels unlike the cranial cervical region. The greater mucins expression levels in these regions is expected to aid in higher sperm entrapment and guidance in middle and caudal cervical regions. The higher mucins levels also provide a higher protection as a physical barrier against harmful pathogens in these regions. In agreement with Pluta *et al.* (2011) neutral mucins were generally less expressed in cervical canal of ewes. However; current investigation recorded that they were mostly expressed and limited to the caudal part of cervical canal in old ewes unlike negative expression in young ewes.

There was no record in available literature for the relationship between collagen fibres' area percentage, density and reproductive state of cervical region of ewes. The current study reported that the submucosal collagen fibre area percentage remodelling was directly associated with the optical density values in examined regions of cervical canal in each group. The middle part of cervical canal demonstrated a higher amount of mature collagen bundles than other cervical regions in different stages of surveyed Rahmani ewes. However, it was evident that the reproductive stage with the expected hormonal state of each group had no significant effect on submucosal collagen bundles remodelling. These findings were in agreement with previous reports on cyclic changes of human cervix (Petersen *et al.,* 1991) and cow cervix (Breeveld-Dwarkasing *et al.,* 2011).

In conclusion, the current study provided a complete illustration about the anatomical configuration and histological architecture of the cervical canal of the Egyptian Rahmani ewes to facilitate the future manufacturing a trans-cervical catheter for this breed.

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