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EFFECTS OF AQUEOUS EXTRACT OF Miswak (*SALVADORA PERSICA)* ON HISTOMORPHOLOGICAL STRUCTURE OF THE UTERUS IN THE FEMALE RATS

SABREEN M. GHAREEB, MAHMOUD ABD-ELKAREEM AND AHMED ABOU-ELMAGD

Department of Cell and Tissues, Faculty of Veterinary Medicine, Assiut University, Assiut 71526, Egypt

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ABSTRACT

The uterus is a very important reproductive organ for implantation and holding embryos till labor. Many drugs and medicinal plants affect the uterus, some researchers reported that *Salvadora persica* (Miswak) is a phytoestrogen plant as it contains flavonoids and can be used as a contraceptive drug; however, no available investigations explain the histomorphological structure of the uterus after Miswak extract administration. Twelve female albino rats (165.3 ± 3.269 g) were divided equally into two groups. In the control group, the animals received normal saline daily for 4 weeks. While in the Miswak treated group, the animals received 900 mg/kg of body weight of the Miswak aqueous extract daily for the same period. Grossly, at the end of the experiment, our results revealed that the uteri in the Miswak-treated group had characteristics of low active organs; they were pale with low weight and high thickness. In contrast to the treated group, the uteri of the control group had the signs of active organs; they were more vascular, relatively thin and edematous. Microscopically, in the control group, the uterine lumen was wide and the mucosa was folded and covered by secretory columnar epithelium. The uterine glands were more active, and the myometrium was thick. Whereas, in the Miswak-treated group, the lumen was slit-like, the uterine glands were less active, and the uterine wall had thick lamina propria and thin myometrium. In conclusion, the oral administration of Miswak extract reduces the uterus activity. It showed a state of anestrous with a narrow slit-like lumen and a decrease in glandular activity.

Keywords: *Miswak, Uterus, Structure, Rat, Endometrium, Myometrium*

Introduction

The uterine wall consists of mucosa that is characterized by the development of simple mucosal folds and consisting of lamina epithelialis and lamina propria mucosae. Its epithelium is between simple and pseudostratified columnar and the myometrium has two layers: thick inner circular and thin outer longitudinal smooth muscle fiber. perimetrium which is loose connective tissue covered by mesothelium (Rabie, F.O. and Haibat, S.M. 2020).

Salvadora persica (S. persica) is a known herbal plant of the family Salvadoracaea that grows in the Middle East and Africa

Corresponding author: Mahmoud Abd-Elkareem
E-mail address: mahmoud.mahmoud3@vet.aun.edu.eg
Present address: Department of Cell and Tissues, Faculty of Veterinary Medicine, Assiut University, *Assiut 71526, Egypt*

(Abdoon *et al.,* 2014). *Salvadora persica* stick (Miswak) is used for teeth cleaning throughout the Arabian, especially Muslims. *S. persica* contains active chemical constituents such as salvadorine, trimethylamine, fluoride, chloride, Sulphur, silica, mustard oil, vitamin C, resins, saponins, tannins, flavonoids, and sterol (Elvin-Lewis, 1982 and Kamil *et al.,* 1999). Miswak aqueous extract has an antifertility action on the activity of the ovary and uterus of female albino Wistar rats. It significantly lowers the ovarian weight, uterine weight, number of ovarian follicles, epithelial cell height, and myometrial and stromal thickness of the uterus (Abdoon *et al.,* 2014). Research concerning the effect of Miswak extract on the reproductive performance of females was scanty. Also, there is little available information about the effect of Miswak on the histomorphological structure of the uterus (uterine epithelium, uterine gland, thickness of endometrium and myometrium). The aim of the present work is to give more information on the effect of the aqueous extract of Miswak on the histomorphological structure of the uterus in rats. In addition, to give more details on the relation between the use of Miswak and the normal healthy properties of the uterus in female albino rats and to examine the contraceptive properties of Miswak.

MATERIALS AND METHODS

Preparation of Miswak extract and dose:
Raw Miswak sticks were cut into small pieces and let them dry at room temperature for a week. Then these pieces were ground to fine powder that soaked into distilled water in a sterile dry screw-capped bottle for 2 days at a temperature of 4°C. After that, this mixture of Miswak was shaken in a shaking incubator (model JSSI-100C) at 200 rotations/ min for 5 hours at a temperature 25°C in the Central Laboratory of the Faculty of Veterinary Medicine, Assuit University. The mixture is then filtered using Whatman filter paper till obtains a clear solution. The solution was lyophilized in a vertical freeze drier (model #6KBTES-55) in Faculty of Science, Assuit University. The extracts of Miswak were stored at 4°C till animal administration. The animals received orally 900 mg/kg of body weight of the Miswak aqueous extract daily for 4 weeks (Ramadan & Alshamrani, 2015).

Experimental design:
Twelve female albino rats with an average weight of about (165.3 ± 3.269 g) were used. The rats were housed in group cages and given free access to food and water. Then they were left for one week before starting the experiment for acclimatization. Prior to the beginning and throughout the experiment, the rats were housed at 24˚C room temperature and 12 hours light: 12 hours dark cycle. The rats were divided randomly into two main groups; untreated control & Miswak-treated groups each containing 6 animals. The experimental protocol was approved by the Local Ethical Committee and by the Institutional Review Board of the Faculty of Veterinary Medicine, Assuit University (Approval Number: 06/2023/0080) and was carried out in accordance with relevant guidelines and regulations. This research was done in compliance with the ARRIVE guidelines and regulations (https:// arriveguidelines.org).

Collection of the Samples:
At the end of the experiment the rats were anesthetized by using ketamine–xylazine this was achieved by a dose of 87 mg ketamine/kg of body weight and 13 mg xylazine/kg intramuscular (Van Pelt, L. 1977) and then euthanized by cervical dislocation. The uterus was dissected, removed and weighted.

Paraffin Sections:
Samples were fixed in neutral buffered formalin and processed for embedding in paraffin. Fixed tissue samples were dehydrated in ascending grades of alcohol

then they were cleared in methyl benzoate and embedded in paraffin wax. Serial sections were cut at 5 µm by a Reichert microtome (Leica RM 2155, Germany), and mounted on glass slides. Sections were kept in an incubator at 40°C for dryness. Then stained by Hematoxylin and eosin (Hx & E) for general histological examinations (Harris, 1900), and Periodic acid-Schiff's reaction (PAS) approach for demonstration of neutral mucopolysaccharides (McManus, 1948).

Semithin Sections:
Very small pieces (2 mm) of uteri were fixed in Karnovsky fixative at 4°C for 24 hours. The fixed specimens were washed several times in phosphate buffer, then post-fixed in 1% osmium tetroxide and dehydrated in ascending grades of alcohol and then embedded in epoxy resin. Semithin sections (1 µm) were stained with 1% Toluidine Blue (Abd-Elkareem *et al.*, 2018).

All stained sections were examined under a light microscope (Olympus, USA) and photos were taken by Olympus DP72 camera adapted into the microscope.

Morphometrical Studies:
Measurements were carried out using Image J 1.53c Software.
The morphometrical studies were applied on sections of uterus for measuring the following:

1- The thickness of endometrium.

2- The thickness of the myometrium

3- The thickness of the whole uterine wall

Statistical analysis:
Data were presented as the mean ± *SEM*. Statistical analysis was performed using unpaired student's t test, to compare Miswak treated group with the untreated control group. Differences were considered statistically significant at *(***p< 0. 001, **p< 0. 01, *p< 0. 05). P> 0.05* was considered not statistically significant. Statistical analysis was performed using Graph Pad Prism (Version 6.05).

RESULTS

Macroscopic examinations revealed that in the Miswak-treated group, the uteri appeared to have thick walls and thread-like pale organs with narrow lumen. While in the control group, they appeared more vascular, transparent, thin-walled and had a wide lumen filled with intrauterine fluid (Fig. 1). But it was found that the uterine weight was non-significantly decreased in the Miswak-treated group (0.32 ± 0.020 g) compared to the control group (0.58 ± 0.12 g).

Histologically, the uterine wall was formed of endometrium, myometrium and perimetrium. The Endometrium displayed glandular mucosa consisting of lamina epithelialis and lamina propria. Its epithelium was formed of simple columnar secretory epithelium. The myometrium consists of inner circular and outer longitudinal smooth muscle fibers with small-sized arteries and veins in between. The perimetrium contained loose connective tissue covered with simple squamous epithelium.

Our results revealed that in the control group, the thin wall surrounding the lumen appeared wide, dilated, and filled with uterine secretion and the endometrial surface was folded. Whereas in Miswak-treated group, the lumen was surrounded with thick wall and appeared narrow slit-like with less or no folding endometrial surface (Fig. 2). Lamina epithelialis in control group was consisted of simple columnar epithelium and in some areas, pseudostratified. The simple epithelium was formed of tall columnar cells with basally located nuclei. Whereas in the Miswak-treated group, it was changed to become simple low cuboidal to flat cells (Fig. 2).

The semithin sections examination revealed that in the control group, the epithelium had light and dark columnar secretory cells, with a marked apocrine secretion that characterizes the estrous phase. While in the Miswak-treated group, the epithelium exhibited vacuolation in the cytoplasm and pyknosis in the nuclei with no secretion (Fig 2E & 2F).

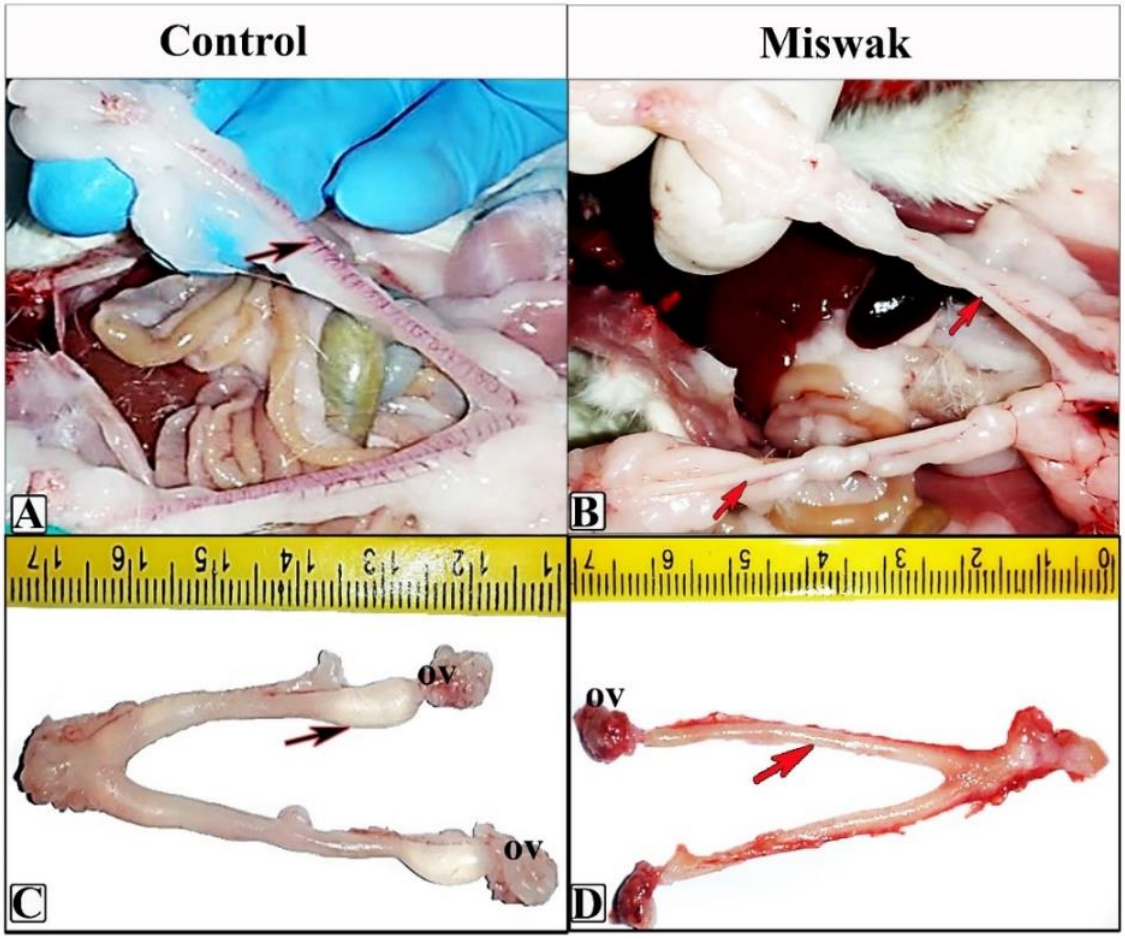


Fig. 1: Uterus at end of the experiment (A & C) control group showed edematous and hyperemic two uterine horns (black arrow), (B & D) Miswak treated group with pale thread-like uterine horns (red arrow).

By light microscopy, gland formation (adenogenesis) was observed in the control group. The gland formation was started by cellular proliferation in lamina epithelialis to form a mass of cells (bud stage). This mass then elongated toward the underlying connective tissue to form a cord of cells (cord stage). Then this cord canalized to form a tubular gland (canalization stage). The uterine glands were lined by a simple columnar apocrine-secreting epithelium, which characterized the secretory stage (fig. 3A & 3C). Whereas the uterine glands in the Miswak-treated group showed signs of the process of declining and degenerative changes including pyknosis of nuclei and in some cases, the epithelium changed to low cuboidal epithelium (Fig. 3B & 3D).

Interestingly, by Periodic Acid Schiff reaction, there were strong PAS-positive materials in the apical regions of the epithelial cells in the untreated control group compared to the weak reaction in Miswak treated group (Fig. 4A & 4B). Semithin sections showed active secretory glands in the control animals, in contrast to inactive glands in the Miswak-treated group (fig. 4C & 4D). Our findings showed that

the myometrium was composed of inner circular and outer longitudinal smooth muscle fibers that were thicker and more compact in the control group than the Miswak-treated one. Numerous blood vessels were found between the two muscle layers. Perimetrium was covered by the mesothelium in both groups. We observed that in the Miswak-treated group, the myometrium showed degeneration, atrophy, vacuolation and necrosis in muscle fibers (Fig. 5).

Morphometrical results indicated that there was a significant increase in the thickness of the endometrium and whole uterine wall at *(P < 0.05)* and a non-significant decrease in the thickness of myometrium in Miswak treated group compared to control (Table 1 & Fig. 6).

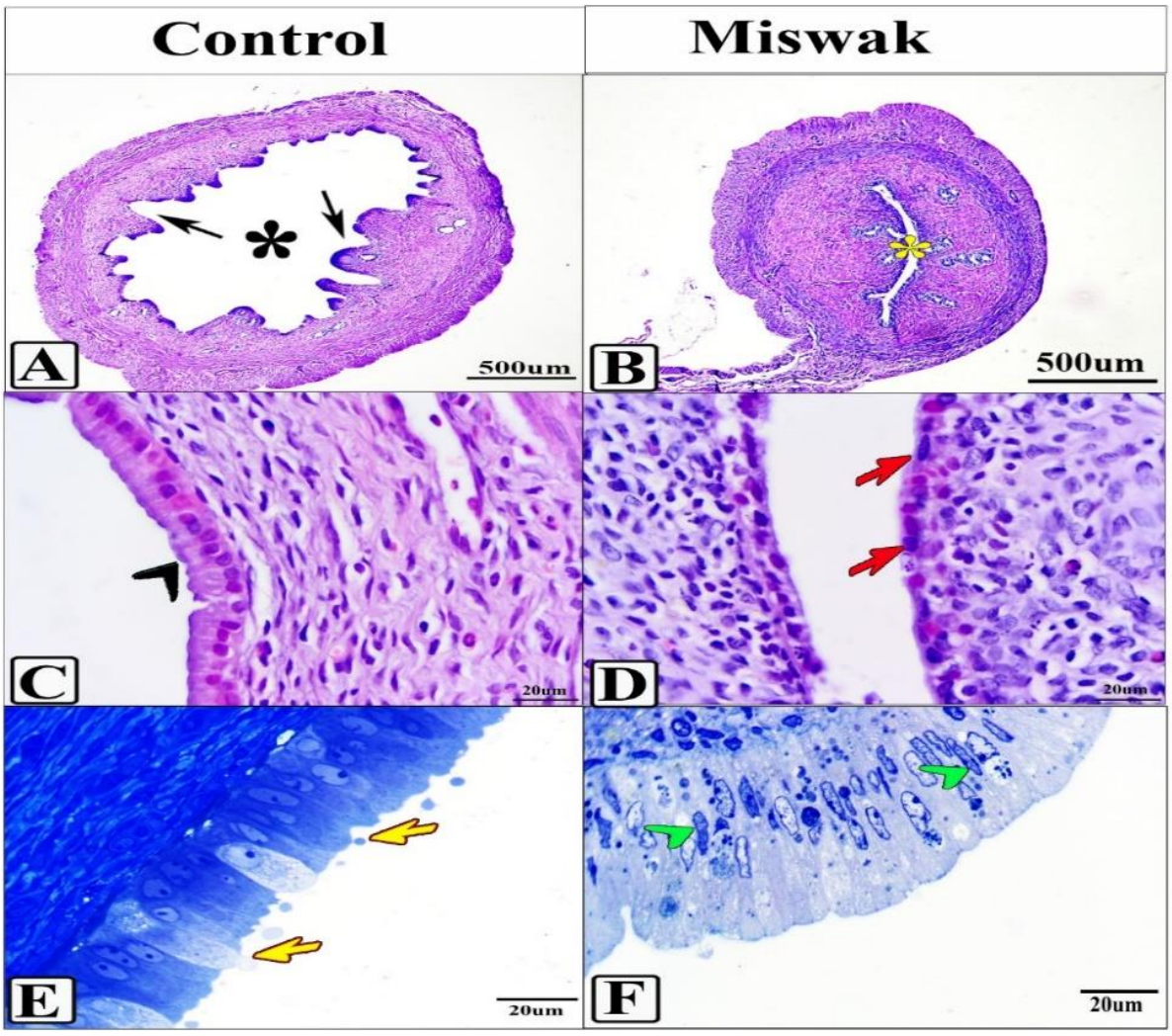


Fig. 2: Paraffin sections stained by HX & E (A-D) and semithin sections stained with toluidine blue (E & F) showed the general view of the uterus and lining epithelium. A: control group with wide lumen (black star) and folded mucosa (black arrow). B: Miswak group with a slit-like lumen (yellow star). C: Control group with simple columnar epithelium (black arrowhead) D: Miswak group with epithelium became simple squamous to simple cuboidal with necrosis and degeneration (red arrow). E: Control group with secretory columnar cells (yellow arrow). F: Miswak group with epithelium exhibit necrosis in nuclei (green arrowhead).

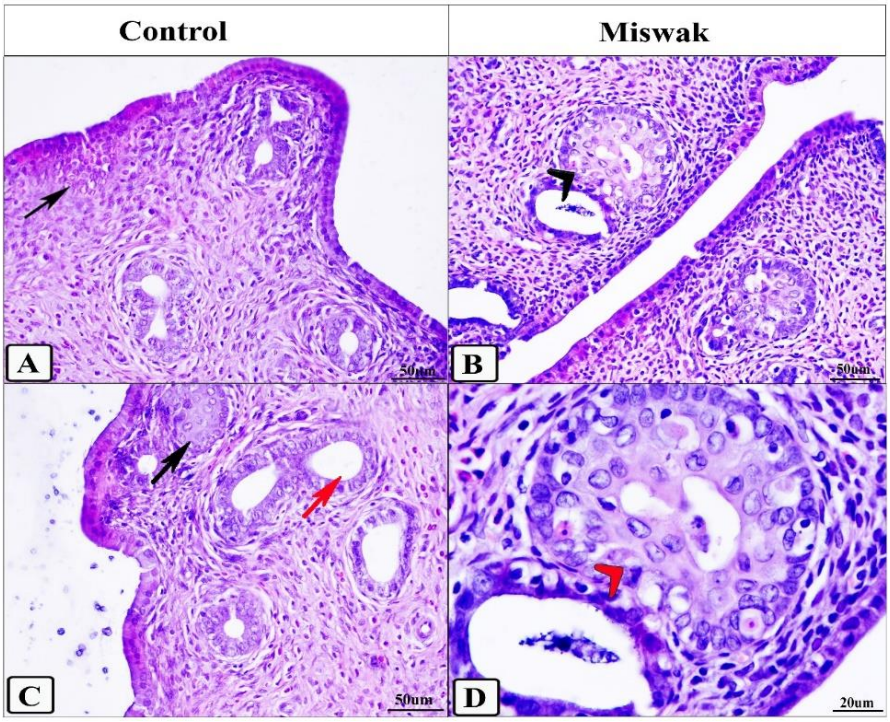


Fig. 3: Paraffin sections stained by HX & E explained gland formation. A: The control group showed cellular proliferation from lining epithelium extending into the underlying connective tissue; cord stage (black arrow). B: The miswak-treated group showed a degenerated mass of cells (black arrowhead). C: The control group showed a mass of cells separate from the epithelium (black arrow) and numerous glands with central lumen (red arrow); canalization stage. D: Miswak-treated group showed necrosis in the cells that formed uterine glands (red arrowhead).

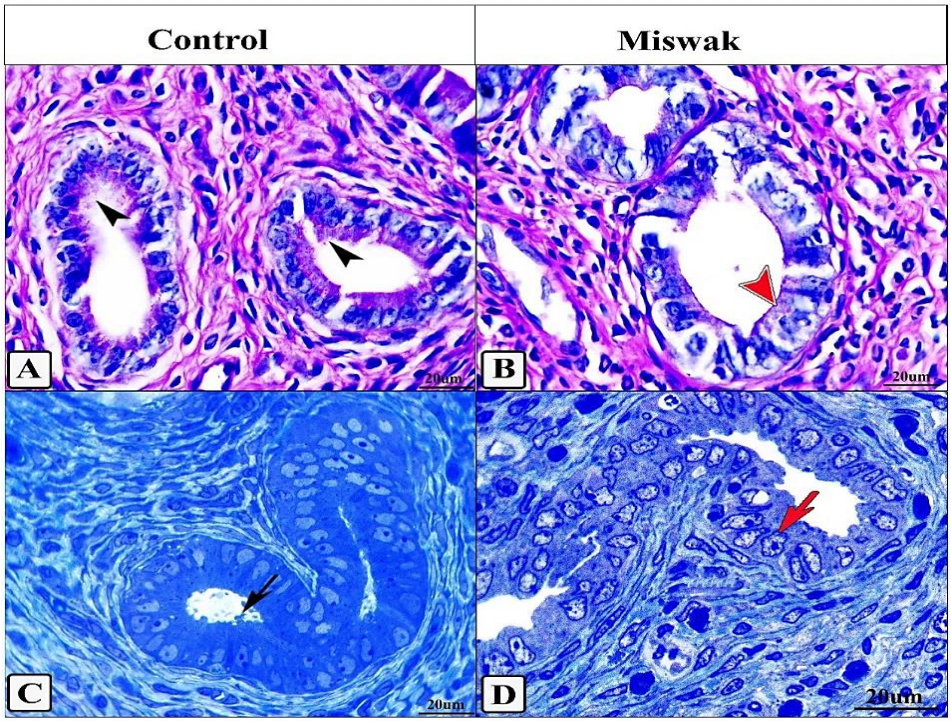


Fig. 4: Paraffin sections stained by PAS & Hx (A & B) and Semithin sections stained with toluidine blue (C & D) for glandular activity. A: Control group with strong positive glandular activity by PAS (black arrowhead). B: The Miswak-treated group showed weak glandular activity by PAS (red arrowhead). C: The control group showed numerous gland secretions (black arrow). D: The Miswak-treated group showed cellular degeneration (red arrow).

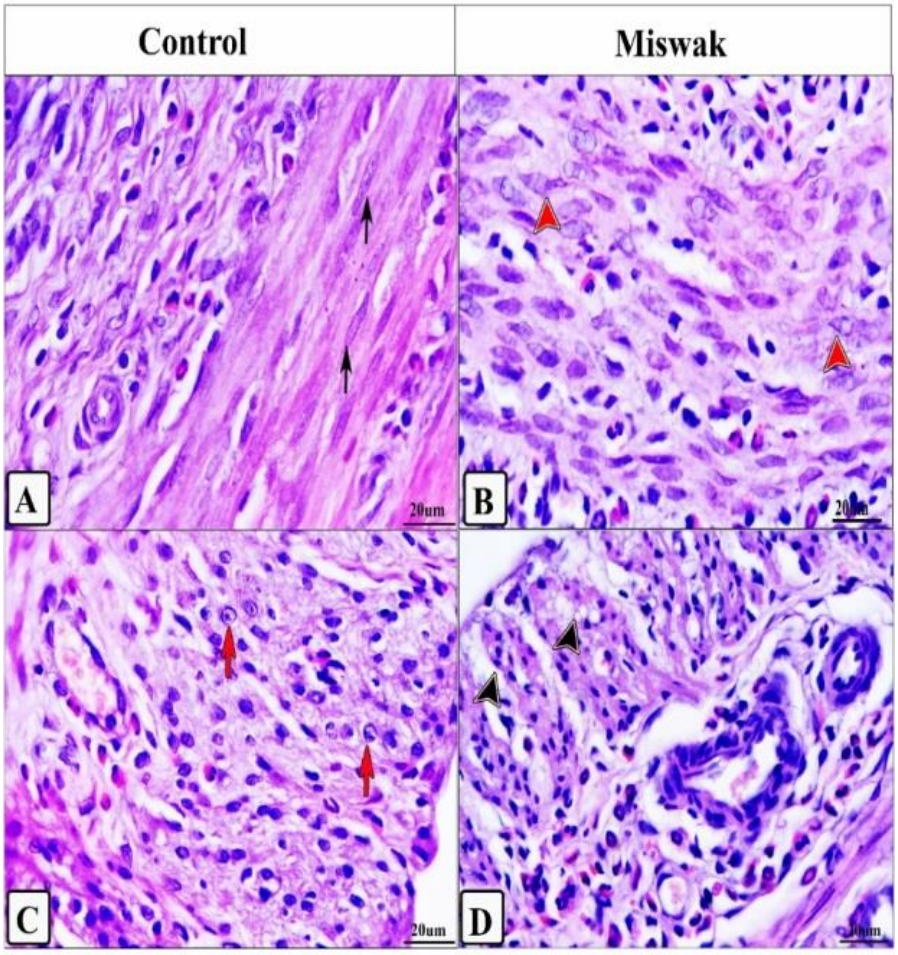
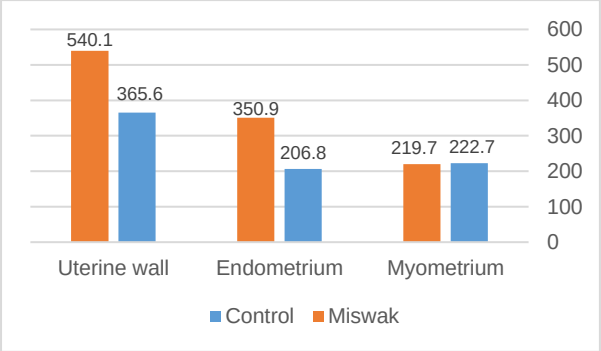


Fig. 5: Paraffin sections stained by HX & E explained differences between myometrium in two groups. A: The control group showed smooth muscle fibers spindle in shape with rod-shaped nuclei. (black arrow). B: The Miswak-treated group showed degeneration in muscle bundles (red arrowhead). C: The control group showed the outer longitudinal smooth muscle fiber with normal structure; rounded structure of different sizes and the largest of them contained a central rounded nucleus (red arrow). D: The Miswak group showed atrophy and vacuolation in muscle bundles (black arrowhead).

Table 1: Showed the thickness (µm) of the endometrium, myometrium and the whole uterine wall in control and Miswak-treated groups.

Groups	Endometrium	Myometrium	Uterine wall
Control	206.8 ± 30.37	222.7 ± 39.19	365.6 ± 57.04
Miswak	350.9 ± 7.88*	219.7 ± 13.91	540.1 ± 11.96 *

Fig. 6: Thickness (µm) of the endometrium, myometrium and uterine wall in control and Miswak group



DISCUSSION

When female albino rats were exposed to 900 mg/kg B.W of Miswak daily for 4 weeks we observed that the uterus became pale, and thread-like, while in the control group, the uterus was hyperemic, dilated, filled with fluid (luminal uterine fluid) that's mean animals in the control group were in estrous phase (Westwood, 2008). Miswak treatment did not significantly decrease the uterus weight and decreased myometrium thickness compared to the control group. This may be due to necrosis, degeneration and atrophy in muscle bundles this result was agreed with Abdoon *et al.* (2014), who reported that Miswak leave and stick extract decrease uterus weight as a result of atrophy of uterine tissue. While Thafar *et al.* (2016) and Darmani *et al.* (2003) reported that Miswak increases uterus weight. Also, endometrium significantly increased in thickness in the Miswak-treated group than control group. We suggest that this was due to the increase in the thickness of lamina propria as a result of its proliferation in the Miswak-treated group.

Mucosal epithelial cells are very important in antigens recognition, bacterial and viral killing, and also signal to underlying immune cells when pathogenic exceeds their protective capacity. This function is controlled by the female sex hormones (Wire *et al.,* 2005). We observed that the uterus in the control group has active endometrial epithelium consisting of tall columnar cells which are two types of light and dark cells (Piano like appearance) as mentioned by (Hassan *et al.,* 2006 and Moselhy W. *et al.* (2016).

Light microscopy showed apocrine secretory epithelial cells in the control group and macroscopically the uterus was edematous and filled with uterine fluid. This observation indicated that the control animals were in cyclicity. While the epithelium in the Miswak-treated group was cuboidal to flat and some were sloughed or necrotic.

Uterine fluid volume differs according to the stage of the estrous cycle. Its secretion by the endometrial epithelium is stimulated by β-estradiol which is increased during estrus (Naftalin *et al.,* 2002 and Salleh *et al.,* 2005) so what, the fluid volume is high at the pro-estrous and estrous phase but low at diestrous (Shih *et al.,* 1940 and Clemetson *et al.,* 1977). Our result revealed that the uterus in the control group was dilated and filled with fluid (uterine luminal fluid), in contrast to the Miswak-treated group in which the uterus was thread-like in appearance. Uterine fluid contains a high concentration of $HCO^{-}{}_{3}$ which plays an essential role in sperm capacitation and fertilization.

It is well-known that the apical membrane of the endometrial epithelium contains two important transport proteins. The first is the apical solute carrier 26 (SLC26) family anion exchangers for secreting $HCO^{-}{}_{3}$ into the uterine fluid. The second protein is the electroneutral $Na^{+}/HCO-3$ cotransporter NBCn1, which is encoded by the *SLC4A7* gene and functions in the reabsorption of $HCO^{-}{}_{3}$. The protein expression of the apical NBCn1 and that of the apical SLC26A4 and SLC26A6 are reciprocally regulated during the estrous cycle in the uterus. NBCn1 is most abundant during diestrus, whereas SLC26A4/A6 is most abundant during proestrus and estrus. This is because the uterine expression of NBCn1 is inhibited by β-estradiol, but stimulated by progesterone, while that of SLC26A4/A6 is stimulated by β-estradiol and inhibited by progesterone (Salleh *et al.,* 2005 and Xie *et al.,* 2018). For these reasons, the activity for $HCO^{-}{}_{3}$ secretion by the endometrial epithelium is significantly higher at estrus than it is at diestrus. Whereas the reabsorption $HCO^{-}{}_{3}$ by the endometrial epithelium is significantly higher at diestrus than it is at estrus. The uterine fluid volume and acid-base homeostasis are regulated by the finely-tuned balance of the activity for $HCO^{-}{}_{3}$ secretion involving the apical SLC26A4/A6 and the activity for $HCO^{-}{}_{3}$ reabsorption involving the apical NBCn1 (Xie *et al.,* 2018). The secretion of uterine luminal fluid initially provides a transport and support medium for spermatozoa, while the absorption of uterine luminal fluid allows blastocyst implantation (Salleh *et al.,* 2005).

El-Rafey and Abdoon (1996) reported that in the buffalo estrous phase, the stroma was highly edematous and blood capillaries were highly congested.

Uterine glands develop as invaginations of luminal epithelium that invade the underlying mesenchyme resulting in an extensive network of epithelial glands throughout the stroma. Endometrial gland adenogenesis in rats is characterized by the formation of simple, tubular glands that, unlike endometrial glands in humans and domestic animals, are neither tightly coiled nor extensively branched (Cooke *et al.*, 2013). Herein we observed the stages of uterine gland formation in the control group: by proliferation of luminal epithelium forming mass of cells (bud stage), downward growth to connective tissue forming cord of cells that later became canalized and started to secret. Whereas in the Miswak-group, there was degeneration and necrosis in the uterine glands.

Uterine glands have an important role in conception, implantation, establishment of uterine receptivity, and stromal cell decidualization**.** The uterine luminal fluid which is secreted by endometrial epithelium and endometrial glands contains amino acids, ions, carbohydrates (glucose, lactate, pyruvate), lipids, and proteins (cytokines, enzymes, hormones, growth factors, proteases and their inhibitors, transporters (Abd-Elkareem, 2017 and Filant & Spencer, 2014).

In this study, the uterine glands were actively secretory in the control group and exhibited a strong positive PAS stain. This might be due to the presence of carbohydrates in the uterine luminal fluid during estrus (Harris *et al.*, 2005). All these results explored the important role of uterine glands during estrous. On the other hand, Miswak causes necrosis and degeneration in the glandular epithelium which becomes less functioning and has no secretion.

This result was similar to hormonal contraceptives that cause endometrial gland regression as reported by Gondos, (1976). Contraceptive as Noristerat (NET-EN)With higher doses causes arrest of endometrial growth and Depo-Provera (DMPA) leads to endometrial tissues becoming inactive and prolonged treatment at high doses results in atrophy of endometrium (Bhowmik and Mukherjea 1988).

Bitzer *et al.* (2011) reported that estradiol oral contraceptive is effective in transforming endometrium into a secretory inactive and atrophic state. Also, continuous use of oral contraceptives is associated with endometrium atrophy as reported by Hee *et al.* (2013) and Eshre Capri workshop group (2001). Phytoestrogen plants as *Cleome gynandra* a medicinal plant have the ability to contract the uterus. Monima *et al.* (2019) studied the plant extract on reproductive organs, they observed that the endometrium of the rats treated with 500mg/kg B.W was lined by simple squamous epithelium with numerous large vacuoles and very few endometrial glands.

CONCLUSION

Miswak affects the uterus in female albino rats through reducing of the uterine size, sloughing of the epithelium, degeneration and atrophy of the glands, and degeneration of the myometrium, in addition to poor vasculature. These results are similar to some contraceptive hormones and some phytoestrogen plants.

Exposure of the albino female rats to Miswak at this dose for this period resulted in a stat similar to persistent anestrous.

It is thought that observed findings of Miswak may be attributed to its direct effect on the uterus or indirect effect on hypothalamus-pituitary-gonadal axis. Further studies should be done to highlight the effect of Miswak on the uterus at the functional and molecular levels.

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تأثير المستخلص المائى للمسواك (سالفادورا بيرسيكا) على التركيب الهستومورفولجي للرحم فى إناث الجرزان

صابرين مصطفى غريب ، محمود عبد الكريم ، أحمد أبو المجد

Email: mahmoud.mahmoud3@vet.aun.edu.eg abdelkareem2006@yahoo.com
Assiut University web-site: www.aun.edu.eg

أجريت هذه الدراسة على اثنتى عشرة أنثى بالغة من إناث الجرزان تم الحصول عليها من بيت الحيوان كلية الطب البيطرى- جامعة أسيوط. قسمت هذه الحيوانات إلى مجموعتين كل واحده تحتوى على ستة إناث, وتزن الأنثى الواحدة حوالى مائة وخمسة وستون جراما. المجموعة الأولى اتخذت كمجموعة ضابطة حيث جرعت بمحلول متعادل. بينما المجموعة الثانية جرعت بالمستخلص المائى للسالفادورا بيرسيكا (للمسواك) يوميا بجرعة 900 ملليجرام لكل كيلوجرام من وزن الحيوان لمدة أربعة أسابيع ثم أخذت العينات فى نهاية التجربة.

أسفرت هذه الدراسة إلى أن المجموعة المعالجة بالمسواك كان الرحم فيها منخفض فى الوزن و شاحب في اللون و ذو جدار سميك على عكس المجموعة الضابطة التى أظهرت نشاطا فى الرحم من حيث زيادة الامداد الدموى الذى جعل لون الرحم وردى وتجويفه مليء بالافرازات وعمل علي زيادة وزن الرحم. كما أظهرت هذه الدراسة أنه تحت المجهر الضوئي كان الرحم فى المجموعة المعالجة بالمسواك له تجويف ضيق شبيهًا بالشق، وكانت الغدد الرحمية أقل نشاطًا، وكانت بطانة الرحم سميكة. بينما في المجموعة الضابطة، كان تجويف الرحم واسعا وكان الغشاء المخاطي مطويًا ومغطى بخلايا طلائية عمودية إفرازية و كانت الغدد الرحمية أكثر نشاطًا. وهذا يظهر أن تناول مستخلص السواك عن طريق الفم يقلل من نشاط الرحم فى الجرزان.