Effect of licorice extract on ovulation induction in immature female mice

تأثير مستخلص عرق السوس على احداث الأباضة في أناث الفئران غير البالغة

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Abstract

The objective of this study was to assess the effect of licorice extract (LE) on induction of ovulation and on the structural changes of ovary, oviduct and uterus. Divided into 6 groups: G1, G2, G3, G4, G5 and G6 (14 animals for each). G1 received 1g/kg bwt/day of licorice extract for 2 weeks. G2 were fed with 0.5g/kg bwt/day of licorice extract for 2 weeks. Mice fed on normal diet and tap water (without LE) were regarded as control group (G3). G4 and G5 received the same doses of G1 and G2 respectively for 4 weeks. G6 received tap water only and considered as a control for G4 and G5. Before the initiation of dosing, vaginal smears were taken daily and ceased when estrus phase of first ovarian cycle appeared. At the end of the experiment, animals were sacrificed and reproductive system were excised and divided into two sides: the left one only was used for histological studies. The results indicated that LE consumption caused an increase in the diameter of the ovary, total number of follicles and their diameters of all mice treated with a higher dose and longer duration. Dose of 1g/kg bwt/day was also more effective upon measuring the diameters of uterine glands and the diameter of the oviductal ampulla, while the lower dose gave higher results only in measuring the height of endometrial epithelial lining cells and the oviductal epithelial lining cells. There was a significant precocious estrus cycle in animals that treated with 1g/kg bwt/day and a non significant precocity with 0.5g/kg bwt/day, as compared to the control group. These results may be attributed to the effect of various components of LE, especially estrogen-like substances in addition to other components like: proteins, amino acids, vitamins and trace elements present in the licorice. The results of the present study indicate that the use of low doses of LE (0.5, 1)g for a short period of time 2-4 weeks improved the reproductive organs, induced earlier estrus cycle and consequently may improve the reproduction. The consumption of 1g licorice extract daily for 4 weeks induced sexual maturity better than the other dose and period.

المستخلص

تهدف الدراسة الى تقييم تأثيرات مستخلص عرق السوس على تحفيز الأباضة ومايرافقها من تغيرات تركيبية في المبيض وقناة البيض والرحم . أجريت هذه الدراسة على إناث فئران غير بالغة بعمر أربعة أسابيع قُسّمت الى ست

مجاميع (14 حيواناً / مجموعة) . تناولت المجموعة الأولى 1غم /كغم من وزن الجسم يوميا ولمدة اسبوعين من مستخلص عرق السوس مذابة في الماء والمجموعة الثانية أعطيت 0.5 غرام / كغم من مستخلص عرق السوس يومياً لإسبوعين بينما أعطيت المجموع الثالثة صفر جرعة من المادة وأتخذت مقياساً للمجموعتين الأولى والثانية . أعطيت المجموعة الرابعة والخامسة الجرع نفسها كما للأولى والثانية على التوالي لكن الفترة أطول (4 أسابيع) أمّا المجموعة السادسة فاعتبرت مقياساً للمجموعتين الرابعة والخامسة (مجموعة سيطرة) . قبل البدء بالتجريع أخذت المسحات المهبلية يومياً حتى ظهور طور الشبق في أول دورة مبيضية . في نهاية التجربة قُتلت الحيوانات وأستا صل جهاز التناسل منها وقسم الى جانبين وحُضّر الأيسر لإجراء الدراسات النسيجية عليه . ظهرت النتائج أنّ تناول مستخلص عرق السوس سبّب زيادة في قطر المبيض وفي العدد الكليّ للجريبات المبيضية وفي اقطارها في كل فئران المجاميع المُعالجة مع زيادة معنوية أعلى في المجاميع التي عوملت بالجرعة الأعلى والفترة الأطول والتي كانت كذلك اكثر فعالية عند قياس قطر الغدّد الرحمية وقطر جراب قناة البيض بينما أظهرت الجرعة الأوطأ نتائج أعلى فقط عند قياس إرتفاع خلايا البطانة الطلائية في بطانة الرحم وخلايا البطانة الطلائية في قناة البيض . كما ظهرت دورات شبقية مبكرة بفرق معنوي في الحيوانات المعالجة بالجرعة الأعلى وبفرق غير معنوي في مجموعة الجرعة الأدنى مقارنة بمجموعة السيطرة . ان هذه النتائج قد تنسب الى تأثير المكونات المختلفة لعرق السوس ، خصوصاً مواد شبه الهرمون المثيرة للدورة النزوية فضلا عن مكونات أخرى مثل : البروتينات ، الأحماض الأمينية ، العناصر النادرة والفيتامينات . ان النتائج المستخلصة من هذه الدراسة تشير إلى أن استعمال جرعات واطئة من مستخلص عرق السوس (0.5 – 1)غم / كغم/يوم ولمدة قليلة (2- 4 اسابيع) أثرت ايجابيا في أعضاء التكاثر وحفزت البلوغ الجنسي في اناث الحيوانات غير البالغة .

Introduction

One of the most important issues in women`s health concerns the risks and benefits of estrogen replacement therapy, however, continual uncertainty and lack of consensus of standard estrogen replacement therapy has driven many women to seek alternative sources of estrogen, including herbal remedies [1], which they thought that it may help in restoring menstruation, some of these herbs are Black Cohosh, Milk Thistle, Wiled Yam, Horsetail and licorice [2]. Many of licorice endocrine properties can be derived from observations of authors of the ancient world, when hormones were not known [3].

A series of studies has been done since the middle of the previous century [4, 5,6] that best illustrates induction of sexual maturity in immature females but they almost use hormones for stimulation like pregnant mare serum. In this study, we use licorice root extract for ovulation induction in immature female mice to assess its effect on some criteria of genital organs like vagina and ovary in addition to study the possibility of using licorice as a dietary supplement to bring about final sexual maturation.

Materials & Methods

The study is performed on 84 immature female Swiss-Webster mice, their ages ranged between 28-30 days with a body weight (bwt) ranged between (9 -14)g. fed with standard diet. Experimental animals were divided into six groups (14 mice/group) as follows: G1: administrated with 1g/kgbwt. /day (0.18mg daily) for 2 weeks, G2: administrated with 0.5g/kgbwt./day (0.09mg daily) for 2 weeks and G3: was given tap water only (without LE) regarded as control for G1 & G2. G4and G5 were administrated with1g/kgbwt./day (0.18mg daily) and 0.5g/kg bwt. /day (0.09mg daily) respectively for 4 weeks and G6: was given tap water only regarded as control for G4 and G5.

Vaginal smears were taken at the beginning of the experiment and continued daily to determine the day of first vaginal estrus (fully keratinized vaginal smear), as described by [7]. Mice were assigned to one of the four estrous stages (proestrus, estrus, metestrus, or diestrus) were visually quantitated under a light microscope, according to [8].

Licorice root extract was dissolved in tap water and administrated orally using a fine plastic stomach tube to animals with dose of 1g/kgbwt./day for G1and G4, and 0.5g/kg bwt./day for G2and G5 according to accepted human dose of 1-4g/kg/day [9], while G3 and G6 were fed with tap water as a control. After 2 weeks, animals of G1, G2 and G3 were anesthetized using diethyl ether then sacrificed and the abdominal cavity was opened, whole reproductive system was quickly excised and immersed in few drops of normal saline. Right ovary with oviduct and right horn of uterus were separated from left side. The left side was fixed with 10% formal saline for subsequent histological study. After 4 weeks, the animals of G4, G5 and G6 were treated in the same way as those killed after 2 weeks.

Parameters used in studying the histological sections:

A- Ovaries:

1. Diameter of ovary.
2. Number and diameter of Graafian (antral) follicles (GF).
3. Number of corpus luteum (CL).

B- Oviducts:

1. Diameter of oviduct (ampulla).
2. Thickness of epithelial layer.

C- Uterus:

1. Diameter of endometrial glands.
2. Thickness of epithelial layer.

Statistical analysis

Data from treated and control groups were expressed as mean ± standard error (SE) and analyzed using Student`s t-test to compare values from experimental and control groups at individual time points. Differences between values were considered significant at $p<0.05$ and highly significant at $p<0.01$ [10].

Results

Effects of 1g and 0.5g /kg bwt /day of licorice extract administration for two weeks duration on histological changes of:

A- Ovaries:

1. Changes in ovaries and ovarian follicles diameters: The diameter of ovary increased significantly ($P<0.05$) in G1 while the increment was non significant ($P>0.05$) in G2 compared to control group. In G1, the diameter of Graafian follicles (antral follicles) (GF), showed a highly significant increase ($P<0.01$), while in G2, the GF exhibit a non significant elevation ($P>0.05$), Table (1).

Table (1): Structural changes in the ovaries associated with administration of licorice extract to immature female mice .

Diameter (µm)	2 weeks treatment G1	G2	G3	4 weeks treatment G4	G5	G6
Diameter of ovary	* 884.25± 3.81	866.48± 5.82	854.18± 6.09	**1109.37± 4.98	**1058.25± 8.98	949.42± 3.06
Diameter of Graafian.Follicles.	** 208.94± 3.27	183.32± 3.46	171.51± 4.27	**253.16± 5.8	**211.01± 6.42	175.13± 2.48
No.of Graafian.Follicles.	** 11.28± 0.49	8.21± 0.7	7.64± 0.24	** 11.57± 0.27	* 11.21± 0.44	9± 0.34
No.of Courps.Luteum.	* 0.21± 0.21	* 0.14± 0.14	0± 0	** 1.85± 0.46	* 0.28± 0.19	0± 0

2. Changes in ovarian follicles numbers: In G1 there is a highly significant increase ($P<0.01$) in no. of GF and significant increase in no. of CL ($P<0.05$) in comparison with that of G3. In G2, GF showed a non significant increase in their numbers ($P>0.05$), while no. of CL increased significantly ($P<0.05$). Table (1), Figure (1).

Table (2): Structural changes in the uteri and oviducts associated with administration of licorice extract to immature female mice

(µm)	2 weeks treatment G1	G2	G3	4 weeks treatment G4	G5	G6
Uterine. Epithelial cells height.	*13.81± 0.51	** 14.58± 0.58	10.56± 0.39	**16.82± 0.74	**18.46± 0.73	12.12± 0.55
Endometrial.Glands.	** 34.21± 0.65	**34.13± 0.76	25.11± 0.57	**43.07± 0.59	**38.68± 0.68	26.19± 0.3
Oviduct Diameter.	* 245.15± 3.05	**216.41± 7.77	150.66± 5.41	**324.71± 14	**268.79± 3.39	196.87± 13.04
Oviduct Epithelial Lining cells height.	* 13.4± 0.46	**13.33± 0.51	10.05± 0.45	**14.61± 0.38	**15.71± 0.31	11.12± 0.36

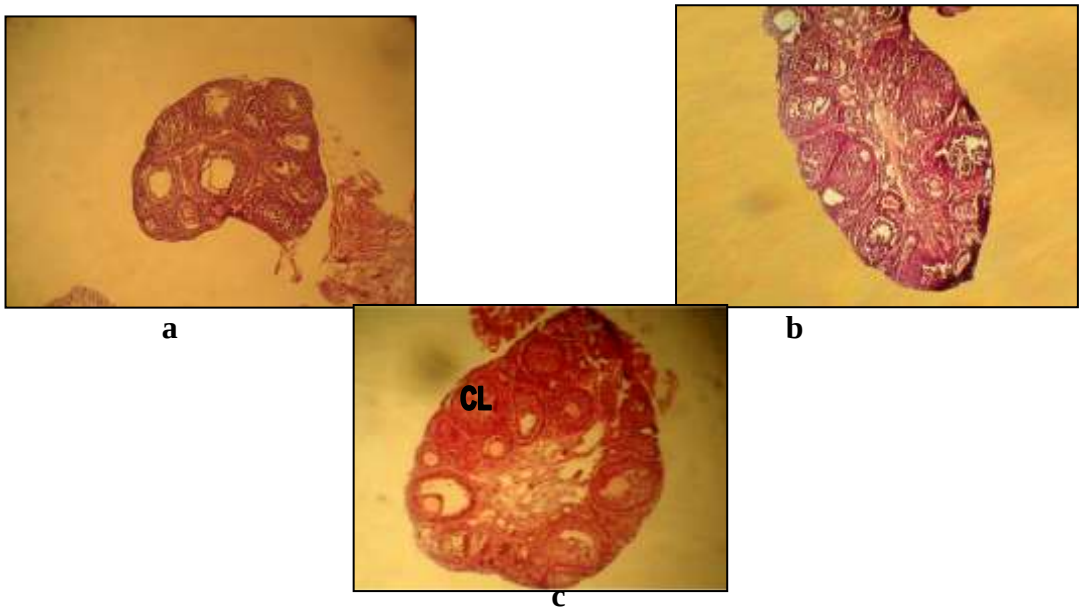


Fig (1) : Ovarian sections of mice aged 6 weeks:(a) control group,(b) treated with 0.5g/kg/day of licorice extract for two weeks ,(c)treated with 1g/kg/day of licorice extract for two weeks (H & E, X100). Note the presence of Corpus luteum (CL) and the increase in the diameter of the ovary & in the number of follicles in treated groups.

B. Uteri and oviducts:

The endometrial and oviductal epithelial lining cells height elevated significantly (P<0.05) in G1, while in G2, they increased with highly significant differences (P<0.01) compared to that of control group. The uterine glands diameters increased highly significantly (P<0.01) in both G1 and G2. The oviducts diameters (ampulla region) showed a significant increase (P<0.05) in both G1 and G2 in comparison with G3, with more elevation in G1 than in G2. Table (2) Figure(2,3).

Table (2): Structural changes in the uteri and oviducts associated with administration of licorice extract to immature female mice

		2 weeks treatment			4 weeks treatment	
(µm)	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**
Uterine. Epithelial cells height.	***13.81± 0.51**	**** 14.58± 0.58**	**10.56± 0.39**	****16.82± 0.74**	****18.46± 0.73**	**12.12± 0.55**
Endometrial.Glands.	**** 34.21± 0.65**	****34.13± 0.76**	**25.11± 0.57**	****43.07± 0.59**	****38.68± 0.68**	**26.19± 0.3**
Oviduct Diameter.	** 245.15± 3.05**	****216.41± 7.77**	**150.66± 5.41**	****324.71± 14**	****268.79± 3.39**	**196.87± 13.04**
Oviduct Epithelial Lining cells height.	** 13.4± 0.46**	****13.33± 0.51**	**10.05± 0.45**	****14.61± 0.38**	****15.71± 0.31**	**11.12± 0.36**

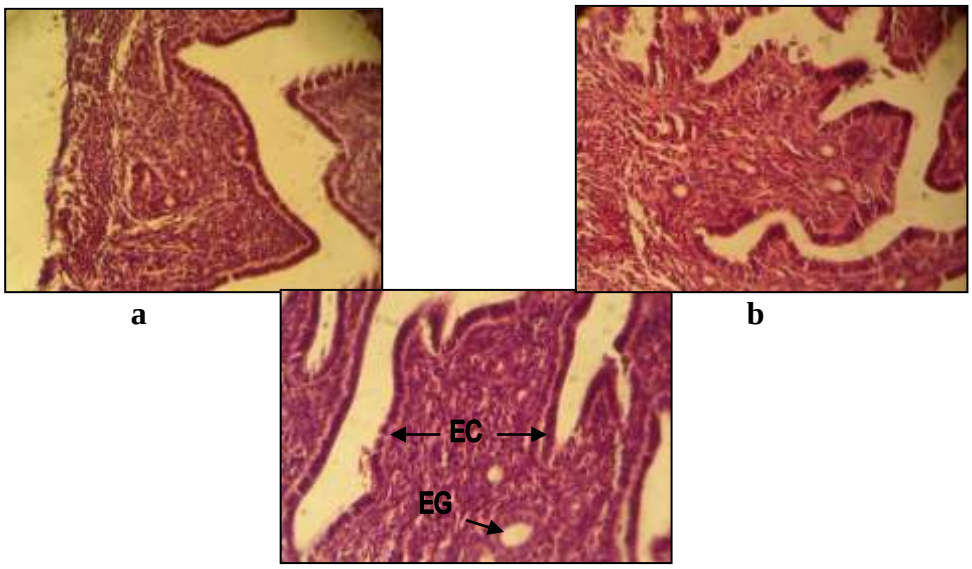


Fig (2): Longitudinal section in uterine horn of mice aged 6 weeks:(a) control group, (b) treated with 0.5g/kg/day of licorice extract for 2 weeks,(c) treated with 1g/kg/day of licorice extract for 2 weeks showing the increase in the endometrial lining cells height (EC) and in the endometrial glands diameter (EG) in the treated groups. (H & E, X 400).

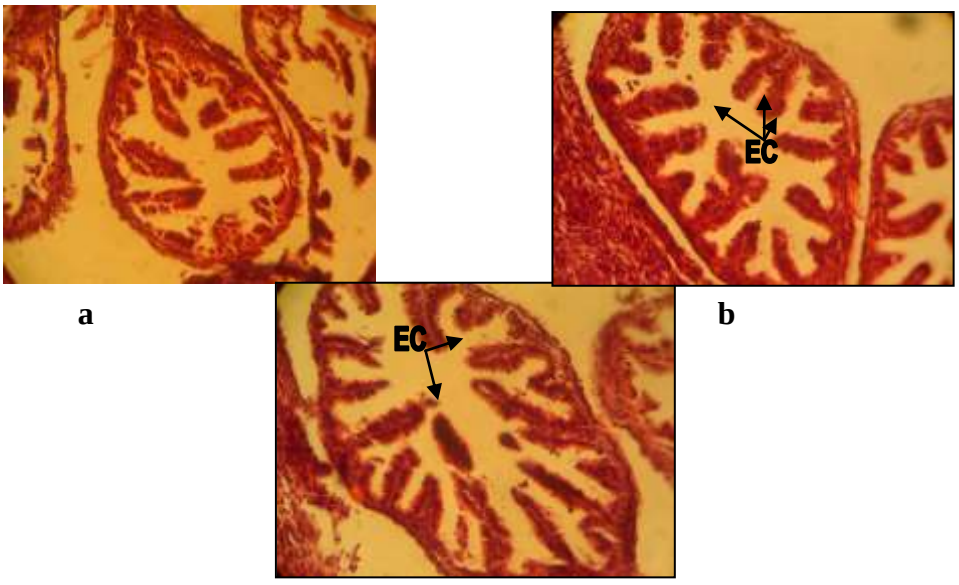


Fig (3): Transverse section in oviduct of mice aged 6 weeks: (a)control group,(b)treated with 0.5g/kg/day of licorice extract for 2 weeks, (c) treated with 1g/kg/day of licorice extract for 2 weeks. Note the increase in the diameter of oviduct & in the epithelial lining cell height (EC) of the treated groups. (H & E, X 400).

C- Effects on estrus cycle induction:

Vaginal smear examination revealed that more than third 33.33% of the females in G1 (which consumed 1g/kg/day) showed an earlier appearance of the first estrus phase of the first ovarian cycle by 2 days i:e on day 38 and all the other two third 66% of them reached

their first estrus on day 39 with a significant difference (P<0.05) as compared to G3 while those in G2 showed lower percentages of response in starting early estrus cycle, and only 25% reached the first estrus on day 38, 33.33% on day 39 and the rest of them on day 40, on the other hand, none of the females in the control group, reached the first estrus phase before day 40 of age. Table (3).

Table (3): Precocious estrus cycle associated with administration of licorice extract to immature female mice

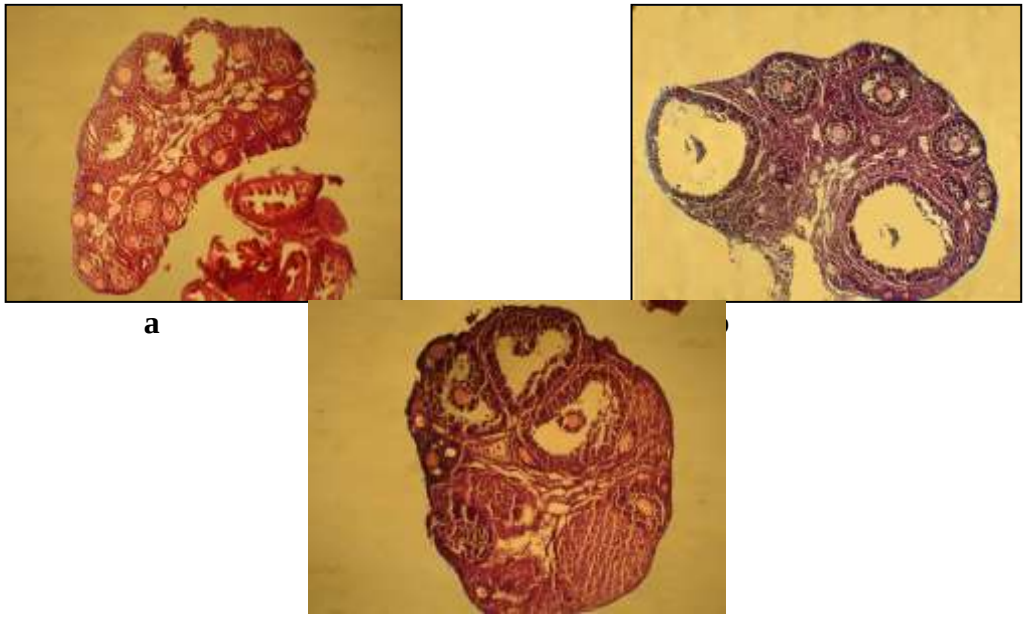
Mice age	Percentage of mice enter first estrus cycle		
	G1	G2	G3
38 days	33.33% ±0.14	25% ±0.13	0
39 days	* 66% ± 0.14	33.33% ± 0.14	0
40 days	0	41.66% ± 0.14	100% ± 0

Values are mean ± standard error (SEM), (n=, 14 animals/group)
*** Significant changes (< 0.05)**
****highly significant changes (P<0.01)**

II. Effects of 1g and 0.5g /kg bwt /day of licorice extract administration for 4 weeks duration on histological changes of :-

A- Ovaries:

1. Changes in ovaries and ovarian follicles diameters: The diameter of ovary exhibited a highly significant increase (P<0.01) in G4 and G5 as compared to G6. In G4 and G5; GF diameters showed a highly significant increase (P<0.01) when compared to the control group. Table (1), Figure (4)



a

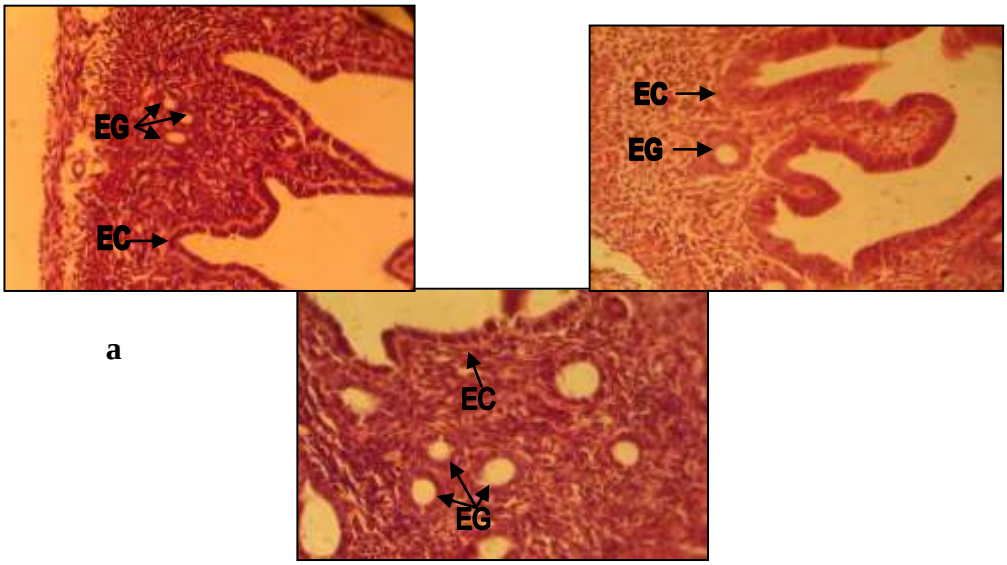
c

Fig (4): Ovarian section of mice aged 8 weeks:(a) control group, (b) treated with 0.5g/kg/day of licorice extract for four weeks,(c) treated with 1g/kg/day of licorice extract for four weeks (H & E, X100). Note the presence of Corpus luteum (CL) and the increase in the diameter of ovary & follicles in the treated groups

2. Changes in ovarian follicles numbers: In G4, number of GF and CL showed a highly significant increase (P<0.01) from that of control group. In G5, number of GF and CL showed a significant increase (P<0.05), as compared to G6. Table (1), Figure(4).

B- Uteri and oviducts changes:

The endometrial and oviductal epithelial lining cells height elevated highly significantly (P<0.01) in both G4 and G5 in comparison with G6 with a higher elevation in G5, also the uterine glands diameters and oviducts diameters increased with a highly significant difference (P<0.01) in both G4 and G5 compared to G6. Table (2), Figure (5 ,6).



Figure(5): Longitudinal section in uterine horn of mice aged 8 weeks:(a) control group, (b) treated with 0.5g/kg/day of licorice extract for four weeks, (c) treated with 1g/kg/day of licorice extract for four weeks showing the increase in the endometrial lining cells height (EC) and in the endometrial glands diameter (EG) in the treated groups. (H & E, X 400).

Fig (6): Transverse section in oviduct of mice aged 8 weeks(a) control group,(b) treated with 0.5g/kg/day of licorice extract for 4 weeks,(c) treated with 1g/kg/day of licorice extract for 4 weeks. Note the increase in the diameter of oviduct & in the epithelial lining cell height (EC) of the treated groups. (H & E, X 400).

Discussion

Results of administration of two doses of (LE) to immature female mice for two durations (2 & 4 weeks) showed important structural changes in the ovaries, oviducts and uteri, as well as improvement in ovarian folliculogenesis and consequent earlier induction of estrus cycle.

The clear structural changes in the ovaries, including the elevation in the ovarian diameters, , number of follicles and in their diameters can be attributed to the effects of estrogen-like substances present in the licorice. [11] confirms the estrogen bioactivity of licorice., this may cause positive feedback action on gonadotropin, probably from both a direct effect of the estrogen on the pituitary gonadotropes to secrete more FSH & LH in response to GnRH and indirectly by stimulating the hypothalamic neurons that secrete GnRH with modulation of the frequency and magnitude of the pulses of GnRH [12,13].

Many researchers [14,15] noticed that serum E2 level tends to rise significantly by ingestion of licorice extract, because it has an action similar to estriol and E2, and it binds to estrogen receptors in genital organs [16,17]. LE also acts directly on the ovarian stroma, affecting estrogen and androgen synthesis [18].It is well known that the stromal tissues of the ovary have the potential to make estrogens and androgens [19]. Estrogens synergize with gonadotropins in the promotion of the ovarian growth [13]. FSH rescues several primary follicles from a pool of these follicles in the ovary each cycle and stimulates the maturation of these follicles, while LH stimulates final follicular maturation and causes follicular rupture and ovulation [20]. Follicular development depends on production of E2, which acts within the follicle in an autocrine and paracrine manner, E2 promotes

proliferation of granulosa cells and increases their responsiveness to FSH, E2 may also stimulate proliferation of theca interna cells, simultaneously, E2 and FSH induce granulosa cells to synthesize receptors for LH, granulosa cells of preovulatory follicles have abundant LH receptors and consequently have acquired sensitivity to LH [21]. Both early and late follicular maturation depend on adequate FSH & LH as well as E2 [22]. FSH stimulates GF to rapidly increase in size and form the mature follicle [23]. Larger follicles need gonadotropic support if they are to reach ovulatory size [22], so in this study, presence of estrogen-like substances in licorice probably causes rapidly accelerating growth of ovary and thrives ovarian follicles that leads to production of more ripe follicles with subsequent earlier ovulation with acquisition of more number of CL in treated groups with licorice extract as compared to control group that did not revealed production of any CL.

The positive results in the uteri concerning the increase in the height of endometrial epithelial lining cells and the diameter of endometrial glands reflect the importance of ingestion this herb at the peripubertal age, since it is well known that estrogens promote growth and development of the oviducts, uterus, vagina and external genitalia, estrogens stimulate cellular proliferation in the mucosal linings as well as in the muscular coats of these structures at puberty [21], so the positive results in uteri of the treated animals may be correlated with the presence of the estrogen-like substance in licorice because the uteri are extremely sensitive to exogenous estrogen [24].

The clear positive effect of (LE) on oviducts can be also related to the phyto-estrogen content of licorice. The influence of the steroidal hormones on the fallopian tubes appears to be quite significant, which are important for ciliary and muscular activity [25]. The estrogens induce the glandular tissues of this lining to proliferate, and they cause the number of ciliated epithelial cells that line the oviducts and their cellular activities to increase [26] and this is exactly what was detected in the treated animals compared to that of control group.

In this study, treated mice enter estrus cycle earlier than control group. This can be attributed to the probable rise in reproductive hormones and synchronism in these hormones that might lead to improvement in folliculogenesis and subsequent earlier induction in the estrus cycle. Estrogens cause the vaginal epithelium to proliferate and show increased cornification [27]. So the estrogen bioactivity of licorice may be beyond earlier appearance of estrus phase (cornified cells) in the vaginal smears of treated animals. The precocious puberty of the treated groups was in agreement with all the positive effects of licorice on the different parts of the genital organs.

The exact trigger for ovarian cycle is still not fully understood, the ovaries might become more sensitive to gonadotropins, or the anterior pituitary may become more sensitive to the positive feedback of estrogens [25]. LE may stimulate some maturational factors like GnRH because the onset of puberty is initiated by some maturation process that occurs elsewhere in the brain [26].

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