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RESEARCH ARTICLE

The Effect of Ajwa Dates Fruit Extract on Follicle Stimulating Hormone (FSH), Graafian Follicle and Endometrial Thickness in Female Rats Exposed to Arsenic

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Abstract

Background: Arsenic exposure in humans can disrupt the reproductive system and reproductive organs in women, which can cause interference hormonal and fertility disorders. The pathogenesis of arsenic causes oxidative damage to the organs and reproductive system due to an imbalance between *Reactive Oxygen Species* (ROS) and antioxidants in the body. *Ajwa* dates contain powerful antioxidants that act as scavenger ROS as well as inhibit lipid peroxidase which stops reactions associated with oxidative stress. *Objective*: This study aims to determine the effect of Ajwa date extract on Follicle Stimulating Hormone (FSH) levels, *Graafian* follicle and endometrial thickness in female rats exposed to Arsenic Trioxide (As_2O_3) for 30 days. *Methods* : Experimental laboratory with 5 treatment groups, negative control group (without arsenic exposure), positive control (3 mg/kg BW arsenic), T1 (arsenic 3 mg/kg BW + date extract 2ml / kg BW), T2 (arsenic 3 mg / kg BW + date extract 4ml / kg BW), and T3 (arsenic 3 mg / kg BW + date extract 8ml / kg body weight). *Results*: Arsenic exposure significantly reduced FSH levels, Graafian follicle count and endometrial thickness. Conclusion: The administration of Ajwa date extract could significantly provide antioxidant protection in the treatment group with an increase in FSH levels, *Graafian* follicle count and endometrial thickness.

Keyword: *Arsenic, Arsenic trioxide, Endometrium, Free Radical, Follicle Stimulating Hormone, Graafian Follicle, Reactive Oxygen Species, Ovary, Uterus.*

Introduction

Hazardous chemicals in the environment can cause harmful effects to the body, one of which is exposure to the heavy metal arsenic. Form of inorganic arsenic that is considered very toxic is widely distributed throughout the environment through air, water and soil [1]. Arsenic trioxide (As_2O_3) is the most commonly found inorganic arsenic in the air and the most well-known form of arsenic as a poison because it is a white powder that is tasteless, odourless and easy to incorporate into food and beverages and is often used in pesticides [2, 3]. The Agency for Toxic Substances and Disease Registry (ATSDR) The United States has determined that arsenic as number one on the Priority List of Hazardous Substances over the lead, mercury, and Vinyl Chloride [4]. Arsenic exposure is one of the factors causing infertility in women due to the accumulation of free radicals [5]. Arsenic exposure is considered as an Endocrine Disrupting

Chemical (EDC) that can interfere with the reproductive system, especially in hormonal regulation that affects the menstrual cycle and ovulation [6]. Arsenic can interfere with the gonadal endocrine system in the pathway hypothalamic-pituitary-ovarian (HPO) axes that regulate puberty development and reproductive function [7]. Arsenic has high-affinity interaction with the estrogen receptor ligand-binding domain [8] and causes oxidative damage mediated by *Reactive Oxygen Species* (ROS) generated during the metabolism of inorganic arsenic in cells [2].

Arsenic exposure causes impaired endometrial cell proliferation, accompanied by damage to the endometrial structure due to oxidative stress, resulting in decreased endometrial thickness [7]. Dates are widely studied because they contain many nutrients that are beneficial for health and fertility. Many studies have examined the benefits of dates for women's reproductive health because they are believed to affect oocyte quality, sperm and ovum interactions, implantation and early embryonic development through antioxidant mechanisms [9, 10].

The antioxidant properties of Ajwa dates are expected to be able to suppress free radicals through the pathway scavenger *Reactive Oxygen Species* (ROS), which in effect will reduce disease proliferation due to the strong antioxidant content in Ajwa dates [11, 12].

Also, dates can stop the binding reaction associated with oxidative stress [13]. Therefore, researchers are interested in conducting a study on the effects of Ajwa date extract (Phoenix dactilifera L) on the female reproductive system, especially in the uterine organs and ovaries in Wistar rats (Rattus norvegicus) because of the lack of research on the effects of Ajwa date extract in preventing toxicity caused exposure to metal arsenic.

Materials and Methods

Chemical

Arsenic Trioxide (As_2O_3) powder produced by Loba Chemie with amount 405 mg was dissolved in 950 ml 0.9% Normal Saline (NS) then stirred with a magnetic stirrer on heating at 50^0 C for 3-4 hours to make the arsenic powder completely dissolved

Preparation of Extract

The process refers to previous studies [14, 15] with slight modifications. The flesh of the Ajwa dates that have been separated from the seeds, mashed with mortar and pestle, then adds 1200 ml of water (ratio 1: 3, g / ml) then blended to get a thick form of Ajwa date extract. Furthermore, the date palm extract is stored in the refrigerator for 48 hours. After 48 hours, the date extract was centrifuged at 4^0 C for 15 minutes at a speed of 10,000g. After being centrifuged, the water extract supernatant was taken and placed in a tightly closed centrifuge tube, then stored in the refrigerator.

Experimental Animals and Ethics Statement

This research is experimental laboratory research with Post Test Only Control Group Design. Healthy adult female Rattus Norvegicus rats of 8-12 weeks of age and weighing between 150-200 g were procured from Malang Murine Farm, Indonesia. The animals were given standard rat pellet feed and water ad libitum. Rats were acclimatized for 7 days before the start of experiments. The research was conducted at the Laboratory of Bioscience, Anatomical Pathology and Biochemistry Laboratory, Faculty of Medicine, Brawijaya University. There are five groups and five repetitions for each group.

They are negative control group was rats given 1ml Normal Saline only per day/rat, positive control group mice exposed to arsenic 3mg / kg body weight/day, then treatment groups are T1, T2 and T3 rats given date extract with three different doses consecutively 2ml / Kg body weight/day; 4ml / Kg body weight/day; 8ml / KgBB / day [14], then exposed to arsenic with dose 3 mg / Kg body weight/day for 30 days [16]. Cytological samples were taken after 30 days of treatment and examined every day to see the rat estrus phase; samples were stained with methylene blue and observed the morphology of epithelial cells under a microscope.

The proestrus phase of the oestrous cycle was chosen for termination [17]. Rats were anaesthetized with ketamine and organs were collected. The study was conducted with the approval of the Health Research Ethics Committee of the Faculty of Medicine, Brawijaya University.

Follicle Stimulating Hormone (FSH) Assay

Blood samples of female rats were taken during proestrus, the mice were anaesthetized with ketamine injection as much as 0.1 mg/kg BW, then performed a dissection, and blood was taken from the heart's ventricle while still beating as much as 3 ml, then centrifuged at 3000 rpm, at 4^0 C for 15 minutes and serum was taken and stored in a tube. Then the blood serum was assayed using Bioassay Technology Laboratory catalogue number E0182Ra ELISA Kit (Enzyme-Linked Immunosorbent Assay) Rat Follicle-Stimulating Hormone.

Histological Examination

Graafian Follicles Count

Ovarian samples were stained with Hematoxylin Eosin (H&E). Graafian follicles were defined according to criteria: follicle with a diameter of 400 µm to> 2 cm at ovulation has a large antrum containing follicular fluid, in which granulosa cells around the oocyte are called the cumulus cells, while granulosa cells surrounding the antrum generate the mural granulosa cell layer. The antral is a characteristic structural feature of all Graafian follicles [18, 19]. The samples are seen under the microscope Dot slide Olympus XC 10 and the number of follicles was counted with Dot Slide software.

Endometrial Thickness

Examined using staining Hematoxylin Eosin (H&E) where uterine samples were fixed in 10% formalin buffer. The tissue is cut with a thickness of 2-3 mm and then inserted into a tissue cassette, after which it is cut with a microtome; the result is a thin tape then dipped in Haematoxylin. Seen under the microscope Dot slide Olympus XC 10 and then measured with Dot Slide Software. Data Analysis Data were analyzed with SPSS 25 software

Results

Assay of *Follicle Stimulating Hormone* (FSH) Levels

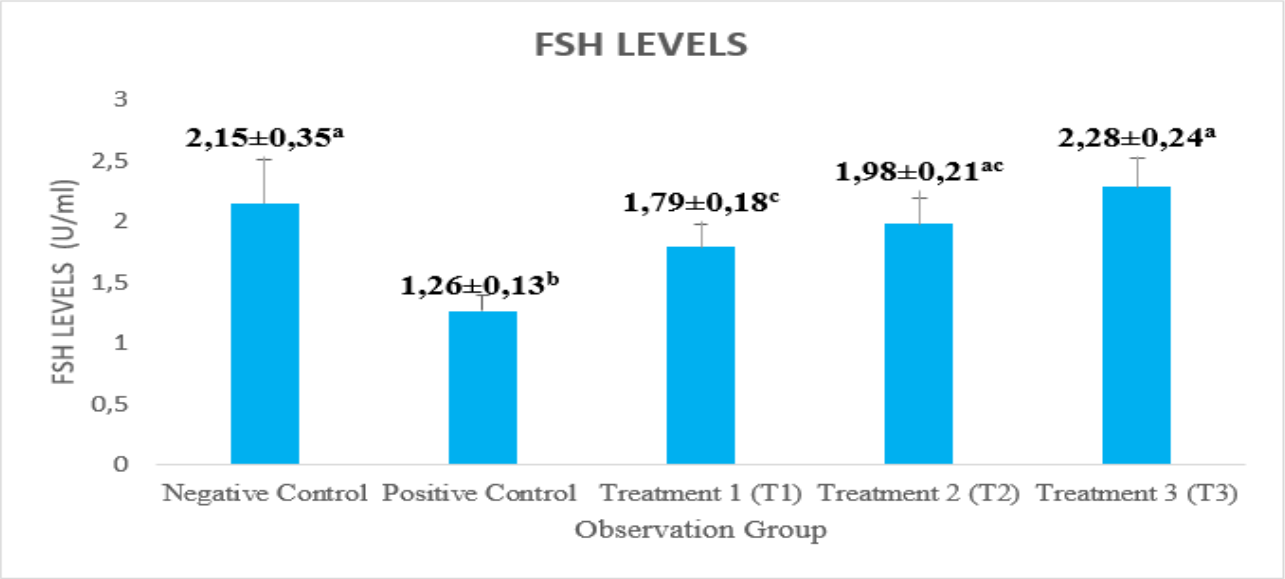

Figure 1: Histogram of FSH Levels.LSD Test. The data represent the mean ± SD. If it contains different letters, it means that there is a significant difference (p≤0.05) and if it contains the same letter, it means that there is no significant difference (p≥0.05)

Based on the LSD test showed that arsenic exposure in female rats caused a significant reduction in FSH levels, it was seen that the mean value of the positive control group was lower than the negative control group. Then, after being given date extracts to female rats exposed to arsenic, the FSH levels increased significantly with increasing doses consecutively. This means that female rats given arsenic and Ajwa date extract in each treatment group will increase FSH levels when compared to female rats exposed to arsenic alone. In group T1 with a dose of date extract 2 ml/kg BW significantly increased FSH levels.

Graafian Follicles Histology Examination

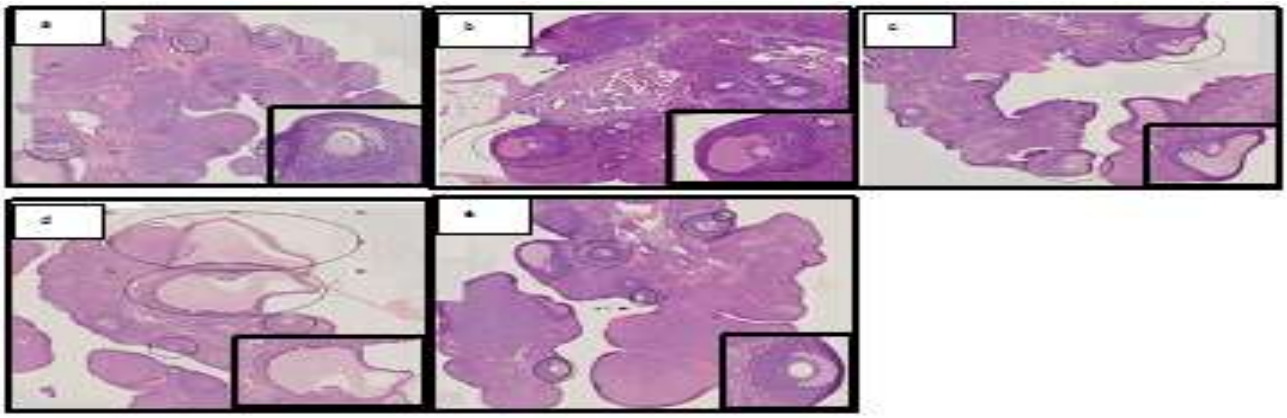

Figure 2: Comparison of Graafian Follicles Count with H&E Staining with 400x Magnification a: Negative control, b: Positive control, c: T1 (arsenic 3 mg/kg BW + date extract 2ml / kgB W), d: T2 (arsenic 3 mg / kg BW + date extract 4ml / kg BW), e: T3(arsenic 3 mg / kg BW + date extract 8ml / kg BW). Seen in group b who were exposed arsenic there was a decrease in the number of *Graafian* follicles, and there was an increase in the number of follicles in groups c, d and e as the dose of Ajwa date extract increased

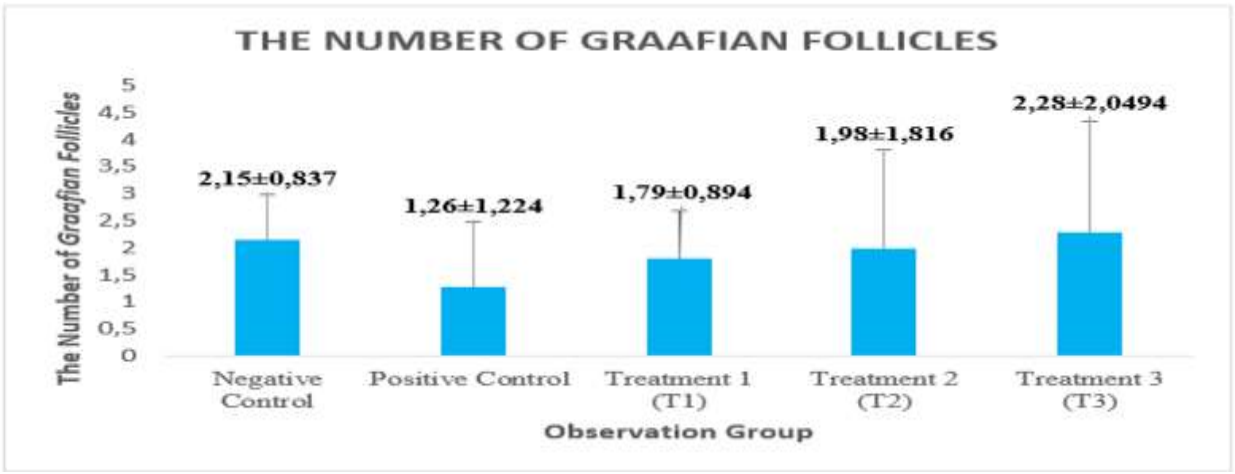

Figure 3: Histogram of the Number of Graafian Follicles. Mann Whitney Test. The data represent the mean ± SD. If it contains different letters, it means that there is a significant difference ($p \leq 0.05$) and if it contains the same letter, it means that there is no significant difference ($p \geq 0.05$)

Based on the Mann Whitney test, there was a significant decrease in the number of Graafian follicles in the positive control group compared to the negative control group. In the treatment given Ajwa date extract and exposed to arsenic, there was a significant increase in group T2 (dose of date extract 4 ml/kg BW) and group T3 (dose of date extract 8 ml/kg BW), but there was no significant difference with group T1 (dosage of dates 2 ml/kg). It can be concluded that the administration of Ajwa date extract at a dose of 4 ml/kg BW (T2) can significantly increase the number of Graafian follicles in female rats exposed to arsenic.

Endometrial Thickness Histology Examination

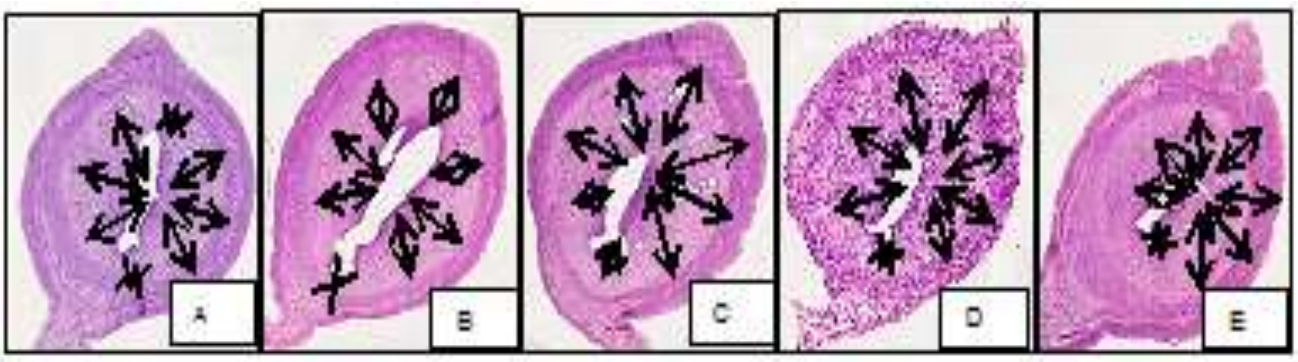

Figure 4 Endometrial thickness Measurement of Female rats by H&E staining with 200x magnification. a: Negative control, b: Positive control, c: T1 (arsenic 3 mg/kgBW + date extract 2ml / kgBW), d: T2 (arsenic 3 mg / kgBW + date extract 4ml / kgBW), e: T3(arsenic 3 mg / kgBW + date extract 8ml / kgBW)

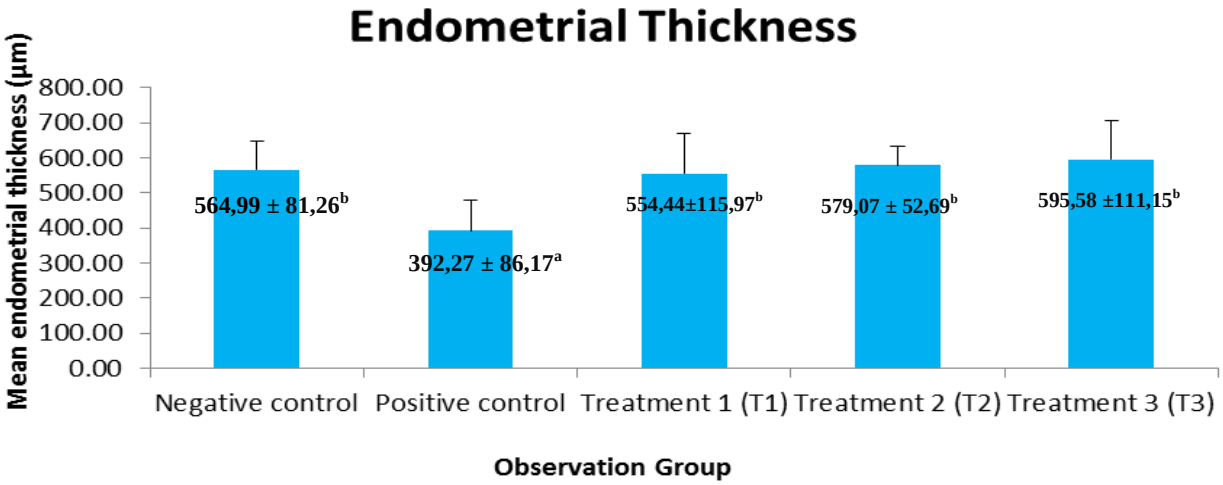

Figure 5 Histogram of Endometrial Thickness. LSD Test. The data represent the mean ± SD. If it contains different letters, it means that there is a significant difference (p≤0.05) and if it contains the same letter, it means that there is no significant difference (p≥0.05)

Based on the results of the multiple comparison tests using the LSD test, it can be seen in the picture above that the endometrial thickness of the positive control group is decreased compared to the negative control group. After being given Ajwa date extract to female rats exposed to arsenic, the thickness of the endometrium increased with increasing dose. The mean endometrial thickness affected treatment group 1 (T1), treatment group 2 (T2) and treatment group 3 (T3) but there was no statistically significant difference between the two doses. It can be said that in this study the T1 group with date extract at dose 2mL / KgBW / day was able to significantly increase endometrial thickness in female rats exposed to arsenic.

Discussion

Arsenic and the Gonadal Endocrine System

It has been observed that subchronic exposure of Arsenic Trioxide on rats at dose 3mg / KgBW [16] for 30 days significantly decreased serum FSH concentrations, which the study has the same result with our study. This study also confirms other studies that exposure to inorganic arsenic (AsIII) can reduce FSH levels in rats, such as in the study of Chatterjee and Chatterji [20] and Mehta and Hundal [21].

Arsenic can be an endocrine disruptor in the endocrine system which is controlled by three axes, namely the hypothalamic-pituitary-gonad (HPG), hypothalamic-pituitary-adrenal (HPA), and hypothalamic-pituitary-thyroid (HPT) [22,23]. The Hypothalamic-pituitary-gonadal (HPG) axis consists of hypothalamic-pituitary-ovarian (HPO) in females and hypothalamic-pituitary-testicular (HPTT) axis in males, which control gonadotropin-releasing hormone (GnRH) and gonadotropins (FSH and LH). GnRH is secreted in the hypothalamus, circulating in the anterior pituitary gland where the FSH and LH hormones are secreted by gonadotrophs and stimulate the production of these gonadotropins [24, 25].

The decrease in FSH levels caused by the effect of arsenic toxicity, which is causing oxidative stress through the accumulation of *Reactive Oxygen Species* (ROS) [26].Arsenic exposure was confirmed that it was accumulated in the anterior pituitary [27] and may induce oxidative stress by increasing ROS in the anterior pituitary cells leading to suppression of FSH and LH secretion [21, 28].

Low plasma gonadotropin levels can be due to overexpression of glucocorticoids due to arsenic exposure, which results in decreased sensitivity of gonadotrophic cells to GnRH [29]. Giving Ajwa date extract can increase FSH levels starting at a dose of 2 ml/kg BW because the antioxidant content of dates can act as a scavenger of ROS [30] to prevent oxidative stress.

Arsenic and Reproductive Organs

Ovary

The decrease in the number of Graafian follicles in the positive control group in this study supports a previous study by Khatun *et.al* [31] and Yu *et.al* [32] and Mehta and Hundal [21], it can be concluded that arsenic

can cause a decrease in the number of Graafian follicles. The mechanism of arsenic toxicity disrupts folliculogenesis because arsenic exposure causes an increase in *Reactive Oxygen Species* (ROS) resulting in oxidative stress on the ovaries [7, 33]. Arsenic also inhibits the peroxidase enzyme in follicular fluid, thereby interfering with folliculogenesis due to high levels of free radicals [29].

Besides the ROS pathway, arsenic toxicity can damage DNA and ovarian cells [34]. Arsenic exposure indirectly interferes with the regulation of the hypothalamic-pituitary-ovarian (HPO) axis, which affects decreasing levels of the Follicle Stimulating Hormone (FSH) and decreasing levels of the steroid estrogen hormone, where the action of this hormone is needed in the folliculogenesis process.

If there is a resistance to these hormones, it can cause follicular regression and low ovarian mass [31]. The increase in follicles in the group T2 and group T3 compared to the positive control group indicates that Ajwa date extract works as an antioxidant that can suppress excessive free radicals in the ovaries due to arsenic exposure [11,12].

After being given Ajwa date extract, there was an increase in the number of follicles from group T1 to group T3 along with increasing doses. The results of this study are the same as research conducted by Moshfegh *et.al* [35] that date extract can increase the number of ovarian follicles. Dates can inhibit lipid peroxidase and thus act as an antioxidant that stops reaction which associated with oxidative stress [36].

The antioxidants contained in Ajwa dates can stop the bond reaction associated with oxidative stress by reducing the formation of ROS [13]. The phenolic content of Ajwa dates, with their redox properties, can act as scavenger ROS, neutralize free radicals, and break down peroxides [30].The vitamin E in Ajwa dates acts as a scavenger for lipid peroxidation radicals (LOO•) and a hydrogen donor to stop free radical chain reactions (*chain-breaking antioxidants*) [37].

Vitamin A contained in dates is essential for maximum egg production [10]. Dates also contain zinc which has a role in improving egg quality through hormone metabolism, organizing DNA and RNA, protein synthesis and stabilization of the bio-membrane of cell division from the chromatin cell nucleus. Apart from zinc, the content of amino acids and fatty acids in dates can directly regulate the secretion of GnRH which further regulates the production of the hormones estrogen and progesterone as well as primary and secondary sex organs [38].

Uterus

The effect of arsenic appears to be on degenerative changes in endometrium and estradiol serum levels, whereas endometrial growth is dependent on the presence of estradiol. Arsenic can be toxic because it produces *Reactive Oxygen Species* (ROS) and causes oxidative damage of several cell components, including denaturation of proteins essential for cell function. Thus, the degeneration of endometrial components may be associated not only with decreased regulation of serum estradiol levels but also with increased production of ROS caused by arsenic exposure [20].

Apart from causing neurotransmitters to be disrupted due to arsenic exposure, an increase in arsenic-induced free radicals can degenerate uterine cells [21, 39]. Dates extract contains isoflavones, which are secondary metabolites produced by plants [40].

The arrangement of isoflavones in dates is similar to endogenous estrogens so that the isoflavones can bind to estrogen receptors in the follicles, resulting in more estrogen hormone which affects the number of glands and the thickness of the endometrial lining [41]. The increase in endometrial thickness is due to phytoestrogen activity in dates which affects increasing the number of cells and endometrial lamina propia.

Uterine weight is also influenced by the thickness of the endometrium because the endometrium is a layer that is very responsive to changes in reproductive hormones, particularly the hormone estrogen. The isoflavone-type phytoestrogen compounds possessed by dates can increase the activity of estrogen receptors increasing endometrial thickness [42].

Conclusion

Firstly, sub chronic arsenic exposure can reduce FSH levels, the number of Graafian

follicles and endometrial thickness in female rats after arsenic exposure.Secondly, giving Ajwa date extract can provide antioxidant protection to the reproductive system and reproductive organs of female rats by increasing FSH levels, the number of Graafian follicles and the endometrial thickness of female rats exposed to arsenic for 30 days.

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