Original Research Article

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Early luteotrophic effect of vaginally administrated Letrozole in ewes

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ABSTRACT: The present study evaluates the luteotrophic effect of letrozole during the metestrus and early diestrus period of the estrous cycle on sheep steroidogenic competency. Letrozole is one of the aromatase inhibitors generations and capable of suppressing aromatase enzyme activity. Aromatase enzyme activity is a unique requirement for the final aromatization of esterones into estrogen. Suppression of aromatase activity results in decreased estrogen concentration in blood which arrests follicular development (after a state of dominance) and delays the emergence of the next follicular wave. Letrozole early luteotrophic potency was tested after the application of letrozole based synchronization protocol, performed by placing an intravaginal sponge containing 7.5mg letrozole for five days, followed by intramuscular injection of prostaglandin at the onset of removal. Compared with standard GPG protocol. Confirmation of predicted ovulation (78.70 ± 0.21 hrs after sponge removal in the letrozole group) by the disappearance of the ovulatory follicle by ultrasound evaluation, followed by blood sampling and detailed transrectal ultrasound evaluation twice daily for five days. By using enzyme-linked immunosorbent assay for detection of progesterone in serum, progesterone concentrations in blood were assayed during early luteal development. The vascularization of the luteal cells was monitored by using transrectal power Doppler ultrasound evaluation. Application of five days letrozole synchronization protocol in ewe yielded increasing early luteal diameter (steroidogenic capacity, besides early luteal vascularization for the letrozole group (20.06 ± 2.22) compared to GPG group (15.52 ± 2.04) conferred by doppler ultrasound evaluation of luteal tissue.

KEYWORDS: Letrozole, Embryonic mortality, luteotrophic effect, Estrus synchronization, Ewe

1. Introduction

Letrozole (MW 285.3 g/mol) has been the focus of most recent studies on the using of aromatase inhibitors in human and veterinary medicine. One of the reasons for making it the focus of studies is that it has a strong inhibitory effect with high specificity for P450arom, and it reversibly inhibits the enzyme without changing the synthesis of progesterone or corticosteroids, because it deprives the body of producing its own estrogen [1]. Letrozole could be the future product for synchronizing ovarian functions in farm animals [1, 2]. Vaginal letrozole can be used as an 'establishment for pregnancy and reduce premature fetal deaths that occur from insufficient progesterone levels in the metestrus and early diestrus. A study of the pharmacodynamic properties of letrozole in healthy postmenopausal women revealed maximum serum estradiol suppression of 78% from baseline at 72 hours after treatment [3]. Letrozole is used to treat postmenopausal breast cancer patients, which has resulted in a decrease in serum estradiol concentrations by 70-80% [4]. Because of its potent inhibition of estradiol production, high oral bioavailability, selectivity, and mild secondary effects, letrozole has become a drug of preference for the treatment of breast cancer, either as a first-line treatment or as neo-adjuvant chemotherapy [5]. Letrozole were used as an adjuvant treatment for hormone- responsive breast cancer in post-menopausal women. A 5-day regimen of letrozole with 2.5 mg daily was reported by Mitwally et al [6]. Moreover, increasing doses of letrozole have used to induce ovarian super-stimulation in women [5, 6]. Although the effects of estrogen deprivation on the reproductive physiology of premenopausal women have not critically examined, letrozole has been applied in assisted reproduction in women based on the concept that estradiol suppresses gonadotropin release through negative feedback effects on the hypothalamic-pituitary

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axis. Theoretically, the removal of circulating estradiol using aromatase inhibition will relieve the suppressive effects of estradiol permitting a surge in FSH, which would, in turn, induce the recruitment of a new wave of follicular development, driving follicular growth, and even trigger the development of more than one ovarian follicle to a preovulatory size [7]. The recent implication of letrozole in synchronization protocols in cows [8] and ewes [9, 2]. Inspiring results obtained after testing five days of letrozole synchronization protocol in ewe trigger our further research potentials for further investigation of the effects of letrozole on early steroidogenic potency around the time of implantation and early embryonic development. Therefore, the present study aims to evaluate the effect of letrozole used in a day synchronization protocol in ewes on diameter, progesterone production, and blood supply of early developed corpus lutea compared with standard GPG protocol. It is also aimed to test whether Letrozole is suitable for normal implantation and early embryonic development [7] as a critical step in further evaluating its effect on early fetal mortality and pregnancy outcomes.

2. Materials and Methods

2.1. Animals and experimental design

This study was done during the breeding season (September- October) at the veterinary teaching hospital in faculty of veterinary medicine, New Valley University, Al Kharga city (latitude 25° 30, N -30°, 35;47 E). Twenty cyclic non-lactating Farafra ewes ranging from 3 to 5 years old and weighing 35-40 kg/BW. Ewes were clinically healthy and kept indoors with outdoor access with concrete floor. Animals were feed with balanced ration; good quality hay add labitum in addition of concentrate mixture as well as $\frac{1}{2}$ kg per head with mineral mixture. They were divided randomly into two equal groups each containing ten ewes. The first group were treatment Group which received Vaginal sponge containing 7.5mg letrozole for five days, then 250 µg of Prostaglandin F2α were given intramuscularly at onset of sponge removal as shown in plate (1), while the other groups named control group and treated with GPG protocol as in plate (2).

2.2. Intravaginal administration of Letrozole

Using locally made vaginal sponges which contain two-centimeter-wide, two-centimeter-thick, three-centimeter-long pieces supplied with 15 centimeters of silk. The vaginal sponges were sterilized through autoclaving then allowed to dry and stored in tightly closed, sterilized glass containers until needed. Before being inserted into a vagina, each sponge was soaked with 7.5 mg of letrozole powder (Femara® Tablets, 2.5 mg; Novartis Pharmaceuticals Corporation). A suitable, sterilized, 2-centimeter glass tube was opened from both sides and the loaded sponge was pushed through the tube until it settled inside the vaginal lumen. To prevent sponge removal, the glass tube, metal rod, and silk were all carefully removed, and the silk was cut one centimeter below the level of the vulva. just after removing sponge, ewes were injected intramuscular with 250 µg of cloprostenol (Estrumate; Mallinckrodt Vet GmbH, Friesoythe, Germany). Ewes were observed and examined for estrus signs through the introduction of ram and observing whether ewes refused ram or searched for its accompany and stand to be mounted. These signs were confirmed through ultrasound examination of ovaries and through disappearance of dominant follicles.

2.3. Ultrasonographic examination of female genital system and measurement of blood area:

The ewes were examined using a Doppler ultrasound scanner (ECM80, Exago, France) outfitted with a 5-10 MHz endorectal linear probe (Lv513) with extension. The lateral recumbent ewe was examined using an endorectal probe. The urine bladder served as a guide to locate the uterine horns. The probe was moved laterally 90∘ clockwise and 180∘ anticlockwise to scan the ovaries and investigate all regions of the genitals. The animals were evaluated daily using ultrasonography to determine the size, shape, and typical echographic appearance of the

Corpora lutea. Ultrasound scanning in letrozole treated group performed at onset of sponge removal, 6 hours later and every twelve hours till ovulation. The examination occurred every six hours to detect color flow mapping in developed corpus luteum. In the group treated with ovisynch protocol, ultrasound scanning was performed after 2nd GnRH injection twice daily till ovulation and subsequent detection of CL, then daily to detected color flow mapping in newly formed corpora lutea. Color signals were used to evaluate the blood flow around the entire perimeter of the corpus luteum. The sectional area (SA) of the corpus luteum was detected by the following equation SA = π 4x (SD)2, where SD is the sectional diameter [9]. The sectional areas were calculated, and the blood flow areas (BA) will be quantified using Image J program (version 1.62) developed at the USA (National Institute of Health (http://rsb.inf.nih.gov/ij).The colored areas in the image that existed at the maximum diameter of the CL is used as a quantitative index to express the blood flow within the wall of corpus luteum. Areas of color represent regions with flow velocity higher than 0.08 m/s called blood flow area (BA), while blood flow area percent (BA%) was estimated by the following equation BA% = BA/SA $\times$ 100. After examination, the findings (images and videos) were saved on a USB Flash Drive (Adata, Adata technology Co., Ltd, Taiwan).

2.4. Blood Sample collection:

Blood Samples were collected from Jugular vein puncture into 5ml plain vacuum tubes (vaccutainer system) at onset of removal the spongy, after 6 hours, and each 12 hours interval till ovulation. The collected samples centrifuged at 2500 rpm for 15 minutes. The serum was separated and placed into 1.5 ml epindorff tubes, labeled, and stored at -20 until assayed. For ovi-synch protocol, sampling was collected every 12 hours after ovulation for five days. Sampling schemes were explained in plates (1,2).

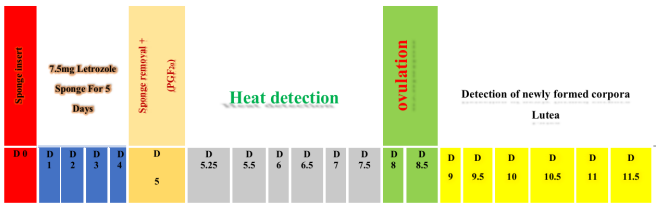
2.5. Hormonal assay

Serum progesterone concentrations were determined using a commercial ELISA Kits (precheckbio,Inc.U.S.A).

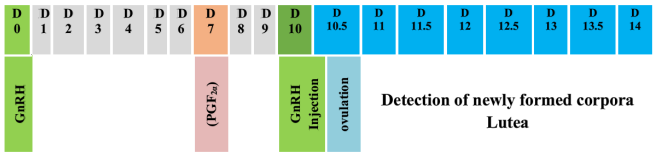
2.6. Statistical analysis

Statistical analysis was performed using Microsoft excel computer program (2010), and the significant difference analyzed by T-Test.

3. Results:



plat 1: Summery of letrozole protocol, sampling and examination.



plat 2: Summery of GPG protocol, Sampling and examination

As seen in (Fig. 1) and Plate (3), the current study's findings demonstrated that letrozole treatment in sheep had an impact on the quantity and width of corpora lutea following ovulation. The letrozole group had a substantially higher number of corpora luteum and its diameter (5.57 ± 0.07 to 7.00 ± 0.00) compared to the GPG group (5.33 ± 0.18 to 5.70 ± 0.20). shown in Plate (4), the blood area of the corpus luteum after four days was greater in the letrozole group (20.06 ± 2.22) than in the GPG group (15.52 ± 2.04). Regarding the effect of Letrozole on the concentration of progesterone in serum, the results showed that the concentration of progesterone (ng/ml) increased slightly during the period of presence of the sponge impregnated with Letrozole in the vagina and then decreased after removing the sponge and injecting

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the solubilizing agent. As well as the serum concentration of progesterone secreted from the developing corpus luteum in the letrozole-treated group was higher than in the GPG group (Fig. 2).

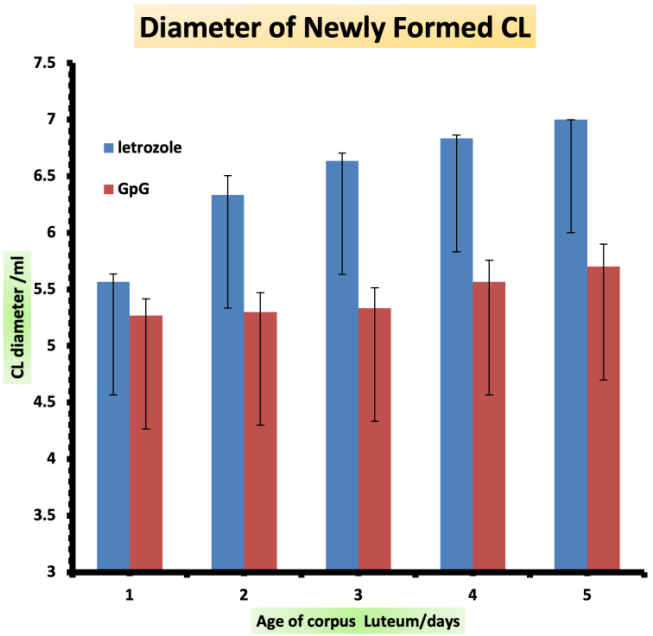


Figure 1: Diameter of newly formed CLs after ovulation in ewes (Mean ± SEM) in Letrozole (n= 10) and GPG (n =10) during early luteinization

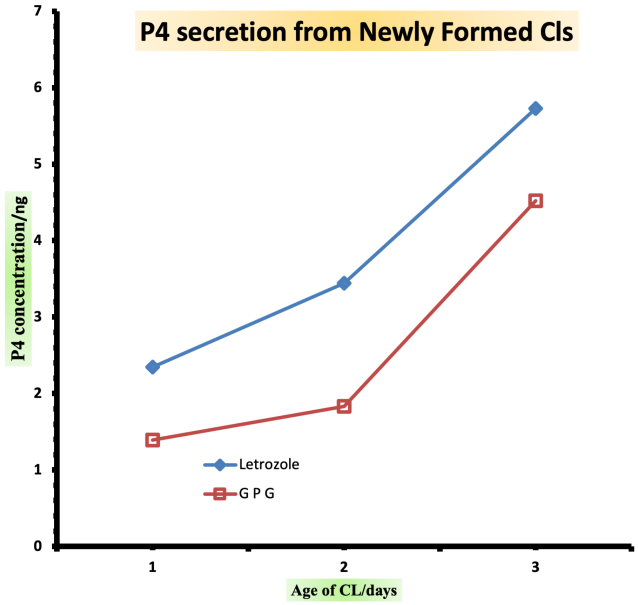
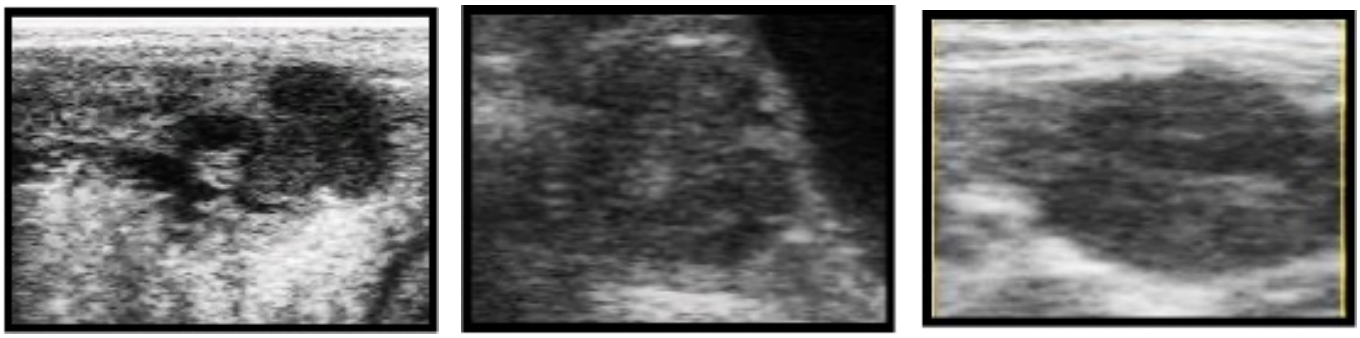


Figure 2: Concentration of Progesterone secreted from CL (Mean ± SEM) in Letrozole (n= 10) and GPG ewes (n=10) during early luteinization.

4. Discussion

Nonsteroidal inhibitors of aromatase enzyme were recently prescribed for control of ovarian functions [3, 10, 11], either alone or in combination with other gonadotropins [12]instead of being only adjuvant anti-estrogenic drugs in treatments [3]. Observed positive effects of nonsteroidal inhibitors of letrozole on reproductive health inspired its application in animals[2, 13, 8]. Administrating letrozole vaginally allows its action to be kept for five days inside the vagina and leads to the continuity of the drug's action [14]. It is better than giving daily doses due to its the half-life of letrozole being two days. In the current study letrozole exerted a positive action on met-estral corpora lutea. This result is compatible with what was observed on cattle [13], and sheep [5]. The increased number of corpora lutea was attributed to an increased number of ovulatory follicles which formed the corpora lutea under a potent luteotropic action of luteinizing hormone [5]. Moreover, super ovulatory brought gonadotrophic response of letrozole on recruited selected follicles which is the main reason for an increased number of corpora lutea in letrozole-treated [2, 15]. The diameter of corpora lutea from letrozole-treated ewes was greater than non-treated ones, these findings are like what was described[5]. It was explained that luteinization of a larger diameter of the ovulatory follicle could result in an increase in the initial diameter of the newly formed corpus luteum [2, 15]. Distribution and growth of lutein cells within initially large cavitation post-ovulatory follicular antrum, result in initially large diameter of ovarian cortex occupied by corpus hemorrhagic. This cavity when examined by ultrasound has different echogenicity delineating it from occupying ovarian echotexture [10]. This description ideally explains the findings in this experiment, thus large-diameter corpora lutea originated from large-diameter ovulatory follicles [11]. Concerning the blood area of five-day corpora lutea, it was



plat (3): Great diameter of corpora lutea in group synchronized using letrozole.

large in letrozole-treated ewes, in fact, these findings attributed to letrozole action weren't described or explained in previous work, either in humans or animals. Significantly highly vascularized corpus luteum was formed after letrozole treatment when compared with that formed after application of GPG protocol at the same age. It may be attributed to the large-sized corpus luteum formed from large-sized follicles after using letrozole [1, 9, 6, 16]. Moreover, it was an increased progesterone hormone produced by the corpus luteum [7] and luteinizing hormone [5], which favor the vascularization of the corpus luteum. These findings coincide with those recorded in cattle by Yapura et al., [6]. Increased vascularity is an indication of the health and activity of the formed corpus luteum which in role is necessary for newly developed embryos [8, 14]. This proven early luteal angiogenic potency of letrozole is attributed to its effect on increasing ovarian angiogenic factors besides endothelial growth factors [17]. In addition, the increased concentration of estrogen has a role in luteal angiogenesis and luteal steroid-producing capacity [13]. We shouldn't ignore the role of increasing progesterone which increases ovarian and endometrial vascular endothelial growth factors [8]. In a condition of the abundance of estrogen produced by the increased number of large diameter follicles, with an increased number of early progesterone-producing lutein cells, together with the presence of letrozole are the best early luteal vascular angiogenic promotors. This theoretical ideal vascular and steroidal endometrial environment should yield ideal implantation and early embryonic development, translated by conception rates. It could improve early embryonic survival, but this finding needs further confirmatory investigations on a large scale before being widely adopted.

Conclusion

We conclude that letrozole has a potent luteotrophic effect in cyclic ewes. Observed in the form of number, diameter, steroidal potency beside luteal angiogenic capacity and hence luteal blood perfusion. which is useful especially when multiple fetuses or ewes with early embryonic mortality are implicated in progesterone deficiency. Moreover, it can increase the responsiveness of corpora lutea to gonadotrophic hormones, which is favorable for the maintenance of a suitable diestral endometrial environment favoring more twining and embryo survival rates. Additional studies are needed on large numbers of ewe flocks to confirm the impact of letrozole on fertility and embryo survival in different tropics.

Declaration of Competing interest

On behalf of all authors, I hereby declare that no conflict of interest may interfere with the publication of the manuscript.

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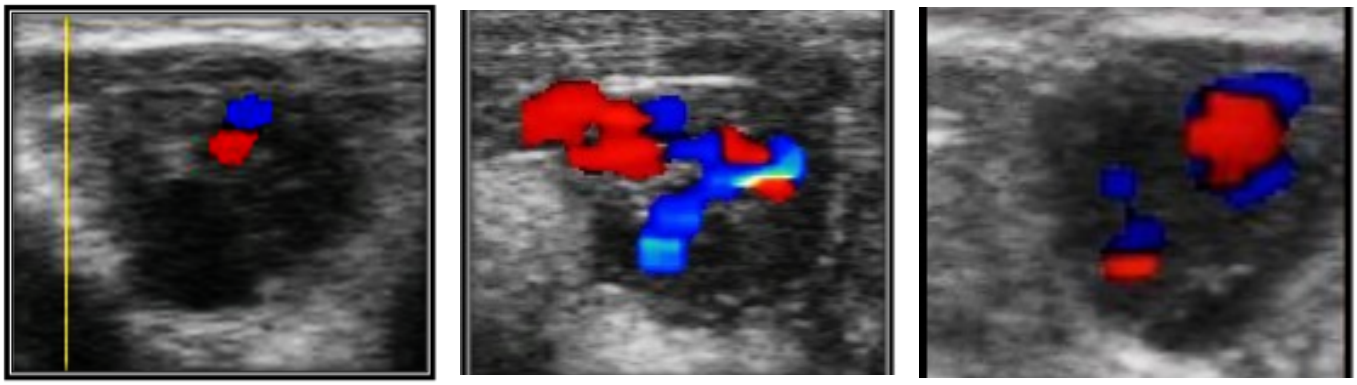

Plate (4): Color doppler images showing blood supply in four days Corpora lutea for letrozole Group.

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