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RESEARCH ARTICLE

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Estradiol benzoate priming during induction of estrus with Vitex-castus extract in dogs

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ABSTRACT

This study compared two methods of estrus induction between dogs (using vitagnus and vitagnus-estradiol). A total of 10 adult cyclic female Shih tzu Terrier mix breed dogs at anestrus stage were selected and divided into two groups. The first group (VAC) received vitagnus for five weeks (90 mg daily, PO). The second group (VAC-E2) was treated with vitagnus and estradiol benzoate. Estradiol benzoate was injected at the beginning of each week (0.01 mg/kg, IM). Blood sampling for evaluation of steroid hormones and vaginal smears were taken weekly. The sings and return to the estrus with the number of puppies were recorded. In the VAC group, 60%, and in the VAC-E2 group, 80% of dogs returned to the follicular phase after five weeks. In the VAC-E2 group, signs of estrus appeared 7 to 10 days and a mating process started 4 to 6 days earlier than those in the VAC group. The average number of delivered puppies was 4. These symptoms were confirmed by the cytology of the vagina. There was no significant difference in the estradiol and progesterone levels between groups. The mean concentration of estradiol significantly changed between weeks 1 and 4, 1 and 5, and 2 and 5 ($p < 0.05$). The progesterone level in the VAC-E2 group on week five was higher than that in other weeks. In conclusion, the administration of estradiol benzoate before vitagnus improved induction of estrus in dogs.

Keywords

estrus induction; estradiol benzoate; Vitex-castus extract; dog

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Abbreviations

VAC: *Vitex agnus castus*
E2: Estradiol
P4: Progesterone
PMDD: Premenstrual dysphoric disorder
PCOS: Poly cystic ovarian syndrome
PMS: Premenstrual syndrome

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Introduction

Training various breeds of dogs for rescuing people, police surveillance, diagnosis of diseases, helping the disabled people and keeping them as pets is increasingly expanded [1]. Dogs are monoestrus and experience an obligate anestrus of 2–10 months following the luteal phase, having an average duration of about 75 days [2]. The anestrous period of the cycle begins when the progesterone plasma concentration falls below 1 ng/ml. Bitches in long anestrus should be treated after the factor leading to anestrus is identified [3]. Therefore, several drugs used to induce estrus in bitches in various programs include bromocriptine, estrogen, GnRH, LH, FSH, hCG, eCG, cabergoline, and herbal ingredients such as *Vitex-castus* extract [1,4,5].

All methods of inducing estrus have advantages and disadvantages. For instance, the use of cabergoline as a dopaminergic compound is not cost-effective. On the other hand, since the drugs are designed for human beings, it is difficult to determine the dosage of the drug for animals. It should also be noted that this drug can affect the central nervous system and leads to nausea and vomiting. The change in the color of the outer skin is another side effect of cabergoline [1].

Administration of deslorelin, PGF2α, and estradiol benzoate can lead to the induction of estrus in dogs, but ovulation is not guaranteed in all of them [6]. Injection of eCG can induce oxidative stress and increase the amount of oxidation in the ovary, hence, it can affect fertility in the long run [7].

The use of GnRH analogues (e.g. Deslorelin, Leuprolide) through hypodermic osmotic mini pumps or implants can lead to estrus induction and pregnancy, however it has some limitations: 1) high cost and lack of availability of implants, 2) formation of immature corpus luteum which shortens diestrus period and reduces fertility rate, and 3) long-term administration of GnRH agonists leads to over stimulation in pituitary and reduced expression of GnRH receptors, which is followed by suppression of LH secretion and in some species it can reduce FSH [8-10].

Considering the adverse effects of hormonal compounds, it is essential to use a safe method. *Vitex agnus castus* (VAC) extract is widely used in the treatment of patients suffering from premenstrual dysphoric disorder (PMDD), menstrual cycle irregularities, polycystic ovarian syndrome (PCOS), lactation difficulties, hyperprolactinemia, cyclical breast pain, menopause-related complaints, premenstrual syndrome (PMS), and associated cyclic mastalgia, acne, digestive complaints, infertility, anxiety, and as antimicrobial, antioxidant, and antifungal [11-13].

Although *Vitex agnus castus* (VAC) extract reduces prolactin secretion by binding to dopamine receptors in the pituitary gland, administration of different dosages of this extract can either stimulate or suppress prolactin secretion [12]. VAC compounds (i.e. linoleic acid) induce expression of ER-α and ER-β receptor genes. Furthermore, VAC stimulates the synthesis of progesterone and presenelin-2 mRNA receptors. This extract can change uterine weight and cytology of the vagina [14]. Estrogenic compounds of the VAC extract, such as flavonoids, penduletin and apigenin, have been identified. This compound normalizes and balances hormone levels. Due to the existence of these compounds, levels of prolactin and cholesterol are diminished [13].

Generally, VAC extracts reduce FSH secretion and increase the secretion of LH. Increasing LH secretion leads to corpus luteum maturation and increases in progesterone levels. On the other hand, reduction in prolactin production leads to the maintenance of corpus luteum structure and function, and it creates a hormonal balance between progesterone and estrogen. However, since FSH is reduced, reaching the end of anestrus and high intake of VAC extract, FSH increased and estrus is engendered through folliculogenesis in dog [1,15]. The VAC extract has several side effects, including gastrointestinal disorders, urticaria, fatigue, dizziness, headache, dry mouth, tachycardia, nausea, and agitation. These side effects are found in less than 2% of the patients. Also, due to a lower cost and higher availability of this compound, it can be used as an alternative for hormonal compounds to induce estrus in dogs [11].

This study aimed to evaluate the effects of a safe and inexpensive herbal extract in order to induce estrus in dogs with the least side-effects compared to hormonal compounds. Since VAC reduces FSH and subsequently reduces estrogen synthesis, this study investigates the effects of combining estradiol and VAC to expedite estrus induction in dogs.

Results

Clinical evaluation

At the end of the fifth week in the VAC group, of the 5 dogs studied, 3 dogs entered their follicular phase. Of these 3 dogs, 2 were in the proestrus phase and 1 was in the estrus phase. At the end of the fifth week in the VAC-E2 group, out of the 5 dogs studied, only one dog was still in the anestrus phase. In the VAC-E2 group, dogs showed signs of estrus and bloody vaginal discharge seven to ten days earlier than the first group. One of the dogs in the group was in the diestrus phase at the end of the fifth week. Dogs were

also monitored for up to three months after the study and their pregnancy and parturition were recorded. In the VAC group, 2 dogs that were in proestrus at the end of the fifth week, mated after 10 ± 2 days (Figure 1). One dog in estrus at the end of the fifth week, mated twice. Of the 3 dogs that were in anestrus, 2 dogs showed bloody discharge after 3 weeks, and 1 dog was still in anestrus. Of the 3 dogs in the VAC group that were in the follicular phase at the end of the fifth week, all 3 gave birth to healthy puppies after average of 62 days. The average number of delivered puppies was 4.

In the VAC-E2 group, 2 dogs that were in proestrus at the end of the fifth week, mated after 6 ± 2 days. One dog that was in estrus, mated twice at the end of the fifth week. One dog that was in diestrus, mated twice from the end of the fourth week until the middle of the fifth week. The anestrus dog did not reveal any mating signs until three months after the study.

Vaginal Cytology

Results related to cellular changes of the vagina are shown in Table 1. As you can see, estrus induction in one of the dogs is performed from the third week and it is performed from the fifth week in the other (Cornified cells were dominant in these dogs). It should be noted that in three other cases of the population, parabasal cells were still dominant at the end of the fifth week, thus, the dogs were in their anestrus phase. It seems that the dogs of the VAC-E2 group, which were in the same sexual cycle as that of the dogs in the first group (sexual non-acceptance), returned to estrus earlier than the first group, since four dogs of this group entered their follicular phase earlier than the fifth week. It seems that estradiol along with Vitagnus can hasten the sexual cycles of the dog. Out of the 10 dogs studied in two groups, only two dogs were in the anestrus phase at the end of the study. We can conclude that administration of Vitagnus alone or along with estradiol can help induce estrus in dogs (Figure 1).

Estradiol Concentration of the Serum

After measuring estradiol by ELISA method, the changes of the hormones were compared in two groups. In statistical terms, in the whole five weeks of the study, there was no significant difference between two VAC and VAC-E2 groups ($p > 0.05$). It seems that these results indicate that Vitagnus-estradiol shortens anestrus period, but it does not have any advantage in increasing estradiol level during the first five weeks compared to Vitagnus group. Hence, Vitagnus alone can help follicular growth induction and subsequent estradiol synthesis at the end of the anestrus. Furthermore, a slight increase in estrogen level of dogs can lead to resurgence of estrus and improving estrus signs. There was no significant difference in estrogen level in the VAC group during different weeks ($p > 0.05$). It seems that, the duration of Vitagnus usage does not affect the amount of estradiol synthesis. But there is a significant difference in estrogen level between the first week and weeks four and five, and between the second week and week five in the VAC-E2 group ($p < 0.05$). Thus, the increase in estradiol level during the last week compared to the initial week

Table 1.
Cyclic changes of dog in two VAC (Vitagnus) and VAC-E2 (Vitagnus+E2) groups based on cytological changes of the vagina.

Drug \ Weeks	1	2	3	4	5
Vitagnus	An	An	An	An	An
Vitagnus	An	An	An	An	An
Vitagnus	An	An	An	An	pro
Vitagnus	An	An	An	An	pro
Vitagnus	An	An	pro	ES	ES
Vitagnus + E2	An	An	An	pro	pro
Vitagnus + E2	An	An	An	An	pro
Vitagnus + E2	An	An	An	An	An
Vitagnus + E2	An	An	pro	ES	Di
Vitagnus + E2	An	An	An	pro	ES

The mating date was set 2 times at 24-hour intervals after entering the estrus.
Anestrus (An); proestrus (pro); Estrus (ES); Diestrus (Di); estradiol benzoate (E2)

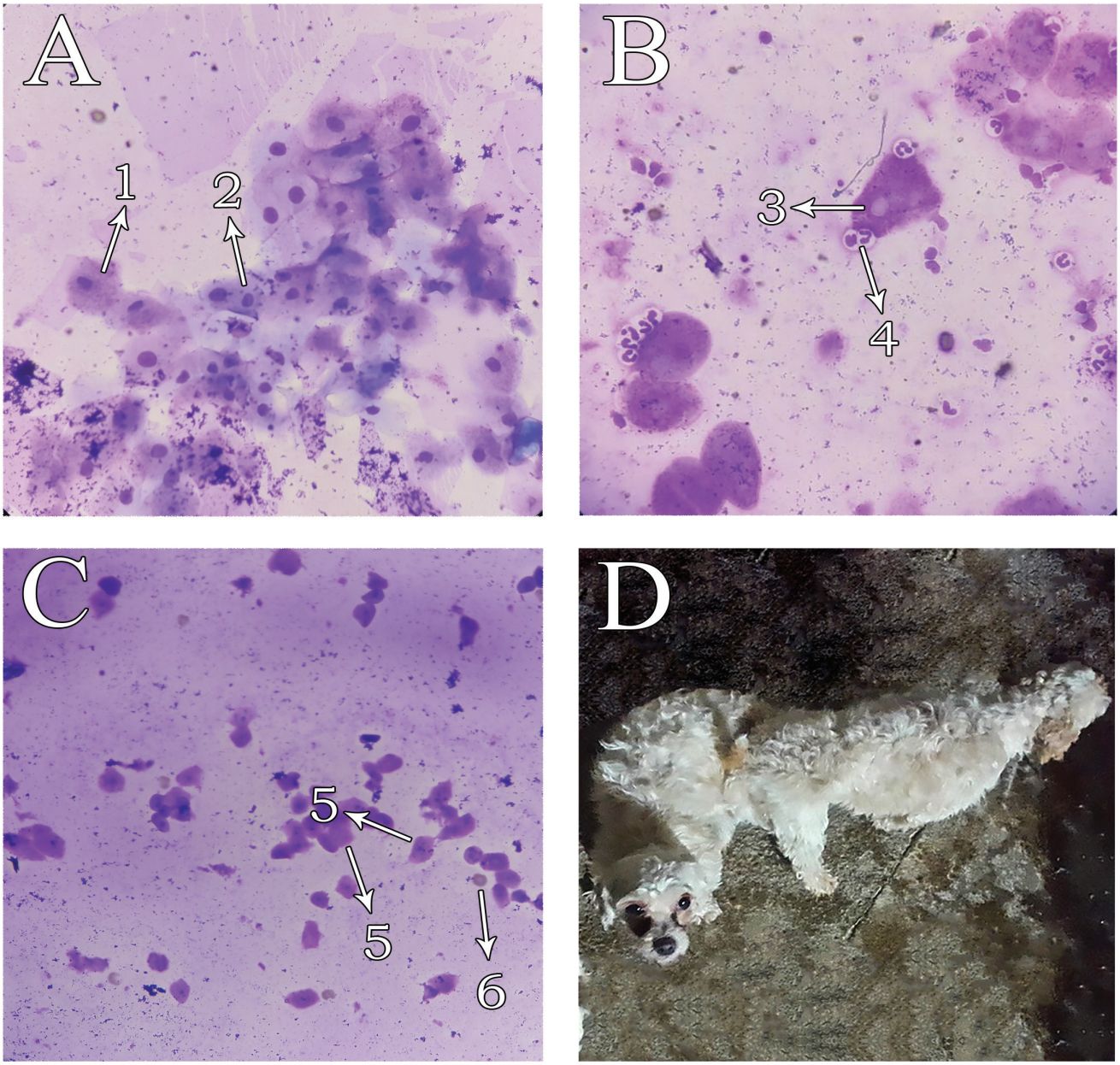

Figure 1.
A-C) Samples of vaginal cytology in the studied groups after Giemsa staining. Numbers 1 to 6 denote the large intermediate cells, parabasal cells, superficial cells, neutrophils, squamous cells, and Red blood cells, rescpectively. Dominant cells in vaginal cytology analysis during proestrus are RBCs and superficial cells, superficial and squamous cells in estrus, intermediate cells and neutrophils in diestrus, and parabasal cells in anestrus. D) Mating of one of the dogs in the VAC group (The copulatory tie in the dog).

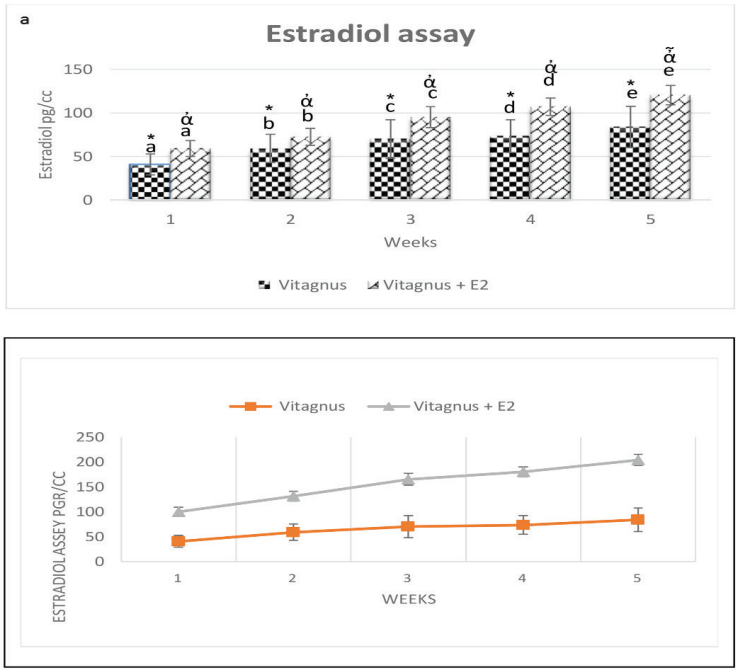

Figure 2.
Evaluation of estradiol changes in the VAC and VAC-E2 groups. The letters a-e show the estradiol changes between the VAC and VAC-E2 groups during the study weeks. There was no significant differences between groups ($p > 0.05$). The asterisk shows the comparison of estradiol changes in the VAC group in different weeks. There was no significant differences between groups ($p > 0.05$). The ά and ἄ show the estradiol changes in the VAC-E2 group in different weeks. There was a significant difference between the first and fifth week ($p < 0.05$).

shows that the duration of estradiol consumption is effective in increasing this hormone, which is quite natural. It can be concluded that since Vitagnus alone can raise estradiol level during the late anestrus phase, hence there is no justification for using it along with estradiol. Returning to estrus is seen earlier in VAC-E2 group, which leads to estrus induction earlier that the VAC group in the studied dogs. Statistical evaluations show that the drugs and the weeks can separately affect the estrogen level, but the reaction of the drug and week on estrogen level is not significant.(Figure 2)

Serum Progesterone Concentration: After measuring progesterone by ELISA method, the changes of this hormone were compared in weekly manner between two groups. From the statistical standpoint, there was no significant difference in progesterone level in the VAC and VAC-E2 groups ($p > 0.05$). From the analysis of progesterone level in two Vitagnus groups, it can be concluded that, at the onset of the estrus, in spite of an increase in progesterone in proestrus and anestrus, there is no significant difference, but passage of the weeks after beginning of estrus, progesterone level increases to a degree that it can lead to significant differences between groups. Analysis of progesterone in different weeks in Vitagnus and estradiol group, it can be concluded that there is a significant difference between progesterone level and five initial weeks of the study ($p < 0.05$). Therefore, it seems that estradiol injection at the onset of each week can help induce estrus in the animal earlier than the VAC group which only received Vitagnus. Early estrus leads to luteinization of the follicular wall. In fact, the procedure of increasing progesterone level in the first groups takes more than five weeks, however it can be reduced to less than five weeks in the second group. As can be seen in the mean rank table, the progesterone level in the fifth week is more considerable in the second group compared to other weeks. In fact, this result is consistent with the increase in estradiol in the final weeks of the VAC-E2 group, since luteinization of the follicular wall happens before the ovulation in dog (Figure 3).

Discussion

After five weeks of treatment in two VAC and VAC-E2 groups, of 10 dogs, 6 dogs entered the follicular phase and only one was in the luteal phase. The results indicate that Vitagnus along with estradiol or alone can help return to estrus in 60% of female dogs. Various compounds with differing dosages have been suggested for returning the female dog to estrus. Dopaminergic compounds are among the main ones. Cabergoline is one of the important compounds in re-

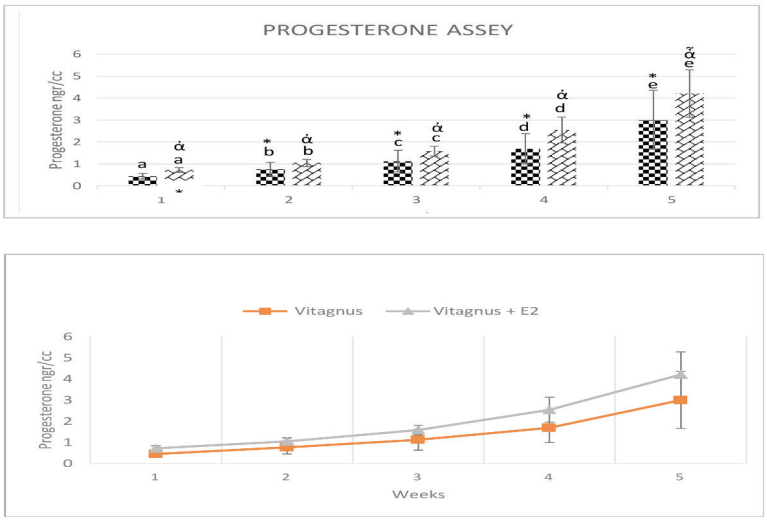

Figure 3.
Evaluation of progesterone changes in the VAC and VAC-E2 group s. The letters a-e show the progesterone changes between the VAC and VAC-E2 groups during the study weeks. There was no significant differences between groups ($p > 0.05$). The asterisk shows the comparison of progesterone changes in the VAC group in different weeks. There was no significant differences between groups ($p > 0.05$). The letters ȧ-ă show the progesterone changes in the VAC-E2 group in different weeks. There was a significant difference between the fifth and first week ($p < 0.05$).

turning the dog to estrus. Mogheiseh et al. (2017) reported that vitagnus can be as successful in returning the dog to estrus just as cabergoline and leads to cytological changes of the vagina and hormonal changes [1]. The results of the current study are in line with the previous studies. An issue that has to be considered in using Vitagnus is that the dog should be evaluated in terms of its reproductive cycle [16].

As stated in the results section, the estradiol level does not increase after Vitagnus, and it only sufficiently increases at the end of the estrus to show signs of the estrus. These results are confirmed in a report provided by Zahid et al. in 2016 [12]. They reported that Vitagnus has a higher tendency to combine with alpha and beta estrogenic receptors. On the other hand, there is a decrease in FSH after administering vitagnus, hence a lack of considerable increase in estradiol especially in the first group (Vitagnus) is seen [12].

There is a considerable difference between progesterone in different weeks of Vitagnus. On the other hand, as Rashidi et al., (2017) reported Vitagnus increases the LH levels [11]. We expect that increasing LH to increase progesterone, but the progesterone level in different weeks showed a significant difference in the VAC group. This seems natural since FSH is low and folliculogenesis is affected. On the other hand, since luteinization of the follicular wall happens before ovulation in the dog, thus no increase in the progesterone level in the first group was observed. However, in the second group, since the return to estrus occurred quickly, and since FSH increases at the end of the anestrus following Vitagnus administration, progesterone level increases considerably in the final weeks.

EEstradiol injection at the beginning of each week led to returning to estrus faster than that in the VAC group. Estrus length was the same in both groups. Estrus signs were more obvious in the VAC-E2 group. Mogheiseh et al. (2017) reported in another study that estradiol injection one week before cabergoline can speed up the dogs returning to estrus compared to the control group [1]. In this study, returning to estrus was faster in the group which received estradiol compared to the VAC group. According to the report provided by Mogheiseh et al. (2017) and the results of the current study, it seems that estradiol can speed up the return to estrus in anestrus dogs [16].

In the anestrus period, due to a decrease in FSH, Inhibin, and lack of negative feedback, there is an amount of estradiol in the serum of the dogs, but the level of alpha and beta receptors in the hypothalamus, pituitary, and ovary is low, hence the estrogen in the anestrus period cannot induce estrus. On the other hand, the main phytoestrogens of Vitagnus are

apigenin, vitexin, and Pendleton, which are bound with ERα and ERβ receptors. Apiginin is the most important due to its higher combination tendency with ERβ. Hence estrogen can bound to its receptors on the hypothalamus, pituitary, and ovary. The result of this bounding is enabling the hypothalamus-pituitary-ovary axis (HPO). With the increase in sensitivity of the pituitary in response to GnRH hormone, LH increases, and progesterone is synthesized. In this study, increasing progesterone level occurs at the end of diestrus and immediately before estrus induction and entrance to proestrus, and as Hatoya et al. (2003) reported, the increase in progesterone level is justifiable and it is in line with the studies conducted by Mogheise et al.[1, 18].

Niroumand et al. (2018) reported that Vitagnus extract can increase estrogen and progesterone level compared to the control group and it reduced prolactin levels. The results of the current study are consistent with that of Niroumand et al. (2018) [14].

The cytological changes of the vagina following hormonal changes especially in the final weeks of the study are obvious. Haji et al. (2018) reported that cytological changes of the vagina are moving toward epithelial and cornified cells following the increase in estrogen level of the blood and it leads to a decrease in neutrophils and parabasal cells. The increase in progesterone level leads to an increase in interstitial cells. In the current study, the follicular phase in anestrus dogs led to a decrease in parabasal cells and increased cornified cells [19].

Various protocols have been used to induce estrus in dogs. Compounds such as Gonadotropin-releasing hormone, gonadotropins, prostaglandins, steroid hormones, dopaminergic compounds, and plant compounds have been used by researchers. All methods of estrus induction have their advantages and disadvantages. For instance, although cabergoline is an effective compound for inducing estrus in the dog, since it is not for veterinary usage, the determination of the exact dose for the dog is difficult [1]. Rodas-Ruiz et al. (2015) used three methods of deslorelin injection, prostaglandin F2α and estradiol benzoate in anestrus dogs and the dogs in the luteal phase and concluded that these methods can induce estrus in dogs, but unlike Vitagnus, they are not able to induce ovulation and pregnancy, hence their study is not in line with the current study [6].

The results of this study show that using the Vitagnus plant alone can induce estrus in female dogs. On the other hand, this compound has fewer side effects than cabergoline and prostaglandin. Due to its availability in the country and also its low cost, this compound can be a suitable alternative for inducing estrus in anestrus dogs which is due to containing dopaminergic compounds. Also administration of estradiol benzoate before Vitagnus improved induction of estrus in dogs. To expedite the estrus in dogs, it is possible to use vitagnus and estradiol. A summary of the function of Vitagnus and Vitagnus-estradiol can be seen in Figure 4.can be seen in Figure 4.

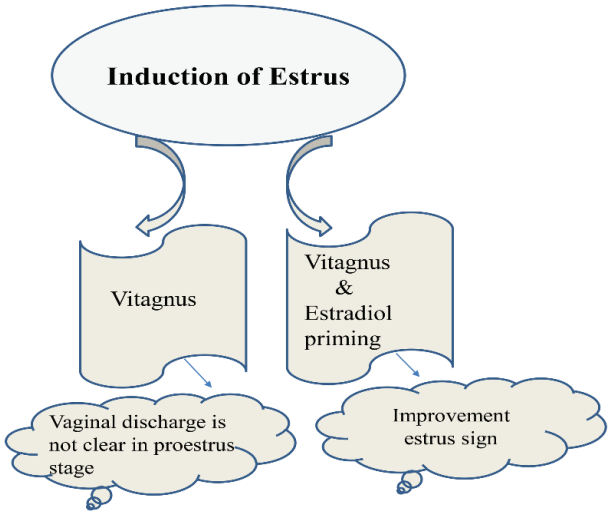

Figure 4.
A summary of the function of Vitagnus and Vitagnus-estradiol.

Materials & Methods

Animals

10 adult non-pregnant dogs with the age of 3-4 years and an average weight of 4 kg were chosen from the Shih tzu Terrier mix breed. Dogs were obtained from a dog training center in Urmia, Iran. Before the initiation of the study, the health status of the obtained dogs was investigated by referral to the Faculty of Veterinary Medicine of Urmia University. Weight and specification were recorded. The dogs were evaluated for their reproductive history. All dogs were in the mid-anestrus stage based on the date of the last estrus signs and observation of vaginal smear by gram staining and serum progesterone assays. Finally, the dogs were given an anti-parasite tablet (Cestal; 1 tablet per 10 kg of body weight). Animal's ID card was evaluated in terms of vaccination status and age. All dogs were kept in Urmia Pet House, Iran.

Study Groups

The animals were divided into two groups: Vitagnus (VAC) and Vitagnus-Estradiol (VAC-E2). Vitagnus is the extract of a plant that is commercially available. In each group, there were five dogs. Before the study, from all dogs, a vaginal smear was prepared to determine the sexual cycle. Serum samples were obtained for the evaluation of steroid hormones.

Estrus induction

To induce estrus in the VAC group, all dogs received oral Vitagnus (Poursina Pharmaceutical Company, Iran) at a daily dose of 90 mg [1]. In the VAC-E2 group, in addition to Vitagnus, Estradiol Benzoate (Abureyhan Pharmaceutical Company, Iran)

was intramuscularly administered at the beginning of the week at a dose of 0.01 mg kg^{-1} of body weight [16]. Meanwhile, the date of starting to use the drug and also the date of observing estrus signs and estrus confirmation based on vaginal cytology were recorded [17]. The occurrence of adverse behavior or side-effects of the drugs were recorded. The duration of the treatment was 5 weeks. At the end of each week, vaginal cytology and evaluation of estrogen and progesterone were conducted in both groups. After the fifth week, the mating date and their delivery time along with the number of puppies were recorded.

Preparation of Vaginal Smear

First, the vaginal area and its surroundings were washed, and then were disinfected with alcohol. After binding the animal, the tip of the sterile swab was smeared with normal saline and entered the dorsal commissure vulva by a tilt of 45 degrees. It was pushed gently in order not to enter the clitoral duct. After passing the ischial arch, by rubbing the swab cotton tip to the vaginal wall taking the contents and wall cells by swab, they were placed on the slide and after drying and fixing with ethanol, they were stained with Giemsa. All of the slides were registered with the name of the case and the sample number and they were investigated with a microscope with a 40X objective. Finally, cytological changes were recorded and the cycle of each animal was estimated [17].

Measuring the Concentration of Steroid Hormones

To measure the progesterone, blood samples were taken from each case while making vaginal smear. The blood samples were placed at a temperature of 37°C for 10-20 minutes to be clotted. It was centrifugated for 10 minutes at 2500 rpm to separate the serum. After serum separation and labeling, the obtained serum was stored at -70 °C. Finally, the ELISA kits (IBL International Germany, Hamburg, GmbH) were used to measure progesterone and estradiol concentrations according to the instructions provided by the manufacturer. After recording the data obtained from vaginal smears and analysis of the data obtained on the estradiol and progesterone concentrations, the VAC and VAC-E2 groups were compared.

Clinical Evaluation

DDogs were evaluated in two groups in terms of returning to estrus, clinical symptoms of estrus, mating after estrus, delivery process, and the number of puppies. The experiments were performed on animals following the guidelines of the ethical committee for research on animals of Urmia University (IR-UU-AEC-1357/DA3-2019).

Statistical Analysis

Statistical analyses were carried out using a mixed design (within and between compared groups). The ANOVA analyses were performed with 95% confidence intervals, independent t-test, and Kruskal Wallis test using SPSS software (version 22.0; SPSS Inc., Chicago, USA). All data are presented as means ± SEM and $p < 0.05$ was considered to be statistically significant.Chicago, USA). All data are presented as means ± SEM and $p < 0.05$ was considered to be statistically significant.

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Authors' Contributions

AG provided some of the materials. EA and AK performed the experiments and wrote the paper. AK analyzed the data.

Conflict of interest

The authors declare no conflict of interest.

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Abstracts (in Persian)

همراه کردن استرادیول بنزوات با عصاره گیاه پنج انگشت در بروز القا جفت پذیری سگ ها

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گروه مامایی، دانشکده دامپزشکی، دانشگاه ارومیه، ارومیه، ایران.

چکیده

در این مطالعه دو روش القا جفت پذیری در سگ ها مقایسه شده است (استفاده از ویتاگنوس و ویتاگنوس استرادیول). ۱۰ قلاده سگ ماده سیکلیک، نژاد شیتزوتریر مخلوط در مرحله آنسترووس انتخاب و به ۲ گروه تقسیم شدند. گروه اول (VAC) به مدت ۵ هفته ویتاگنوس دریافت کردند (۹۰ میلی گرم، روزانه، خوراکی). گروه دوم (VAC-E2) با ویتاگنوس و استرادیول درمان شدند. استرادیول بنزوات در شروع هر هفته تزریق شد (۰/۰۱ میلی گرم به ازای هر کیلوگرم، عضلانی). هر هفته نمونه خون جهت ارزیابی هورمون های استروئیدی و اسمیر مهبلی اخذ گردید. علایم و برگشت به جفت پذیری همراه با تعداد توله های به دنیا آمده ثبت شد. در گروه VAC ۶۰ و در گروه ۸۰ VAC-E2 درصد سگ ها بعد از ۵ هفته به فاز فولیکولار برگشتند. در گروه VAC-E2 علایم جفت پذیری ۷ تا ۱۰ روز و پروسه جفت گیری ۴ تا ۶ روز زودتر از گروه VAC رخ داد. میانگین تعداد توله های متولد شده ۴ تا بود. این علایم با سیتولوژی مهبل تایید شد. تفاوت معنی داری بین گروه های مورد مطالعه در سطح استرادیول و پروژسترون مشاهده نشد. تغییرات میانگین غلظت استرادیول بین هفته های ۱ و ۴، ۱ و ۵، ۲ و ۵ به طرز معنی داری مشاهده شد ($p < ۰.۰۵$). سطح پروژسترون در گروه VAC-E2 در هفته پنجم بیشتر از سایر هفته ها است. در نتیجه، استفاده از استرادیول بنزوات قبل از ویتاگنوس منجر به بهبود القا جفت پذیری در سگ ها می شود.

واژگان کلیدی

القا جفت پذیری، استرادیول بنزوات، عصاره گیاه پنج انگشت، سگ