

CHANGES IN THE VAGINAL CYTOLOGY AND PROGESTERONE AND ESTROGEN SE-RUM CONCENTRATIONS AFTER TREATMENT OF ANESTRUM BITCHES WITH A COMBINATION OF FSH AND LH

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Abstract: The current study was designed to evaluate the changes in vaginal cytology and serum progesterone and estrogen concentration in anestrus bitches treated with a combination of FSH and LH. Seven healthy Mongrel anestrus bitches were treated with daily administration of 75 I.U. FSH plus 75 I.U. LH for consecutive nine days and another three bitches were kept as a control without any treatment. Treated and control bitches were examined for the percentages of different vaginal-cytology cells, serum concentration of estrogen and progesterone, and signs of proestrus and estrus in a scheduled timeline. Five of the seven treated bitches (71.4%) showed a gradual decrease in the percentage of parabasal cells, a gradual increase in the percentage of cornified and RBCs, a rapid decrease in the estrogen concentration, and an increase in the progesterone concentration. These five bitches showed signs of proestrus and four of them showed male acceptance and were conceived. These five bitches had high estrogen concentrations before initiation of the treatment. The other two bitches had a low estrogen concentration before the initiation of the treatment and after treatment, they showed a gradual increase in the estrogen concentration, but they did not show characteristic changes in vaginal cytology or progesterone concentration. Control bitches had high estrogen concentrations before treatment, but they did not show characteristic changes in vaginal cytology, estrogen and progesterone concentrations, or signs of proestrus up to one month after the end of the treatment. In conclusion, treating anestrus bitches with a mixture of FSH and LH induced characteristic changes in vaginal cytology and serum estrogen and progesterone concentrations in the majority of bitches. These changes were associated with the induction of fertile estrus in more than half of the treated bitches, and this percentage increased to about 80% in bitches having initial high estrogen concentration.

Key words: bitch; estrus-induction; FSH-LH, vaginal cytology, progesterone; estrogen

Introduction

Domestic bitches spontaneously ovulate once or twice per year (1). The inter-estrus intervals range from 5 to 12 months in fertile, non-bred, and non-fertile cycles (2), and some bitches may enter into either primary or secondary pathologically anestrus (3). The inter-estrus interval consists of about 2 months of the luteal phase (diestrus) followed by a prolonged anestrus phase which

extends to 3 to 10 months (4). During anestrus phase, progesterone and basal concentration LH is low, but the basal FSH concentration is somewhat high, ranging from 50 – 400 ng/ml (5). Moreover, there is follicular growth in the ovary, but small follicles usually regressed without progressing to ovulatory size (6). The main factors that maintain the bitch in the anestrus phase are the low concentration of the LH and the high concentration of prolactin (7). Therefore, the induction of estrus depends mainly on inducing a drop in prolactin concentration or inducing an increase in the gonadotrophins mainly LH concentration.

A practical method to induce estrus may be applied to reduce the inter-estrus interval in the cases of missed breeding opportunities or conception failure, and for treatment of primary or secondary anestrus (8). Several treatment protocols using different medications such as anti-prolactin, gonadotropin-releasing hormones, and gonadotrophin active preparations have been developed to induce estrus in the bitches (8). Administration of anti-prolactin results in a reduction in prolactin concentration that indirectly leads to an increase in LH pulse frequency which stimulate follicular development up to ovulation in bitches (9, 10). Administration of anti-prolactin to anestrus bitches has successfully induced estrus within 14 to 40 days with a success rate ranging from 80 to 83% and a conception rate ranging from 60 to 80% (11-14). The administration of different GnRH agonists has successfully induced estrus in anestrus bitches within 7 to 10 days, with an estrus induction rate ranging from 70 to 80% and a conception rate ranging from 58 to 100% (15-17). Thus, administration of GnRH-agonist elicits the release of a sufficient concentration of endogenous gonadotropins.

Gonadotropin-active preparations such as purified LH, purified FSH, hCG, and/or eCG provide a stimulus similar to that provided by the endogenous LH pulses during the natural estrus cycle and may stimulate further development and maturation of the follicles up to ovulation (18 – 21). Administration of gonadotropin active preparations to anestrus bitches safely induces estrus within 5 to 7 days with the estrus induction rate ranging from 70 to 80% and a conception rate ranging from 50 to 80% (19-22). All the studies that employed gonadotropin-active preparations have focused on the estrus induction rate without studying the detailed changes in the vaginal cytology and the serum estradiol and progesterone concentrations in response to the treatment with gonadotropin-active preparations. Examination of detailed changes in the vaginal cytology and the serum estrogen and progesterone concentration will be helpful to understand the detailed response of bitches to the treatment protocol (23). Therefore, the current study was designed to investigate the detailed changes in the vaginal cytology and the estradiol and progesterone concentrations in anestrus bitches treated with a combination of FSH and LH.

Material and methods

Management of Bitches

The experimental design was approved by the Institutional Animal Care Use Committee Zagazig University (ZU-IACUS, approval number ZU-IACUC/2/F/161/2021). Ten healthy mongrel bitches with average age of 15 to 36 months and weights from 25 to 30 kg were used. All bitches were housed in the experimental animal unit faculty of veterinary medicine, Zagazig University from two weeks before the initiation of the treatment protocol up to 30 days after the end of the treatment protocol. Before initiation of the treatment, bitches were dewormed by using Drontal tablets with a dose of 1 tab/10kg (Praziquantel 50 / Pyrantel embonate 144 / Febantel 150 mg tablet for dogs, Bayer, Germany). To overcome tick infestation, the bitches were dipped in Mitaban Solution for dogs (Amitraz 10.6 ml, Zoetis, USA). During the whole experiment, bitches were supplied with a continuous source of clean water, fed on cooked meat with vegetables, and regularly exposed to natural light.

Defining the stage of the estrous cycle

There was no data about the previous fertility or the number of days since the previous estrous. To define the stage of the estrous cycle, bitches were subjected to progesterone hormonal assay and vaginal cytology examination 2 weeks before (day -14) and just before initiation of the treatment (day -2). An anestrus phase was defined by the dominance of parabasal cells with the presence of a few proportions of RBCs, WBCs, and intermediate cells in the vaginal cytology, and progesterone concentration <1 ng/ml, (2, 24).

Treatment Protocol

Bitches were randomly allocated to either the treated (n=7) or control group (n=3). Bitches in the treated group were treated with daily intramuscular administration of 75 I.U. FSH plus 75 I.U. FSH (Epigonal 75, Eipico, Cairo, Egypt) for nine days according to the method described by (18). Control bitches did not receive any treatments. Control bitches or treated bitches that did not respond to the treatment were observed

for the signs of proestrus until one month after the end of the treatment protocol.

Assessment of the serum estrogen and progesterone concentrations

Blood samples were collected from both treated and control bitches on day -14 and then day after day beginning from day -2 till day 12 (with day 0 being the day of treatment initiation). The serum was separated and stored at -20 °C until the assessment of the serum progesterone and estrogen concentrations. The serum concentrations of progesterone and estrogen were measured using the electrochemiluminescence immunoassay "ECLIA" (Cobas e 801 immunoassay analyzers). The procedures were applied according to the guidelines of the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany). The intra-assay coefficients of variation were below 6.81% and 9.39% for the estradiol and progesterone, and the inter-assay coefficients of variation were 2.3% and 3.66% for estradiol and progesterone, respectively. The sensitivities were 5 pg/ml and 0.3 ng/ml for estradiol and progesterone, respectively.

Assessment of vaginal cytology

Vaginal smears were collected on day -14 and every 2 days beginning from day -2 until the beginning of the proestrus or day 12 according to the method described by (24).

After the preparation of a stained film, 100 cells were examined and categorized into different types. Parabasal cells which are small, round, or ovoid with a marginal big nucleus, occasionally, contain cytoplasmic vacuoles or neutrophil granulocytes in the cytoplasm. Intermediate cells are characterized by a polygonal border and a well-shaped nucleus. Cornified cells are large squamous none nucleated cells that take dark stains. Leucocytes which are polymorph nuclear-granulocytoid cells, and finally the RBCs. The proportion of each type of cell was calculated as a percentage of the total number of cells.

Monitoring the behavioral and external signs of proestrus and estrus

Bitches in treatment and control groups were examined daily for the external signs of proestrus. The beginning of the proestrus was characterized

by the appearance of pronounced vulvar swelling and the presence of bloody tinged discharge from the vulva. The beginning of the estrus was defined based on changing the vaginal discharge into straw-colored and receptivity to the male, and the end of estrus was defined by the disappearance of male acceptance.

Mating and pregnancy diagnosis

Bitches that showed the signs of the proestrus were teased daily by a male beginning from the first day of proestrus to test their male acceptance. Bitches that showed male receptivity were mated with a proven fertile three males on the first day of male acceptance and every 2 days until the refusal of the male. Bitches were examined for pregnancy diagnosis using ultrasound on day 25 of post-coitus. Pregnant bitches were maintained up to the day of birth. The pregnancy period was estimated from the day of natural mating up to the day of parturition, conception rate and the number of puppies were recorded.

Statistical Analysis

The statistical differences for the effect of examination time points within the same variable and the same group were analyzed using a linear intercept mixed-effects model. The models included each measured variable as a fixed-effect. These variables include the percentages of different vaginal cytology cells and the concentrations of estrogen and progesterone. Bitch was included as a random effect in the models to account for the dependent nature of the data where variables were measured at multiple time points in each bitch. The statistical differences between treated and control groups or respond and non-respond bitches were evaluated using a t-test. The descriptive statistics were calculated for all variables over the different time points and the results are presented as mean \pm SE. All analyses were performed in R software version 3.5.3, and the critical probability was set at $P < 0.05$ for all analyses (25).

Results

The changes in the vaginal cytology

On days -14 and -2, the vaginal cytology of treated bitches showed a dominance of parabasal

cells ($81.3 \pm 2.7\%$) and few percentages of RBCs ($8.5 \pm 1.3\%$), WBCs ($2.8 \pm 1.2\%$), and intermediate cells ($7.4 \pm 2.4\%$). After the initiation of the treatment, the vaginal cytology revealed a significant decrease in the mean percentage of the parabasal cells beginning from day 6, and the lowest percentage was recorded on day 12 ($22.7 \pm 38.7\%$). Meanwhile, there were marked increases in the mean percentages of the RBCs and cornified cells beginning from day 10 (26.7 ± 12.8 and $30 \pm 20.8\%$, respectively). The mean percentage of the intermediate cells showed a significant increase on days 6 and 8 followed by a reduction on days 10 and 12 (Figure 1 a).

The untreated control bitches showed a significant decrease in the mean percentage of parabasal cells during the examination time points, however, the parabasal cell was the dominant cell up to day 12 ($79.6 \pm 0.5\%$) (Figure 1 b). Significant differences between the control and treated groups in the percentage of parabasal, cornified, and RBC cells were recorded beginning from days 4, 8, and 10, respectively. Untreated bitches did not show any cornification in the vaginal cytology. Before the appearance of a significant increase in the percentage of cornified

cells in the treated group, there was a significant increase in the percentage of intermediate cells on days 4,6, and 8 days.

Examination of the vaginal cytology of individually treated bitches revealed the presence of two groups of bitches. Five of the treated bitches showed characteristic changes in the vaginal cytology that ended with the appearance of the signs of proestrus, but the other two bitches did not show pronounced changes in the vaginal cytology during the examination period. The changes in the vaginal cytology in the five respond and the two non-responded bitches are present in Figure 2.

Estrus induction and conception

Five out of the 7 treated bitches (71.4%) showed the external signs of proestrus. These bitches were the bitches that had high estrogen concentration before initiation of the treatment. The other two bitches (28.6%) did not show any signs of proestrus. These two bitches were the bitches that had low estrogen concentration before initiation of the treatment. One bitch showed the signs of the proestrus before the end of the treatment (day 8),

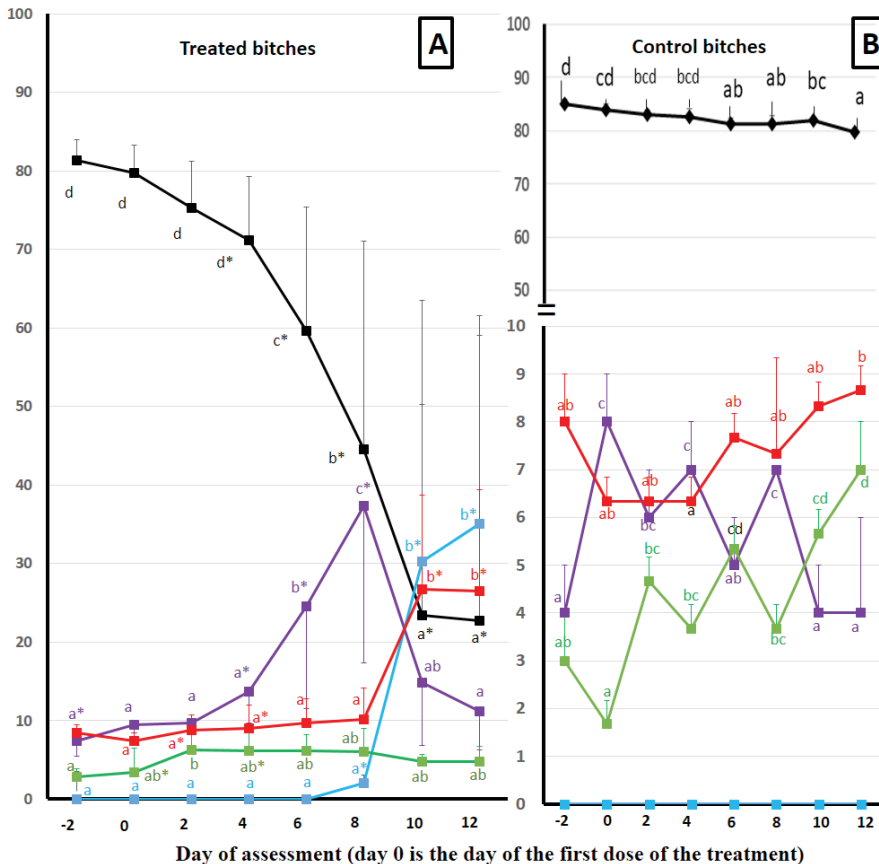


Figure 1: The mean percentages of different superficial vaginal cells in the vaginal smear of treated (A) and untreated bitches (B) at examination time points. Parabasal cells (black line), intermediate cells (violet line), cornified cells (blue line), WBCs (green line), and RBCs (red line). Values that have different superscripts are significantly different between different time points in the same type of cells at a p-value <0.05. * Indicate significant differences between treated and control groups at the same examination time point in the same type of cells. Note that the y-axis in untreated bitches was split

and the interval from the initiation of treatment until the appearance of the signs of the proestrus ranged from 8 to 13 days with a mean of 11 days. The duration of the proestrus ranged from 7 to 10 days with a mean of 8.4 days. Four treated bitches (4/7 57.1%) showed male receptivity and were mated. The duration of the estrus ranged from 8 to 18 days with a mean of 13.5 days. All mated bitches get conceived (conception rate of 100% (4/4) and pregnancy rate of 57.1% (4/7)). All pregnant bitches maintained the pregnancy till parturition, and the duration of pregnancy ranged from 59 to 65 days with a mean of 62 days. The litter size ranged from 2 to 6 puppies with an average of 4 puppies. There were no side effects recorded from the drug during or after the end of the treatment protocol.

None of the untreated control bitches showed signs of proestrus up to one month after the end of the treatment

The changes in estrogen and progesterone concentrations

On day -2, the mean concentration of progesterone and estradiol in the serum of treated

bitches were (0.5 ± 0.14 ng/ml and 76.5 ± 51.9 pg/ml). Examination of the serum estradiol and progesterone concentration of individual bitches before the initiation of the treatment revealed that five bitches showed a high estrogen concentration with a mean of 101 pg/ml and the other two bitches showed low estrogen concentration with a mean of 5.5 pg/ml. After the initiation of treatment, treated bitches showed a rapid decrease in the mean serum concentration of estradiol beginning from day 2, and the mean serum estradiol concentration continue low up to day 12 (16.5 ± 4.3 pg/ml). Meanwhile, there was a gradual increase in the mean serum concentration of progesterone till reached a significantly high concentration on days 10 and 12 (2.7 ± 0.8 and 5.9 ± 1.9 ng/ml) (Figure 3). The untreated control bitches did not show a pronounced change in the mean concentration of estradiol (ranging from 111.7 to 116.7 pg/ml) and progesterone (ranging from 0.34 to 0.38 ng/ml) during the examination period although there were some significant differences. The concentrations of estrogen and progesterone in treated bitches were significantly different from that of untreated bitches beginning from day 2 and 6, respectively (Figure 3).

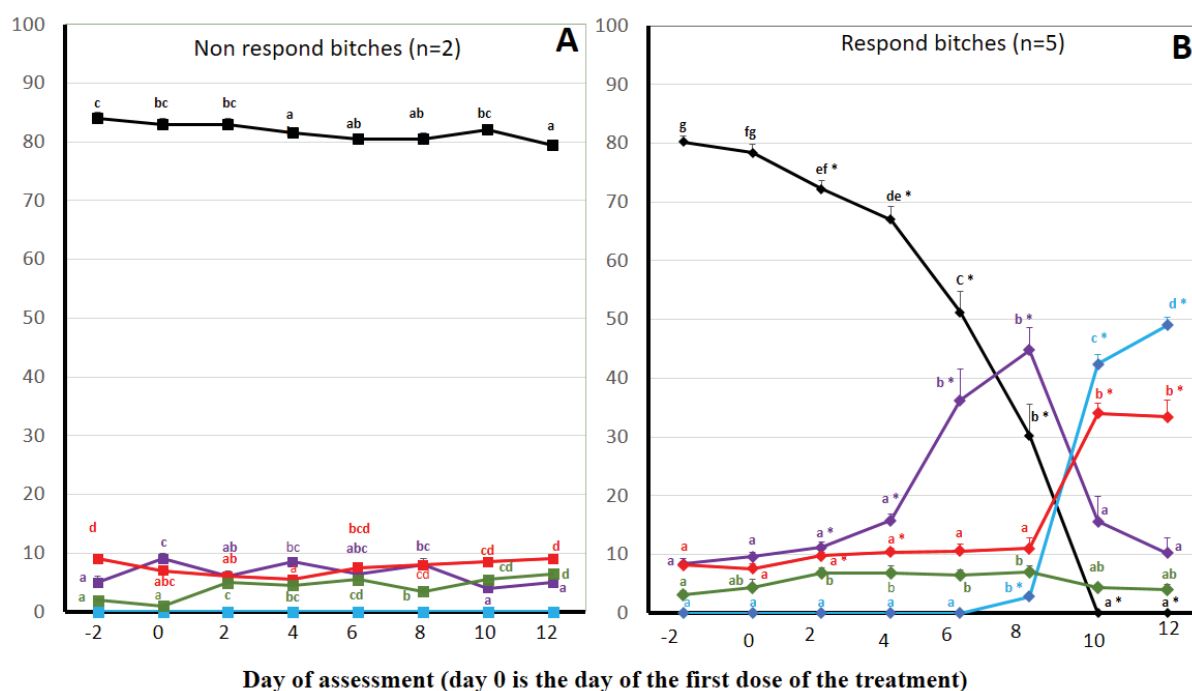


Figure 2: The average percentages of different superficial vaginal cells in the vaginal smear of the two treated non-responded bitches (panel A) and the five treated responded bitches (panel B) at examination time points. The responded bitches showed the signs of proestrus but the non-responded bitches did not show the signs of proestrus. Parabasal cells (blue line), intermediate cells (red line), cornified cells (grey line), WBCs (violet line), and RBCs (yellow line). Values that have different superscripts are significantly different between different time points in the same type of cells at a p-value <0.05. * Indicate significant differences between responded and non-responded bitches at the same examination time point in the same type of cells.

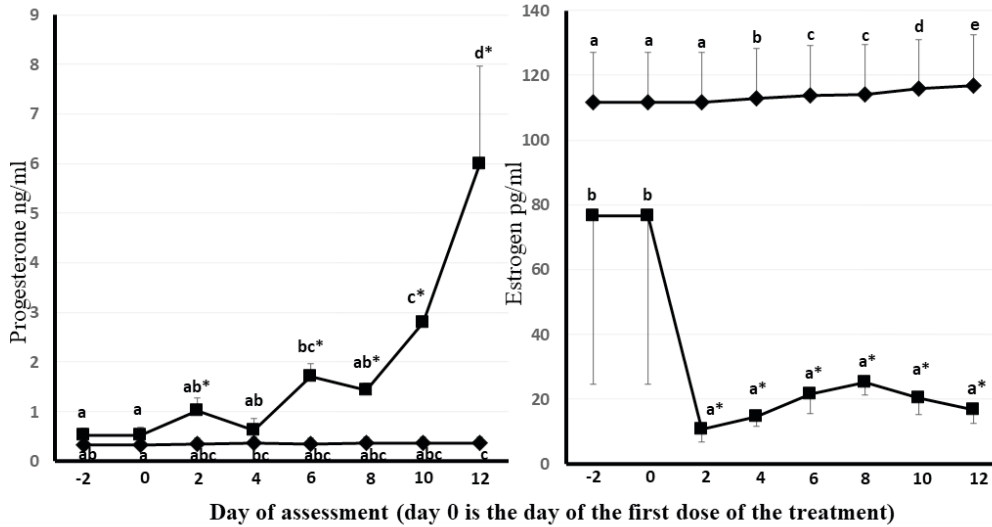


Figure 3: The mean concentrations of progesterone and estradiol in serum of treated (■) and untreated control bitch-es (◆) at examination time points. Values have different superscripts that are significantly different between different time points at a p-value <0.05. * Indicate significant differences between treated and control bitches at the same examination time point in the same hormone

Examination of the hormonal changes in individually treated bitches revealed that five bitches showed the previously described changes, these five bitches were the bitches that had a high estrogen concentration before the initiation of the treatment. But the other two bitches that had low estrogen concentration before the initiation of the treatment showed a different pattern of hormonal changes. These two bitches showed an increase

in the estrogen concentration beginning about six days after the initiation of the treatment followed by a decrease in the estrogen concentration at about day 10 after the initiation of the treatment. These two bitches showed a moderate increase in the progesterone concentration beginning from day 6 (Figure 4). The changes in the estrogen and progesterone concentrations in the five respond and the two non-respond bitches are present in Figure 4.

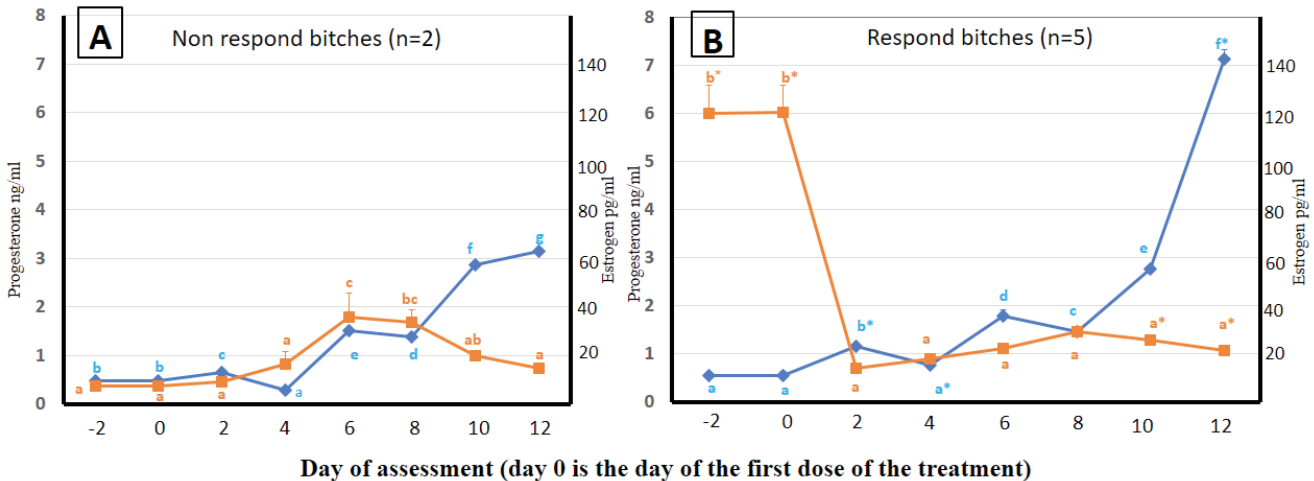


Figure 4: The average serum concentration of estrogen (orange line) and progesterone (blue line) in the two treated non-respond bitches (panel A) and the five treated respond bitches (panel B) at examination time points. The responded bitches showed the signs of proestrus but the non-responded bitches did not show the signs of proestrus. Values that have different superscripts are significantly different between different time points in the same type of cells at a p-value <0.05. * Indicate significant differences between responded and non-responded bitches at the same examination time point in the same hormone.

Discussion

In the current study, we investigated the detailed changes in vaginal cytology, estradiol, and progesterone concentration in anestrus bitches treated with a combination of FSH and LH.

In the current study, 5 out of 7 treated bitches showed a considerably high estradiol concentration before the initiation of the treatment but the other two bitches showed low estrogen concentration. This may indicate differences in the steroidogenic activity of the follicles that are present in the ovaries before initiation of the treatment (6, 26). The five treated bitches that had initial high estradiol concentration showed a rapid decrease in the serum estradiol concentration from the second day of the treatment. However, the other two treated bitches that had low initial estrogen concentration showed an increase in the estrogen concentration for 4 to 6 days followed by a decrease in the estrogen concentration. Regardless of the concentration of the estradiol before the initiation of the treatment, treating anestrus bitches either with pure porcine LH or eCG and hCG combination was accompanied by an initial increase in the serum estrogen concentration followed by a decrease in the estrogen concentration (27, 28). The variations in the estradiol concentration before the initiation of the treatment may reflect differences in the nature of the gonadotropin receptors in the ovarian follicles at this time (29, 30). These differences may be responsible for the different responses to the treatment as reviewed by (8).

The two treated bitches that showed an increase in the estrogen concentration after treatment may respond to the administration of FSH and LH combination by the development of new follicular growth or higher estrogen production from the current follicles (31). However, the five treated bitches that show a rapid decrease in the estrogen concentration may respond to the treatment by early luteinization of the current follicles (31, 32). This theory was supported by the fact that the five treated bitches that showed a rapid decrease in estradiol concentration showed a gradual increase in progesterone concentration. The increase in the progesterone concentration was reported after treatment of anestrus bitches with porcine LH or a combination of eCG and hCG (18, 27, 28, 32). The interval from the initiation of the treatment until detection of a high progesterone concentration varied among studies. In the current study,

treated bitches showed a somewhat early increase in the progesterone concentration which reached above 5 ng/ml on day 12 after the initiation of the treatment with the interval from initiation of the treatment till the proestrus was 11 days, which agrees with a previous result after using eCG (32).

The five treated bitches that showed a remarkable increase in the progesterone concentration showed an increase in the percentage of RBCs and cornified cells in the vaginal cytology in addition to the appearance of a serosanguinous vulvar discharge that indicates the proceeds of these bitches to the proestrus at about day 9 of the treatment. The proestrus induction rate in treated bitches reported in the current study was 71.4% which is comparable to the 70 to 80 % reported after using different combinations of the eCG and hCG (31), but it is lower than the 100% reported after using purified pig LH (28). The interval to the proestrus reported in the current study was 9 days which is longer than the 4-6 days previously reported after using different combinations of eCG and hCG (32), but it was shorter than the 14-15 days reported in another study used eCG and hCG combination (22). Four out of five bitches that showed signs of proestrus showed male acceptance, but the fifth bitch did not show signs of male acceptance. This indicates that this bitch may respond to the treatment by follicular growth, but the follicles did not reach the required stage to induce male acceptance. Twenty-five percent of the anestrus bitches that were treated with porcine LH and showed the signs of proestrus did not proceed to estrus (28). The estrus induction rate reported in the current study was 57% which is comparable to the 64% reported after using a combination of eCG and hCG (32), but it is lower than the 80-100% reported after using eCG and hCG combinations (33, 34)

The current study did not have a clear indication of ovulation, but four treated bitches conceived which indicates ovulation of good-quality ova. The pregnancy rate reported in the current study was 57% which is comparable to the 40 to 57 % recorded after treating anestrus bitches with cabergoline (0.6µg/kg/day), deslorelin, Lutrelin, and hCG injections (13, 16, 18, 35, 36), but it is lower than the 67 to 100 % reported after treatment of the bitches with deslorelin vulvar implant, metergoline and cabergoline (5µg/kg/day) (14, 34, 37, 38), respectively. In the

current study, all treated bitches that conceived maintained the pregnancy until the full term which excludes the luteal insufficient suggested after treatment of anestrus bitches with eCG and hCG combination (39). In the current study, all the three untreated control bitches had a high initial estrogen concentration, but none of them showed any change in the hormonal profile or vaginal cytology during the sampling period and none of them showed any signs of proestrus or male acceptant up to one month after the end of the treatment. This indicates that the treatment applied in this current study is efficient to induce estrus in anestrus bitch that had initial high estrogen concentration and that this treatment shortened the inter-estrus interval by at least 40 days.

The efficiency of the current treatment on the two bitches that had an initial low estradiol concentration was completely different from the five bitches that had an initial high concentration of estrogen. The two bitches that had an initial low estradiol concentration showed a marked increase in the estrogen concentration but fail to show any pronounced changes in the progesterone profile or the vaginal cytology. These findings agreed with a previous result that treatment of anestrus bitches with a combination of eCG and hCG increases the estrogen concentration in all five bitches but only two bitches showed an increase in the progesterone concentration (31). This may indicate that the treatment protocol applied in the current study may be efficient to induce follicular growth but unable to achieve follicular maturation and proceeding to ovulation, and growing follicles are mostly regressed (31). It seems that the initial concentration of estrogen may be related to the success of the hormonal treatment. In the current study, the number of bitches is few a further study to define the threshold concentration of estrogen that may predict the successful response after treatment will be helpful. Moreover, administration of a higher dose of LH may be required to induce follicular maturation and ovulation in bitches that have initial low estrogen concentration, further investigation is required to prove this hypothesis.

In conclusion, treating anestrus bitches with a mixture of FSH and LH induced characteristic changes in the vaginal cytology and the serum estrogen and progesterone concentrations in the majority of bitches. These changes were associated with induction of fertile estrus in more than half of

the treated bitches, and the estrus induction rate increased to about 80% in bitches having initial high estrogen concentration.

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