



## Induction Estrus in Local Anestrum Bitches by using GnRH, PMSG and hCG Combination

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**T**HIS study was performed to compare the ability of GnRH alone and combination of PMSG-hCG hormones to induce of estrus in anestrum female dogs (bitches). Hence, a total of 17 local dogs; 15 bitches of 2-4 years old and 2 fertile males (2-5) years old, were selected, prepared and inseminated naturally from December 2022 to June 2023. Firstly, all bitches were divided into three groups; G1 that represents the control and leaves without treatment, G2 that received GnRH at a dose of 0.0042 mg/animal I.M for two days, and G3 that received PMSG at a dose of 40 IU/animal for 7 days and then hCG at a dose of 150 IU/animal on the 8<sup>th</sup> day I.M. During anestrum, the vaginal smears showed that the predominant cells were parabasal and small intermediate cells; while in estrus phase, superficial cells were the most common. Ultrasound examination for 10 treated bitches in G2 and G3 groups was made during the 19<sup>th</sup>, 30<sup>th</sup>, 47<sup>th</sup> and 59<sup>th</sup> days post mating. Furthermore, pregnancy rate was recorded 80% in G2 (GnRH) group and 75% in G3 group (PMSG / hCG). While, insignificant variation ( $P < 0.05$ ) was seen in gestational period between G2 and G3. In conclusion, induction of estrus by using GnRH or PMSG with hCG is safe and effective.

**Keywords:** Bitch, Estrus, GnRH, PMSG, hCG, Ultrasound examination.

### Introduction

For decades, the utilization of deslorelin implants has played a significant role in the induction of estrus in bitches [1, 2]. The bitches have one, two, or sometimes three estrus cycles per year and no obvious seasonality, the fertility is lower during the warm season [3]. The stimulation of estrus in bitch has several benefits such as controlling the timing of pregnancy, treating of prolonged infertility or anestrum, failure of conception or losing of breeding, and synchronizing of ovulation for embryo transfer programs [4- 6]. For this purpose, different protocols have been used to induce of estrus in bitches such as the utilization of dopamine, exogenous gonadotropins (e.g. PMSG and hCG), GnRH agonists, and synthetic estrogens [7, 8]. The majority of studies in the bitch have investigated anti-Mullerian hormone (AMH) utilization as diagnostic instrument for reproductive problems like granulosa cell tumors, ovarian cysts, and ovarian remnant syndrome [9, 10]. However, there was little data in literature on

the correlation among GnRH, PMSG and hCG, the study Iraqi bitch local breed to investigate the ability of GnRH and the combination of PMSG-hCG hormones to induce estrus in anestrum bitches [11,5].

### Material and Methods

#### Animals

A total Normal fertile of 17 dogs; 15 local bitches of two to four years old and 2 fertile males of 2-5 years old were used for natural insemination from December 2022 to June 2023 (during the low fertility season) at the College of Veterinary Medicine, University of Baghdad (Baghdad, Iraq). Study bitches were divided as follows; G1 represents the control and leaves without treatment, G2 that received GnRH at a dose of 0.0042 mg/animal intramuscularly for two days, and G3 that received PMSG at a dose of 40 IU/animal for 7 days and then hCG at a dose of 150 IU/animal on the 8<sup>th</sup> day, intramuscularly (12; and 13). To obtain the vaginal sample, a swab was utilized, which was

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dampened with a saline solution to aid in the collection of the sample within the vaginal cavity. Additionally, a speculum was employed to prevent any adhesion between the swab and the labia minora to obtain a sample of cells, it is necessary to insert the swab into the caudal vagina and carefully maneuver around the fossa of the clitoris, twisting the swab a full turn before withdrawing it. Placed the swab on the slide and kept rolling it across the surface in accordance with [14]. The process involved the application of the vaginal smear onto a clean microscope slide, followed by air-drying and subsequent fixation with methanol for 1 minute, before allowing it to dry once more. Subsequently, the slide was immersed in an Eosinophilic red stain for 5-10 dips, with each dip being of one-second duration [15]. The natural insemination after estrus detection, and The pregnancy was detected through the utilization of an ultrasound (DP-10 Germany), which was conducted around 15 days post-breeding. The curved and linear abdominal probe were employed to determine the pregnancy rate. A gel was given to the inspection area, which stretched from the belly to the pelvic region, after it had been clipped and sheared.. During the parturition of the litter, a water-based gel was utilized and the ultrasound

transducer probe, along with the scanner (EDAN Vet) and printer were employed to document the process [16].

**Biochemical assay:** A biochemistry analyzer device (Humalyzer 3500) was used to determine serum total iron (Fe). Blood collection during day 0 and between days (35-45) from pregnant animals.

#### Statistical analysis

GraphPad Prism Software was used for the statistical analysis of the data. To evaluate significant differences between means, a one-way ANOVA and least significant differences (LSD) post hoc test were used [17].

#### Results and Discussion

According to the outcomes of the present study, the pregnancy rate in the GnRH-treated group was significantly  $P < 0.05$  higher compared with group treated with PMSG / hCG; where the pregnancy rate recorded 80% in G2 (GnRH) and 75% for G3 group (PMSG / hCG). From the other hand, the results revealed no significant  $P < 0.05$  differences in gestational period between G2 and G3 when recorded  $60.75 \pm 1.18$  and  $60.33 \pm 0.33$  days respectively (Table 1).

**TABLE 1. Effect of GnRH and PMSG /hCG on animal response, pregnancy rate, gestation period, No. offspring, and mortality rate in local female bitches**

Groups	No. of animal response	Pregnancy rate No.(%)	Gestation period/ days	No. of offspring	Sex of offspring		Mortality No. (%)
					Male	Female	
Control	-	-	-	-	-	-	-
GnRH	5	4	80 <sup>a</sup>	$60.75 \pm 1.18^a$	28 <sup>a</sup>	12 42.8% <sup>b</sup>	16 57.1% <sup>a</sup> 10.71% <sup>a</sup>
PMSG / hCG	4	3	75 <sup>b</sup>	$60.33 \pm 0.33^a$	18 <sup>b</sup>	10 55.5% <sup>a</sup>	8 44.4% <sup>b</sup> 5.55% <sup>b</sup>
Total	T.9/11 C.2/11	7		46	22 / 46 47.82%	24 / 46 52.17%	

Different small letters refer to significance ( $P < 0.05$ )

Our findings were agreed those recorded by some researchers [12] who found that the pregnancy rate was 75% in all groups treated with eCG / hCG. In addition, our findings were consistent with findings of other researchers. Some studies [7,18] reported that the pregnancy rate was 77% and 82%, respectively. These findings agreed with those found by some authors [19] who recorded that gestation period of  $61.5 \pm 1.19$  in bitches treated with PMSG / hCG. On the other hands, the data recorded by this study demonstrate highly significant number  $P < 0.05$  of offspring in group treated with GnRH (28 off spring) than

PMSG treated group which recorded 18 offspring. From the other hand; the results of sex of offspring recorded in the GnRH group high significant  $P < 0.05$  female (57.1%) than male babies (42.8%); while significant  $P < 0.05$  recorded male (55.5%) than female (44.4%) in group PMSG. The results of the number of offspring agree with [20] who claimed that the majority of his research's data came from huge litters, with a mean litter size of roughly 7 puppies and just three stallions having just one youngster; the rest all had 5 or more. Additionally said that increased follicular recruitment in response to pituitary stimulation by

the GnRH agonist may have contributed to the large litter size in the majority of fawns [21]. In addition, [13] eCG and hCG treatment resulted in a newborn baby with 38 whelps being reported. 29 whelps (67.3%) were alive whereas only 9 (23.7%) were lost to death. The Mortality rate was recorded significantly  $P < 0.05$  in GnRH when recorded (10.71%) than in the PMSG that reported (5.55%). The result of the present study disagrees with Fontaine *et al.* [20] when recorded stillbirth puppies were (6.4%) previously treated with a GnRH agonist (Deslorelin). The deficiency in iron may be associated with increased maternal and neonatal morbidity and mortality [21]. During anestrus phase, smears were taken during anestrus; parabasal and small intermediate vaginal epithelial cells are the predominant cells, the neutrophils might be present or not (Figure 1 A and B). These results were consistent with those of other studies

[2, 23-25]. During anestrus, superficial cells decrease, intermediate cells increase, and parabasal cells predominate [26]. In estrus phase, the superficial cells were predominant and these cells are a nuclear with the presence of superficial cells during the estrus phase (Figure 2 A and B). These findings were consistent with previous research [27, 28, 24, 25]. In these studies, all cells of the vaginal cytology during estrus were cornified and anuclear, and no polymorphonuclear cells. The superficial cells increased a lot during estrus, and very a smaller number of intermediate cells [23]. During pro-oestrus, oestrus, and early metoestrus, a significant number of neutrophils infiltrate the epithelium and lamina propria; these neutrophils eventually escape into the vaginal lumen. A large number of polymorphonuclear neutrophil leucocytes are visible in the smear toward the conclusion of oestrus [29, 30].

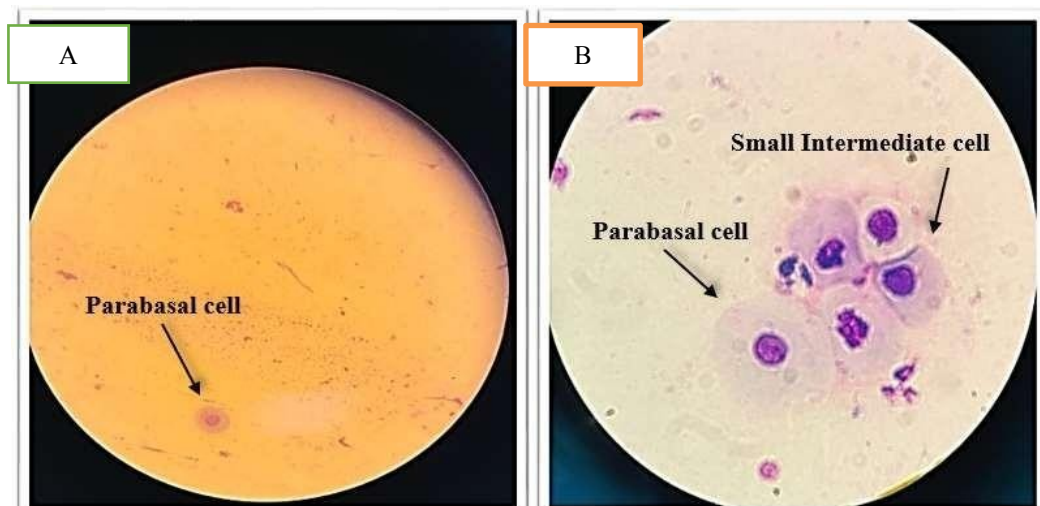


Figure (1 A and B). Microscopic anestrus smears show parabasal cells (40× and 100x).

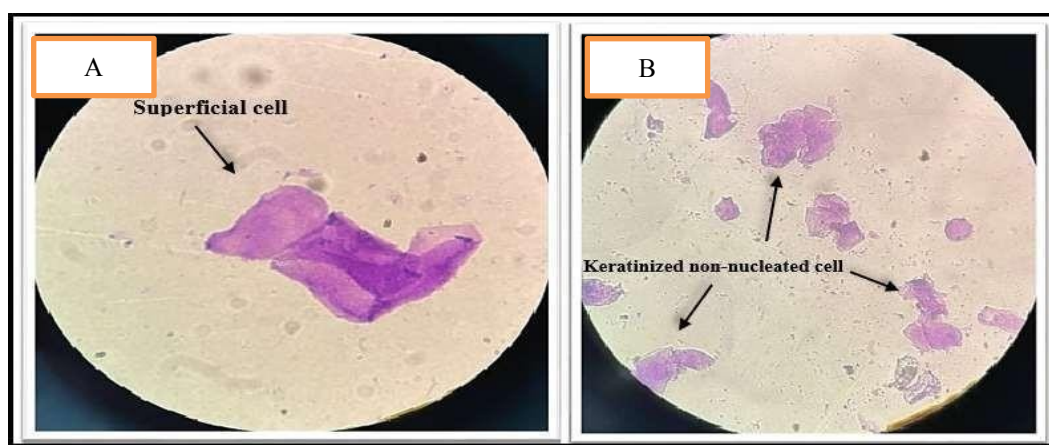
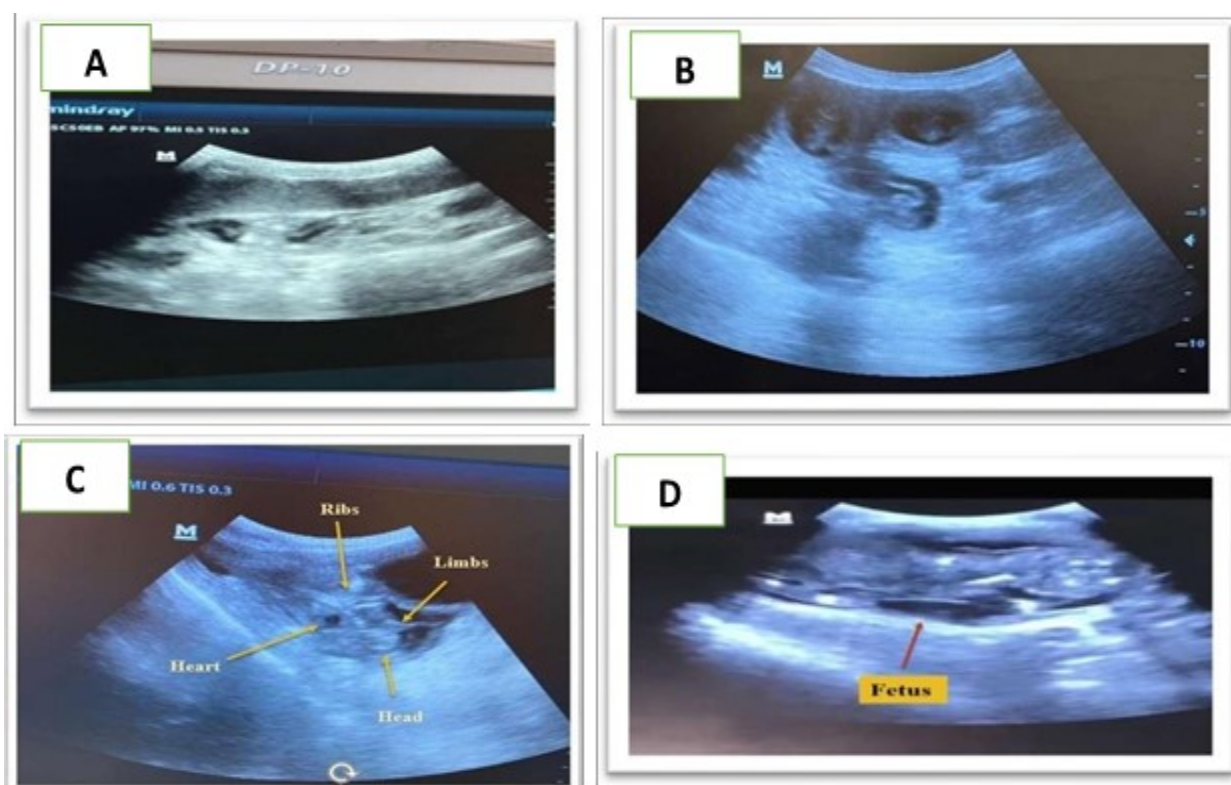


Figure (2 A and B). Microscopic estrus smears show superficial cells (100× and 40x)

Ultrasound examination was applied to diagnosis of pregnancy rate among 10 treated bitches in G2 and G3 groups at the 19<sup>th</sup> day of mating. The findings showed an existence of embryonic vesicles at early stage of pregnancy (Fig 3, A). Post 10 days of first examination, the second examination was done to confirm pregnancy showed the presence of fetus in pregnant bitch (30 days), (Fig 3, B). 47 days of gestation in bitch, third examination was done to

follow up the pregnancy status (Fig 3, C). Another ultrasound examination of fetus was made at 59 days of gestation in bitch, (Fig 3, D). The results of the Ultrasonography examination were proven with other author who reported that the accuracy of ultrasound for these groups was 100% [31]. These outcomes were consistent with research that demonstrated embryonic vesicles appeared in dogs between 18 and 23 days [32] and 20 days after ovulation [33].



**Figure 3.** Ultrasonography of fetus in different stages. A, embryonic vesicle at 19 days of gestation period in bitch / Transabdominal ultrasound (5 MHZ). B: Fetus at 30 days of gestation period in bitch/Transabdominal ultrasound (3.5 MHZ). C: Fetus at 47 days of gestation in bitch/ Transabdominal ultrasound (4 MHZ). D: Fetus at 59 days of gestation in bitch/ Transabdominal ultrasound (5 MHZ)

Our observations as the embryonic vesicles were found between 13-21 days after the mating were according to the observations of [34] pregnant dogs of various breeds and another animal [35].

#### Biochemical parameters (Iron and CBC)

The result appeared in the table (2) revealed non-significant  $P < 0.05$  differences in (iron, WBC and RBC) in all groups; while Hb recorded significantly  $P < 0.05$  differences between G2 and G3 when recorded  $17.50 \pm 0.63$ ,  $17.14 \pm 0.45$  g/dl

compared with G1 that recorded  $14.98 \pm 1.04$  g/dl. Finally, the platelet recorded high significant differences in G1 ( $415.40 \pm 54.57$ ) compared with G2 & G3 when recorded  $248.40 \pm 18.33$ ,  $307.00 \pm 21.25$  respectively. From the other hand the data reported in the Table (3) demonstrate non-significant  $P < 0.05$  differences among all groups after pregnancy between days 35-45.

**TABLE 2. Evaluation of biochemical parameters (Iron and CBC) for three groups before treatment in local female bitches**

Groups	Iron(Mg/dl)	WBC	RBC	Hb(g/dl)	PLA
<b>G1(Control)</b>	83.05±6.24	8.20±0.26	5.65±0.43	14.98±1.04 <sup>b</sup>	415.40±54.57 <sup>a</sup>
<b>G2 (GnRH)</b>	106.53±6.77	9.83±1.65	6.45±0.40	17.50±0.63 <sup>a</sup>	248.40±18.33 <sup>b</sup>
<b>G3 (PMSG + HCG)</b>	93.61±12.59	9.65±0.60	6.45±0.26	17.14±0.45 <sup>a</sup>	307.00±21.25 <sup>ab</sup>
<b>LSD</b>	<b>27.77 NS</b>	<b>3.16 NS</b>	<b>1.15 NS</b>	<b>2.32</b>	<b>109.18</b>

Means with a different letter in the same column are significantly different (P<0.05).

**TABLE 3. Evaluation of biochemical parameters (Iron and CBC) after pregnancy between days (35-45) for pregnant animals only in local female bitches**

Groups	Iron(Mg/dl)	WBC	RBC	Hb(g/dl)	PLA
<b>GnRH</b>	97.68±11.25	14.67±0.95	5.52±0.36	14.32±1.06	368.00±46.43
<b>PMSG + hCG</b>	91.92±15.90	8.70±2.69	3.44±1.19	9.53±3.48	540.00±137.04
<b>P-value*</b>	<b>0.78 NS</b>	<b>0.14 NS</b>	<b>0.21 NS</b>	<b>0.30 NS</b>	<b>0.33 NS</b>

Independent-test

**TABLE 4. compared (Iron) between non-pregnant and pregnant animals**

Groups/Iron	Non-pregnant	Pregnant
<b>GnRH</b>	A106.53±6.77 <sup>a</sup>	B97.68±11.25 <sup>a</sup>
<b>PMSG + HCG</b>	A93.61±12.59 <sup>b</sup>	A91.92±15.90 <sup>a</sup>
<b>LSD</b>	7.5	

Means with a different small letter in the same column are significantly different (P<0.05)

Means with a different capital letter in the same row are significantly different (P<0.05)

Hematological markers are helpful to provide further assurance that a pregnancy is healthy. It is normal for females to have mid-pregnancy alterations in the total blood count and normochromic normocytic anemia; also, the hematocrit significantly drops by the end of pregnancy [36]. The results illustrated in the Table (4) that compare of iron level between non-pregnant and pregnant bitch, revealed significant P<0.05 decrease in iron concentration in pregnant bitch (97.68±11.25 Mg/dl) that previously treated with GnRH than non-pregnant bitch in the same group (106.53±6.77 Mg/dl). Prevalences of iron, cobalamin, and folate deficits fluctuate significantly amongst women depending on socioeconomic status and dietary history, which has a significant impact on the prevalence of pregnancy-related anemia (PRA) in women [22]. The authors showed that neither serum cobalamine nor iron was linked to the development of PRA in pregnant stallions; however, the same authors found a significant rise in iron concentration in the late stages of pregnancy [37,38].

Using GnRH or PMSG with hCG is safe and effective in inducing fertile estrus in bitch. Furthermore, using two doses of GnRH was better

than PMSG-hCG in relation to the animal response and pregnancy rate in bitch.

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#### Novelty statement:

The novelty of the study is its focus on physiologically active GnRH hormone that can be employed as a stimulating hormone for the induction of estrus and reduce anestrus period in a bitch.

#### Authors contribution

These authors each contributed equally.

#### Conflict of interest

The authors declare that they have no competing interests



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## استحداث الشبق في الكلاب المحلية عديمة الشبق باستخدام هرمون GnRH ، PMSG و hCG

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تم إجراء دراسته لمقارنة فعالية هرمون GnRH لوحدة وفعالية كل من هرمونات PMSG-hCG على استحداث الشبق في إناث الكلاب. وبالتالي، وكانت الدراسة على مجموع 17 كلبًا محليًا؛ تم الاختيار على 15 من إناث الكلاب تتراوح أعمارها بين 2-4 سنوات و2 من الذكور القادرين على الإنجاب (2-5) سنوات، وكانت عملية التلقيح بشكل طبيعي، والفترة الدراسة تراوحت من كانون الأول 2022 إلى حزيران 2023. في البداية، تم تقسيم جميع إناث الكلاب إلى ثلاث مجموعات؛ مجموعة الأولى التي تمثل مجموعة السيطرة وتترك دون علاج، المجموعة الثانية التي كانت تحقن بهرمون GnRH بجرعة 0.0042 ملغم / حيوان في العضل لمدة يومين، والمجموعة الثالثة التي كانت تحقن بهرمون PMSG بجرعة 40 وحدة دولية / حيوان لمدة 7 أيام ثم يتم حقن هرمون hCG بجرعة 150 وحدة دولية / حيوان من اليوم الثامن أثناء العضل، حيث أظهرت المسحة المهبلية أن الخلايا السائدة كانت عبارة عن خلايا شبه قاعدية وخلايا وسطية صغيرة؛ بينما في مرحلة الشبق، كانت الخلايا السطحية هي الأكثر شيوعًا. تم إجراء الفحص بالموجات فوق الصوتية لعشرة من الكلاب المعالجة في مجموعات الثانية والثالثة خلال الأيام 19 و30 و47 و59 بعد التزاوج. علاوة على ذلك، تم تسجيل معدل الحمل 80% في مجموعة الثانية (GnRH) و 75% في مجموعة الثالثة (PMSG / hCG). بينما لوحظ وجود اختلاف غير معنوي ( $P < 0.05$ ) في فترة الحمل بين الثانية والثالثة. يمكن ان نستنتج، بان التحفيز الشبق باستخدام GnRH أو PMSG مع hCG آمن وفعال.