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Immunization of Rats Against Inhibin Using Bovine Follicular Fluid

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Abstract. Inhibin is a protein hormone that inhibits FSH secretion from the pituitary gland. Immunization of animals against inhibin resulted in increased ovulation rate. Mature Wistar albino rats were randomly assigned into two groups of eight treatments each using 6 rats per treatment. The treatments were: untreated control, 5 g GnRH, 50 i.u. PMSG, and 5, 10, 20, 40 or 80 l charcoal extracted bovine follicular fluid (bFF) to remove steroid hormones. Rats were treated daily for four days. Group 1 animals were killed on day 5 post-treatment. The second group was killed on day 8 post-treatment. Blood samples were collected during killing. The ovaries were removed and weighed. Blood serum was separated 24 hours after collection and stored at -20 °C until assayed for hormones. Rats injected with PMSG had larger ovaries ($p<0.001$) and greater ($p<0.001$) serum progesterone (P4) concentration (ng/ml; determined by RIA) than other rats in the same group. Between treatments comparisons showed that serum P4 concentration was lower ($p<0.001$) in the PMSG treated rats of group 1 than group 2. Rats from group 1 treated with 20 l bFF had larger ovaries ($p<0.001$) than those in group 2 with the same treatment. Group two rats treated with 10 l bFF or 20 l bFF had greater ($p<0.001$) serum P4 concentrations than their counterparts in group 1. Serum P4 concentration in group two rats treated with 80 l bFF was greater ($p<0.001$) than those treated with 5 l bFF. We conclude from these results that treated rats with PMSG or bFF results in increased ovulation rate as reflected by increased P4 concentrations secreted from the corpora lutea, and that increased P4 concentration is not necessarily associated with ovarian enlargement.

Introduction

Inhibin is a glycoprotein hormone produced by granulosa cells of large antral follicles [1,2]. It is made of two dissimilar subunits, and β, with molecular weight of 32 kDa. Administration of steroid-free inhibin preparations (i.e., charcoal extracted follicular fluid) suppresses serum follicle stimulating hormone (FSH), but not luteinizing hormone (LH), to baseline levels [3,4]. The major action of inhibin is its negative feed back effect on the anterior pituitary gland in a variety of species including rats and sheep [5-7]. In sheep, passive and/or active immunization against inhibin increased the synthesis of FSH, and hence, ovulation rate [8,9]. In addition, immunization of ewes against bovine

follicular fluid (bFF) was found to produce a three-fold increase in FSH concentrations over normal levels, while the ovulation rate was significantly increased by 40% [10].

Pregnant mare's serum gonadotropin (PMSG) is synthesized by the endometrial cups of pregnant mares [11] and used for inducing superovulation in laboratory animals and large domestic species [12]. There is a rapid reduction in peripheral blood inhibin levels after bilateral ovariectomy in PMSG-stimulated immature rats [13] and following ovariectomy in adult rats [14]. In addition, injection of low doses of LH stimulated inhibin production from granulosa cells after induction of its receptor with FSH [15].

Gonadotropin releasing hormone (GnRH) is a decapeptide hormone synthesized in the mediobasal hypothalamus [16,17] and is responsible for the release of FSH and LH from the anterior pituitary [18]. Through the release of FSH and LH, GnRH controls folliculogenesis in female animals.

The objectives of this study was to test the effect of different doses of charcoal extracted bFF on ovarian weight and blood serum progesterone (P4) concentration, and compare bFF treatment to PMSG and gonadotropin releasing hormone (GnRH) treatments.

Materials and Methods

Experimental animals

Ninety six mature Wistar albino female rats with a mean weight of 179.6 ± 20 g. (SEM) were obtained from the Center of Laboratory Animals at the College of Pharmacy, King Saud University. The rats were randomly assigned into two groups of eight treatments each using 6 rats per treatment. Treatment one was injected s/c with saline (vehicle) to serve as control. Treatment two received 5 g GnRH (Fertagyl, Intervet International; Holland). Treatment three received 50 i.u. PMSG (Synchro Part, Sanofi; France). Treatment four to eight received 5, 10, 20, 40 or 80 l charcoal extracted follicular fluid, respectively. All treatments were given daily for 4 days. Half of the rats were killed on day 5 (group 1), and the second half on day 8 (group 2) post treatments. Blood samples were recovered immediately after killing and the ovaries were removed and weighed. Blood serum was separated 24 hours after collection by centrifugation at 3000 rpm for 30 minutes and stored at -20 °C until assayed for P4 concentrations.

Hormone analysis

Serum progesterone concentration was determined in duplicate by specific double-antibody radioimmunoassay (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA). Assay sensitivity for P4 (95% binding) was 0.02 ng/ml. Intra- and interassay coefficients of variation were 4.7 and 6, respectively.

Follicular fluid collection

Bovine follicular fluid was obtained by aspirating all visible follicles (>1 mm in diameter) from cow ovaries obtained from a local slaughter house. Ovaries were kept at 4°C until aspiration of follicles (within 12 h) using different size needles and disposable syringes. The resulting fluid was frozen at -20 °C until charcoal extraction to remove steroid hormones.

Removal of steroid hormones from follicular fluid

Charcoal extraction was performed as follows; a known volume of follicular fluid sufficient for all injections was thawed and mixed in a sterile beaker with charcoal added to a concentration of 5 mg/ml of follicular fluid. The mixture was stirred at room temperature for 1 h, followed by centrifugation at 4 °C for 30 min at 3000 rpm. The decanted fluid was double filtered through a filter paper and stored at -20 °C in 20 ml aliquots. It is reported that 99% and 98% of the original steroids are removed by this technique [19,20] respectively.

Statistical analysis

All data were analyzed by analysis of variance using the general linear model of SAS [21]. Main effects included in the model were group, treatment and interaction between group and treatment. The effect of ovarian weight was calculated as percentage of body weight to correct for differences in animals' weights and multiplied by 1000 for easier interpretation of the data.

Results

Cross groups ovarian weight was significantly ($p<0.001$) higher in PMSG treated rats than in other treatments. Treatment with PMSG resulted in five-fold and three-fold increases in serum P4 and ovarian weight, respectively, in comparison to control. The significant ($p<0.001$) increase in P4 concentrations in PMSG treatments (Fig. 1) coincides with the increased ovarian weights. Overall, ovarian weights were positively correlated ($R^2=0.79$) with serum P4 concentrations (Fig. 2). Rats treated with PMSG in group 2 had significantly ($p<0.001$) larger and heavier ovaries, (02.13 ± 0.06) when compared to those under the same treatment in group 1 (1.58 ± 0.06; Fig. 3). Ovarian weights did not differ between groups 1 and 2 under other treatments. There were differences in P4 concentrations between group 1 and 2. Treatment with PMSG, 10 and 20 1 bFF resulted in greater ($p<0.001$) serum P4 in group 2 rats than in group 1 (Fig. 4). Within group 2, 80 1 bFF treatment resulted in greater ($p<0.001$) serum P4 (14.63 ng/ml) than 5 1 bFF(6.64 ng/ml) treatment. Results of other treatments did not differ between group 1 and 2 (Table 1).

Table 1. Ovarian weights and P4 concentrations for the different treatments in the two groups of rats

Treatment	Group one*		Group two*	
	Ovary weight	P4 (ng/ml)	Ovary weight	P4 (ng/ml)
Control	0.61^{b}	8.56^{b}	0.71^{b}	9.04^{bc}
GnRH	0.72^{b}	6.67^{b}	0.57^{c}	13.29^{bc}
PMSG	1.58^{a}	26.07^{a}	2.13^{a}	44.73^{a}
5 ul FF	0.73^{b}	10.19^{b}	0.60^{bc}	6.64^{c}
10 ul FF	0.65^{b}	6.14^{b}	0.67^{bc}	13.91^{bc}
20 ul FF	0.73^{b}	2.24^{b}	0.56^{c}	11.45^{bc}
40 ul FF	0.71^{b}	11.13^{b}	0.60^{bc}	8.35^{bc}
80 ul FF	0.62^{b}	9.55^{b}	0.62^{bc}	14.63^{b}

Values represent Least-Square means for ovarian weight in group 1and 2 with ± SEM of 0.06, and for P4 in group 1 and 2 with ± SEM of 2.50. Ovarian weights are percentage from rat weights multiplied by a 1000.
* Means within group with different postscripts are different ($p<0.001$).

Discussion

In this study, the effects of GnRH, PMSG and different doses of charcoal extracted bFF on ovarian weight and serum P4 were evaluated. Treatment with PMSG had a significant positive effect on ovarian weights and serum P4 concentrations. In agreement with this result, administration of large doses of PMSG (50 i.u.) to immature female rats caused a greater than ten-fold increase in ovarian weight within 72 h [22]. Cycling rats injected with PMSG during estrus had, on the same day, three times as many follicles larger than 55 m than in untreated animals [23]. The amino acid sequence and activity of PMSG resemble largely FSH and LH [24,25]. For this reason, PMSG not only stimulates ovarian follicles, as FSH does, but also stimulates luteal P4 production [26,27]. In addition, PMSG treatment was shown to produce a rapid reduction of peripheral blood inhibin in ovariectomized adult rats [28].

GnRH did not affect ovarian weight in both groups of rats. Previous studies [29-31] reported that GnRH inhibited hCG-stimulated and FSH-stimulated ovarian and uterine weight gains and P4 production in hypophysectomized rats. In immature, hypophysectomized, estrogen-primed rats, GnRH treatment was reported to induce atresia in preantral follicles [32]. In our study, overall treatment with GnRH did not affect ovarian weight nor serum P4 concentrations. This might be due to over-stimulation by GnRH (5 g) which leads to atresia of follicles [33]. However, within group 2, ovarian weights decreased when compared with control. While there was no significant difference in P4 concentrations between group 1 and 2 (6.67 vs. 13.29 ng/ml), GnRH treatment tended to increase serum P4 concentrations in the latter group.

Treatment with 10 and 20 l bFF resulted in increased serum P4 concentrations, but not in ovarian weight, in group 2 in comparison to group 1 (Table 1). The increased P4 concentration can be explained by the removal of the suppressing agent, inhibin, from

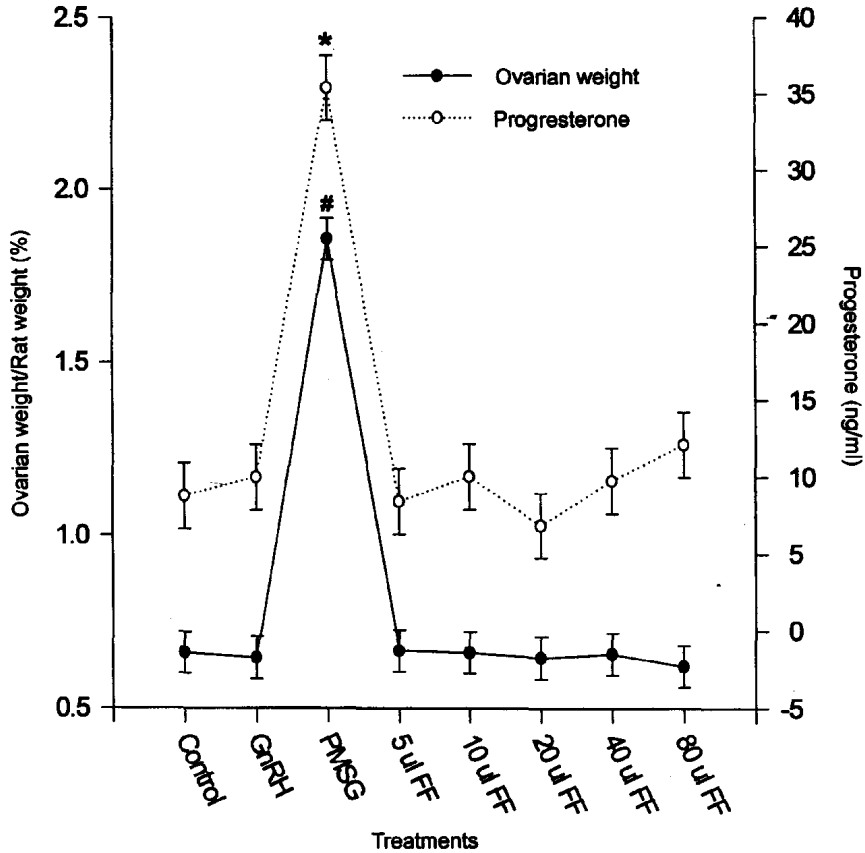

Fig. 1. Overall ovarian weights and serum P4 concentrations under different treatments in rats. * and # indicate significant effects of PMSG treatment ($p<0.001$) on P4 concentrations and ovarian weights, respectively. Notice that the heavier the ovaries, the greater the P4 concentrations.

the animals that resulted in increased follicular growth, ovulation and formation of corpora lutea. Our results indicate that immunization with 80 l bFF gives the highest P4 concentration, even though, it was not significantly different between group 1 and 2. However, we have no explanation for the indifference in ovarian weight, while there was a greater change in P4 secretion.

We conclude from this study that ovarian weight is highly correlated with P4 secretion and that immunization with charcoal extracted follicular fluid results in increased P4 concentration.

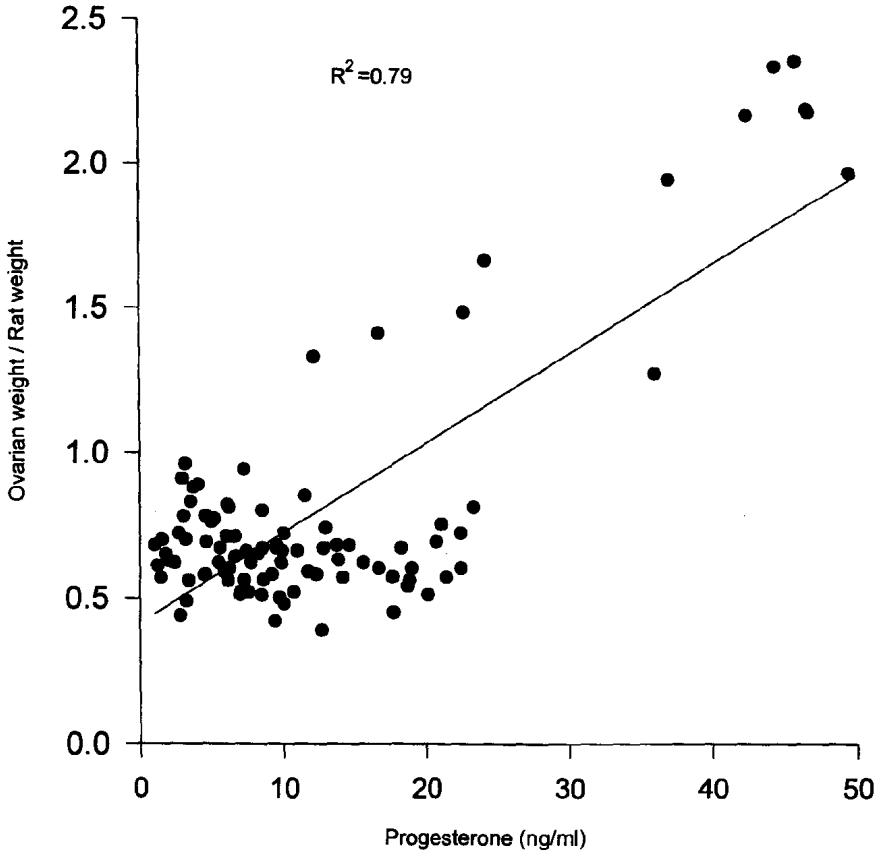

Fig. 2. Correlation between ovarian weights and P4 concentrations across treatments in rats. The correlation is highly significant (R^2=0.79) which reflects the changes in the ovaries (formations of corpora lutea) that lead to P4 production.

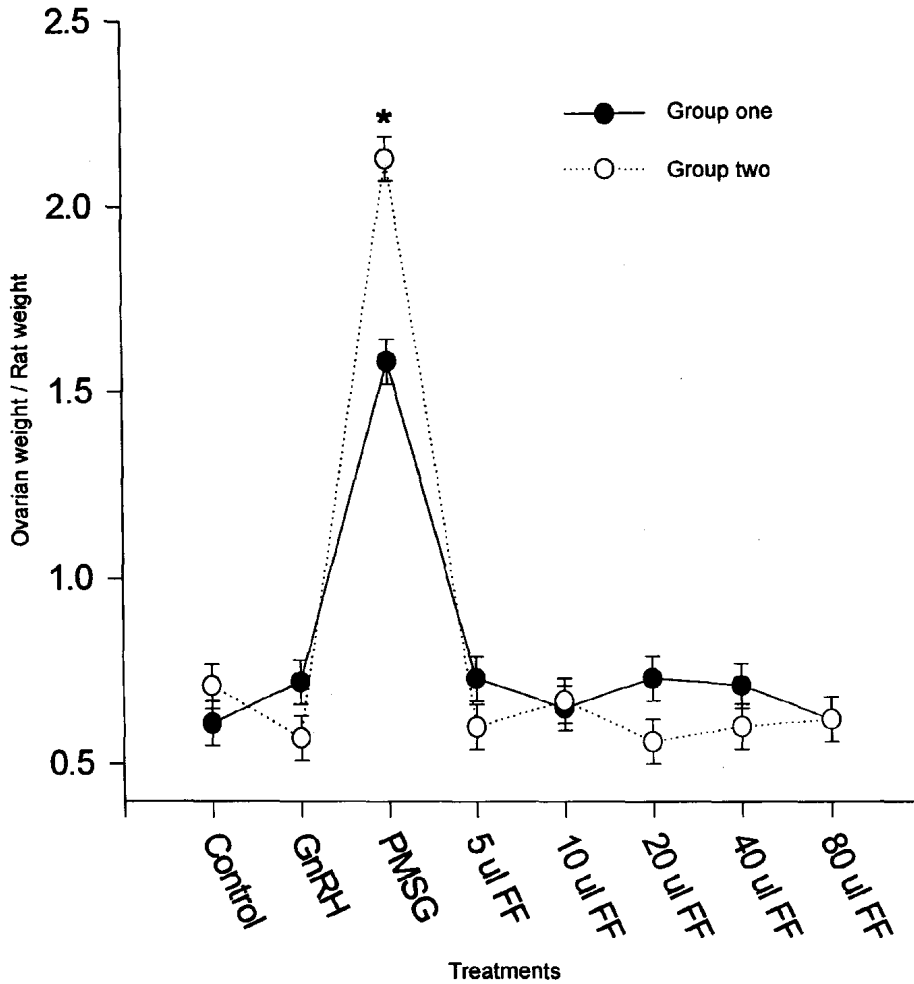

Fig. 3. Differences in ovarian weights between group 1 and group 2 rats. * indicate a significant effect ($p<0.001$) of group where PMSG treated rats in group 2 had heavier ovaries than their encounter parts in group 1. Notice that within group, PMSG had a significant ($p<0.001$) effect on ovarian weights.

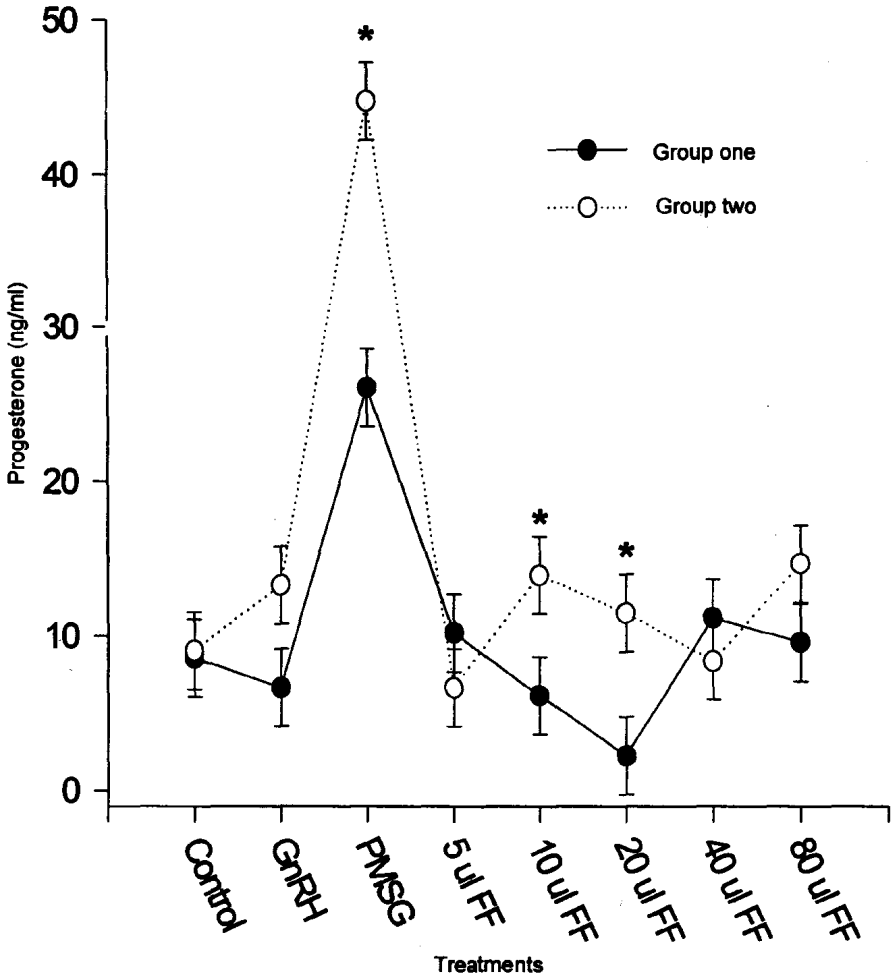

Fig. 4. P4 concentration differences between group 1 and group 2 rats. * indicates a significant effect ($p<0.001$) of group where PMSG, 10 and 20 bFF treated rats in group 2 had heavier ovaries than their encounter parts in group 1. Notice that within group, PMSG had a significant ($P<0.001$) effect on P4 concentrations. In addition, within group 2, treatment with 80 bFF had resulted in greater ($p<0.001$) P4 concentration that 5 1 bFF treatment.

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تحصين الجرذان ضد الإنهيبين باستخدام سائل جريبات مبايض البقر

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(قُدم للنشر في ١٤١٧/٧/٢٣ هـ ؛ وقبل للنشر في ٣ / ٧ / ١٤١٨ هـ)

ملخص البحث. يمكن تثبيط إفراز الهرمون المنبه لنمو الجريبات المبيضية بواسطة هرمون بروتيني يعرف بهرمون الإنهيبين (Inhibin) حيث أنه عند تحصين الحيوانات ضد الإنهيبين فإن معدلات الإباضة تزداد. لهذا أجريت تجربة على مجموعتين من الجرذان البيضاء البالغة وقسمت عشوائيا كل مجموعة إلى ثماني معاملات، كل معاملة ستة جرذان، وحقنت كالتالي: المعاملة الأولى (الشاهد)، الثانية عوملت بـ ٥ ميكروجرامات من الهرمون المحرر لمنبهات المناسل (GnRH)، الثالثة عوملت بـ ٥٠ وحدة دولية من مصل الفرس الحامل (PMSG) ومن المعاملة الرابعة حتى الثامنة حقنت الجرذان بـ ٥، ١٠، ٢٠، ٤٠ و ٨٠ ميكرولترا من سائل جريبات المبايض المستخلص منه الهرمونات الإستيرودية. عوملت تلك الجرذان يومياً لمدة أربعة أيام وفي اليوم الخامس قتلت المجموعة الأولى، والثانية في اليوم الثامن من بداية الحقن. جمعت عينات الدم ووزنت المبايض بعد القتل مباشرة. تم فصل مصل الدم وحفظه بعد ذلك على درجة -٢٠°م لحين تحليل الهرمونات بواسطة المعايرة الإشعاعية (RIA). ومن النتائج اتضح أن الجرذان المعاملة بهرمون مصل الفرس الحامل أوزان مبايضها أكبر وتركيزات مرتفعة لهرمون البروجسترون وكانت عالية المعنوية عن باقي المعاملات. كما أن تركيز البروجسترون داخل تلك المعاملة أقل انخفاضا في المجموعة الأولى عن الثانية والفروقات كانت معنوية. أما الجرذان التي عوملت بـ ٢٠ ميكرولترا من السائل الجريبي فكانت أوزان مبايضها أكبر عنها في الجرذان لنفس المعاملة في المجموعة الثانية والفروق كانت معنوية. كما أن المعاملتين بـ ١٠ و ٢٠ ميكرولتر من السائل الجريبي في المجموعة الثانية لها تركيزات أكبر من البروجسترون عن المجموعة الأولى والفروق كانت معنوية. كما لوحظ ارتفاع تركيز البروجسترون في المعاملة التي حقنت بـ ٨٠ ميكرولترا عن التي حقنت بـ ٥ ميكرولترات في المجموعة الثانية. وخلاصة النتائج أن التحصين بهرمون مصل الفرس الحامل أو بجرعات مختلفة من السائل الجريبي يعمل على زيادة الإباضة وهذا ما يشير إليه زيادة تركيز البروجسترون المفرز من الأجسام الصفراء، ولكن ليس بالضرورة زيادة تركيز البروجسترون يشير إلى زيادة أوزان المبايض.