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The Effect of Different Hormonal Supplementations on Pregnancy Support and Outcome in Mice

Muhammad Baqir M. R. Fakhrildin, Ayad M. A. Fadhil, M. A. K. Ibrahim, Jafar M. R., B. S. Al-Temimie, H. A. Z. Al-Ibrahimi and J. I. Asa'ad
Baghdad Research Center for Biotechnology, University of Baghdad, Baghdad, IRAQ.
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Abstract

The effectiveness of different hormonal supplementations and durations of injection on early embryonic implantation and pregnancy outcome in mice were investigated. One hundred and ninety six mothered mice were divided into two major groups depending on hormonal stimulation of estrous cycle of females either stimulated or non-stimulated. Further, each of major group was subdivided into two minor groups depending on period of injection during gestation either for 1st week or 1st and 2nd weeks. Each minor group was divided according to type of hormone supplement including human chorionic gonadotrophin (3 IU), progesterone (0.07 mg), estradiol (0.035 mg) or other combinations between progesterone and human chorionic gonadotrophin or estradiol. The main parameters were studied are gestation period, litter size and endometrium thickness of uterine horn.
The results of the present study observed that the administration of hormones have insignificant differences in the period of gestation as compared to its control groups. Also, litter size and endometrium thickness of uterine horn were significantly ($P< 0.05$) increased as a result of hormonal treatments when compared to its control groups. However, progesterone supplementation for 1st and 2nd weeks of gestation period was best and significantly ($P<0.01$) increased litter size and endometrium thickness of uterine horn without noticeable side effects. It was concluded that the synchronization of ovarian stimulation and hormonal supplementation by using either progesterone alone or in combination with human chorionic gonadotrophin may be have good results to improve normal embryonic implantation rate and enhance pregnancy outcome. Further hormonal and histological studies are recommended to investigate the effects of different hormonal supplementations on endocrine and reproductive organs of pregnant female.

الخلاصة

بُحث تأثير اختلاف التزويد بالهرمونات وفترات الحقن من بداية مرحلة انغراس الاجنة ونتائج الحمل في الفئران. قسمت مائة وست وتسعون فأرة إلى مجموعتين كبيرتين اعتماداً على التحفيز الهرموني للمبايض ضمن دورة الشبق وهي إما محفزة أو غير محفزة هرمونياً. بالإضافة إلى ذلك قسمت كل مجموعة كبيرة إلى مجموعتين صغيرتين اعتماداً على فترة حقن الهرمونات وهي أما في الأسبوع الأول وأما في الأسبوعين الأول والثاني من بداية فترة الحمل. قسمت كل مجموعة صغيرة تبعاً لنوع الهرمون الذي تم تزويده للفئران وهي أما هرمون الغدد المشيماني (hCG; 3 IU) أو البروجستيرون (Progesterone; 0.07 mg) أو الإستراديول (Estradiol; 0.035 mg) أو لمزيج من هرمون البروجستيرون مع هرمون الغدد المشيماني أو مع هرمون الإستراديول. درست الصفات التالية وهي فترة الحمل (Gestation period) وحجم الولادة (Litter size) وسمك غشاء بطانة قرن الرحم (Endometrium thickness).

أظهرت نتائج البحث الحالي وجود اختلافات في فترة الحمل لم تصل إلى درجة المعنوية بالمقارنة مع مجموعة السيطرة. وازداد حجم الولادة وسمك غشاء بطانة قرن الرحم بشكل معنوي ($P<0.05$) باستخدام المعاملات الهرمونية مقارنة بمجموعة السيطرة. وعلى أي حال فان الحقن بهرمون البروجستيرون خلال الأسبوعين الأول والثاني من فترة الحمل حقق أفضل النتائج من حيث حجم الولادة وسمك غشاء بطانة الرحم وبدون وجود أي تأثيرات ظاهرية سيئة. نستنتج من نتائج الدراسة الحالية أن تزامن تنشيط المبايض والتزويد بهرمون البروجستيرون سواء بمفرده أو بمزجه مع هرمون الفند المشيمائي حسن معدلات انغراس الأجنة ويعزز نتائج الحمل. نوصي بإجراء المزيد من الدراسات الهرمونية والنسيجية لتقصي تأثيرات التزويد بهرمونات مختلفة على الغدد الصماء وأعضاء التناسل للأنثى الحامل.

Introduction

The peri-implantation period is a critical time during embryonic development (1,2). It was reported that the implantation is arguably the most critical stage in the establishment of pregnancy (3). In humans, it has been estimated that between (30-70)% of conceptuses are lost before or at the time of implantation. Mainly, successful rate of embryonic implantation is affected by formation and maturation of endometrium and deciduas (4). Furthermore, blastocyst implantation involves a complex series of events occurring over time. It requires synchronized development of the conceptus and a receptive uterus (5). Progesterone appears to be necessary for implantation and maintenance of an early intrauterine pregnancy and this partly has been the basis for recommending luteal phase supplementation with progesterone following oocyte fertilization *in vitro* and embryo transfer (6, 7). More recently, Pritts and Atwood (8) stated that the "i.m. progesterone is favored as hormonal supplementation to support pregnancy with the addition of estrogen". Furthermore, human chorionic gonadotrophin (hCG) is useful tool to overcome the luteal phase inadequacy and early abortion (9).

Certainly, large numbers of materials and factors are support normal embryonic implantation and pregnancy including: β-1 integrins (10), platelet-activating factor (PAF) (11), placental protein-14 (4), progesterone (12-14) and estradiol (E2) (15, 16). Interestingly, several treatments were suggested to prevent the extra-uterine discharge of *in vivo* and *in vitro* transferred embryos and improve the rate of embryonic implantation and successful pregnancy including: nitric acid (1), gonadotrophin releasing hormone analogue (GnRH-a) (17, 18), cyclooxygenase-2 (19) and human chorionic gonadotrophin (20). Therefore, the current study was designed to select the effective hormonal supplement including human chorionic gonadotrophin (hCG), progesterone, estradiol or other combinations and period of injection either for 1st week or 1st and 2nd weeks to support embryonic implantation and successful pregnancy outcome in female mice with natural cycle (non stimulated ovaries) or stimulated cycle (stimulated ovaries) by using chorionic gonadotrophin, progesterone, estradiol or other combinations between progesterone and human chorionic gonadotrophin or estradiol..

Materials and Methods:

1- Animals:

One hundred and ninety six healthy, female, mothered mice (age: 15-16 weeks) of Swiss albino strain were kept in an air-conditioned room at a temperature of (26±2) °C and exposed to 12-14 hour day light. Females were weighting (25-30) g and each three females were housed in small plastic cages measuring (29X12.5X11.5) cm. Bedding of the cages were lining with soft crushed wood shaving, and washed one time weekly with soap, disinfectant and tap water and sterilized with 70% ethyl alcohol throughout the period of the present study (21).

2- Chemicals and drugs:

Table 1 show the drugs and chemicals were used in the present study.

3- Experimental design:

Mainly, one hundred and ninety six females were divided into two major groups according to hormonal stimulation of estrous cycle as following: 1-natural cycles (non-stimulated females), and 2-superovulated cycles (stimulated females). Also, each major group was subdivided into two minor groups according to the length period of injection during gestation as following: I- first week of gestation period, and II- first and second weeks of gestation period. Furthermore, each minor group was divided into several groups according to the types of hormones were injected. In details, table (2) shows the experimental design of the present study.

4- Vaginal smears:

To diagnose and determine the stage of estrus cycle of adult female, repeated vaginal smears were done. Mainly, the procedure was applied depending on the research of Zarrow and associates (22) as following: one drop of sterile normal saline was placed by the loop in the vagina, aspirated this loop several times and then transferred the mixture of sterile normal saline and scraping vaginal cells onto a clean slide and dried in the air. Thenafter, stained with methylene blue and examined under the light microscope (40 X). Presence of large cornified cells and remnant of epithelial cells was considered that this female within estrus stage and mating with active male should be take place, and vaginal plug is resulted within 16-24 hour. Occurrence of vaginal plug was considered the first day of gestation.

5- Hormonal stimulation (superovulation) of females:

Each female was intraperitoneally (IP) injected with 15 I.U. of PMSG (Folligon). After 72 hour of the first injection, same female was IP injected with 15 I.U. of chorionic gonadotropin (Chorulon), and mated with active fathered male. Occurrence of vaginal plug was considered the first day of gestation (23).

6- Histological sections:

At day fourteenth of gestation period, only six pregnant mice was killed by cervical dislocation, and dissected to obtain both uterine horns. Both uterine horns were cleaned and fixed in Bouin's solution for 24 hour. Then, series of dehydration by ethanol alcohol was performed, and cleared by xylene. Both uterine horns were embedded and blocked in paraffin wax, and sectioned (5μ thickness) serially. Sections were mounted and stained with Erlich haematoxylin and eosin (24). To measure the endometrium thickness of uterine horn, serial histological sections were examined under light microscope (40 X).

7- Statistics:

In addition to the standard statistical methods to determine the mean and standard error of mean (S.E.M.), analysis of variance (ANOVA) to report the level of statistical significance among the means of groups (25).

Results

1- Gestation periods:

The results of the present study appeared that the periods of gestation have insignificant difference among all groups injected with different hormone supplements and control of non- stimulated females (Table 3). Similarly, the same results were registered among groups injected with different hormone supplements and control of stimulated females (Table 4). In general, the periods of gestation for all groups injected with different hormone supplements and control of 1^{st} week and 1^{st} and 2^{nd} week's injection of non-stimulated females were longer than the stimulated females. However, these differences were considered insignificant (Figures 1and 2).

2- Litter size:

Progesterone injection for the 1^{st} week of gestation period in non-stimulated females was either significantly ($P<0.01$) or insignificantly increased the litter size as compared to groups of control and human chorionic gonadotrophin (hCG) injection, respectively (Table 3). Also, in the same table, injections of progesterone either with /or without hCG for 1^{st} and 2^{nd} weeks of gestation period were statistically ($P<0.01$) increased the litter size as compared to its control group of non- stimulated females. However, non significant differences were observed in the litter size in all groups of female mice were injected with hCG and /or progesterone throughout 1^{st} week and 1^{st} and 2^{nd} weeks of gestation period (Table 3).

Data are shown in Table (4) observed a significant ($P<0.01$) increased in the litter size of hCG group and progesterone group in the 1^{st} week of gestation period as compared to the control and estradiol combined with progesterone injection groups of superovulated females. Meanwhile in the same injection period of gestation, non significant differences were occurred between hCG and progesterone groups (Table 4). Significant ($P<0.01$) differences were assessed in the litter size between the control group and all groups of different hormonal supplementations for 1^{st} and 2^{nd} weeks of gestation period of superovulated females (Table 4). Also in the same injection period, the litter size of group injected with estradiol combined with progesterone was statistically ($P<0.01$) decreased as compared to groups of female mice were injected with progesterone alone or combined with hCG (Table 4).

The litter size was significantly ($P<0.01$) increased for groups of superovulated females injected for 1st week of gestation period with either progesterone or hCG as compared to non-superovulated females (Figure 3). Also, the litter size of superovulated females injected for 1st and 2nd weeks of gestation period with progesterone only or in combination with hCG was significantly ($P<0.01$) increased when compared to non-superovulated females (Figure 4). However, insignificant differences were noticed in the litter size of control groups of non-superovulated and superovulated females (Figures 3 and 4).

3- Endometrium thickness of uterine horn:

The endometrium thickness of non-superovulated females injected for either 1st week or 1st and 2nd weeks of gestation period with different hormonal supplements were significantly ($P<0.01$) enlarged as compared to the control group (Table 3). Same results were obtained for superovulated females (Table 4). After 1st week injection of gestation period, the endometrium thickness of superovulated females of all groups was statistically ($P<0.01$) enlarged as compared to non-stimulated females (Figure 5). Significant ($P<0.01$) increased in the endometrium thickness of superovulated females were recorded when injected for 1st and 2nd weeks of gestation period with different hormone supplements when compared to non-superovulated females. Meanwhile, insignificant differences were calculated among control groups of both non-superovulated and superovulated females (Figures 5 and 6).

Discussion

The results of the present study appeared that the periods of gestation for non-superovulated and superovulated females of control and hormone treated groups have insignificant difference (Tables 3 and 4). Two reasons may be explain this result, first all hormones were injected with low quantity dose and no obvious side effects, and second all females were injected for no longer two weeks, and don't continuous until gestation period ended. These results are an agreement with results were reported by Crosby *et al.* (26), and they indicated insignificant differences between hormone treated and the control groups. Furthermore, the periods of gestation of non-superovulated females have longer periods than the superovulated females, which support two hypotheses were mentioned above. It's known that the partially reduction of gestation period as a result of increased the litter size (27, 28), or hormones – treated females needs larger dose (14, 29).

Data are shown in the tables (3 and 4) and figures (3-6) appeared an improvement in the litter size and endometrium thickness of uterine horn when non-superovulated and superovulated females treated with different hormones as compared to the control group. These results were considered important for supporting early embryonic implantation and normal pregnancy outcome. Certainly, several reports were achieved to support pregnancy and prevention of abortion human and other mammals (6,8,9,29). Generally, females were treated with progesterone was proved the best results for litter size and endometrium thickness (Tables 3 and 4). These results may explain the essential role of progesterone throughout progression of gestation. Lalitkumar *et al.* (4) and Duffy *et al.* (13) they concluded that the progesterone prepares the lining of the uterus for early developed embryo and deciduas, as well as progesterone is required for ovulation, luteinization and the maintenance of luteal structure and function in primates. Therefore, each combination between progesterone and another hormone supplement involving human chorionic gonadotrophin (hCG) or estradiol has superior improvements of litter size and endometrium thickness. Also, these hormonal supplements have significant improvements as compared to the control group. It's known that the injection of progesterone during mid-luteal phase of females with luteal phase defect or polycystic ovarian syndrome enrolled in program of *in vitro* fertilization (IVF) to increase the rate of embryonic implantation and birth babies (30-32). Similarly, estradiol combined with progesterone or hCG may be inhanced results of pregnancy as documented by several researches (6,8,16,33,34).

Different hormonal supplementations for both non-superovulated and superovulated females caused an increased thickness of endometrium as compared to the control group (Tables 3 and 4). Segal and Casper (7) reported that the enlargement of endometrium thickness causes further stability for early implanted embryos and decreases early or habitual abortion (35). Therefore, best synchronization of litter size and enlargement endometrium thickness was combined with progesterone injection (Tables 3 and 4). Also, it's noticed that the period of

progesterone injection has a direct effect on increase thickness of endometrium and size of litter. Several investigators reported that the progesterone administration is related to several factors including: number of implanted embryos (17), age of female which received embryos (36,37), history and physiology of female health (38-40), psychology of females (41), and environmental changes (42-43).

Mostly, the thickness of endometrium of superovulated females was larger than non-superovulated females in hormone treated and control groups (Figures 5 and 6). Certainly, ovarian stimulation increases the number of growing ovarian follicles as compared to the non-stimulated ovaries (23). Consequently, production of both estrogen and progesterone from growing ovarian follicles were increased (15,29). Therefore, further enlargement in endometrial thickness of hormonal treated females (7). In conclusion, we suggest that the synchronization of ovarian stimulation and hormonal supplementation may be has good results to improve normal embryonic implantation rate and enhance pregnancy outcome. Further hormonal and histological studies are recommended to investigate the effects of different hormonal supplementations on endocrine and reproductive organs of pregnant female.

Table 1: drugs and chemicals were used in the present study

	Drugs or Chemicals	Company and source country
1.	Pregnant Mare Serum Gonadotrophin (PMSG)	Folligon 1000 I.U.; Intervet, Boxmeer, Holland.
2.	Chorionic gonadotrophin	Chorulon 1500 I.U.; Intervet, Boxmeer, Holland.
3.	Progesterone 25mg	Biologici Italia Lab., Novate, Milano, Italy.
4.	Human Chorionic Gonadotrophin	Profasi 5000 I.U.; Serono, Italy.
5.	Estradiol benzoate	5 mg / ml ; Intervet, Boxmeer, Holland.
6.	Methylene blue stain	BDH, England.
7.	Picric acid	BDH, England.
8.	Glacial acetic acid	BDH, England.
9.	Formaline (40%)	BDH, England.
10.	Haematoxyline stain	Fluka, Switzerland.
11.	Eosine stain	BDH, England.
12.	Paraffin wax	Merck, Germany.
13.	Xylol	Merck, Germany.
14.	Ethanol	Merck, Germany.

Table 2: Experimental design was applied in the present study

<table>
<tr><td colspan="8">One hundred and ninety six pregnant mice</td></tr>
<tr><td colspan="8">Group 1: Eighty four natural cycle (non-stimulated) females</td></tr>
<tr><td colspan="3">Injections throughout 1st week of gestation period</td><td colspan="5">Injections throughout 1st and 2nd weeks of gestation period</td></tr>
<tr><td>Control (normal saline injection)</td><td>hCG injection</td><td>Progesterone injection</td><td>Control (normal saline injection)</td><td colspan="2">hCG + progesterone injection</td><td>Progesterone injection</td></tr>
<tr><td>0.1 ml of 0.09 % NaCl</td><td>3 I.U.</td><td>0.07 mg</td><td>0.1 ml of 0.09 % NaCl</td><td colspan="2">3 I.U. + 0.07 mg; respectively</td><td>0.07 mg</td></tr>
<tr><td colspan="8">Group 2: One hundred and twelve superovulated cycle (stimulated) females</td></tr>
<tr><td colspan="4">Injections throughout 1st week of gestation period</td><td colspan="4">Injections throughout 1st and 2nd weeks of gestation period</td></tr>
<tr><td>Control (normal saline injection)</td><td>hCG injection</td><td>Progesterone injection</td><td>Estradiol benzoate + Progesterone injection</td><td>Control (normal saline injection)</td><td>hCG + progesterone injection</td><td>Progesterone injection</td><td>Estradiol benzoate + Progesterone injection</td></tr>
<tr><td>0.1 ml of 0.09 % NaCl</td><td>3 I.U.</td><td>0.07 mg</td><td>0.035 mg + 0.07 mg; mixture</td><td>0.1 ml of 0.09 % NaCl</td><td>3 I.U. + 0.07 mg; respectively</td><td>0.07 mg</td><td>0.035 mg + 0.07 mg; mixture</td></tr>
<tr><td colspan="8">Each group consist of fourteen pregnant females were divided into eight females for study of gestation period and litter size, and other six females for histological studies</td></tr>
</table>

Table 3: The effect of different hormonal supplementations on gestation period, litter size and endometrium thickness of uterine horn of non-superovulated mice

Parameters	Types of injections during gestation period					
	First week			First and second weeks		
	Control	hCG injection	Progesterone injection	Control	hCG + progesterone injection	Progesterone injection
Gestation period (days)	20.875 ± 0.125	21.0 ± 0.189	21.0 ± 0.267	20.875 ± 0.226	21.0 ± 0.267	21.125 ± 0.295
Litter zise	5.0 ± 0.267	5.875 ± 0.350	6.125 * ± 0.440	4.875 ± 0.350	6.125 * + 0.295	6.625 * ± 0.460
Endometrium thickness (µm)	252.5 ± 6. 748	420.0 * ± 16.90	460.0 * ± 15.118	242.5 ± 6.196	462.5 * + 22.815	488.75 * ± 14.322

*: Significant (P<0.05) difference as compared to its control group.

Table 4: The effect of different hormonal supplementations on gestation period, litter size and endometrium thickness of uterine horn of superovulated mice

Parameters	Types of injections during gestation period							
	First week				First and second weeks			
	Control	hCG injection	Progesterone injection	Estradiol + Progesterone injection	Control	Progesterone injection	hCG + Progesterone injection	Estradiol + Progesterone injection
Gestation period (days)	20.0 ± 0.267	20.125 ± 0.295	20.375 ± 0.323	20.375 ± 0.375	20.125 ± 0.226	21.0 ± 0.267	20.50 ± 0.327	20.125 ± 0.350
Litter zise	5.125 ± 0.350	7.875 * ± 0.479	8.125 * ± 0.610	5.250 ± 0.250	4.875 ± 0.313	9.375 * ± 0.625	8.875 * ± 0.440	5.750 *# ± 0.366
Endometrium thickness (µm)	365.0 ± 14.51	642.5 * ± 31.94	778.75 * ± 8.951	458.75 # ± 8.75	272.5 ± 11.76	1597.5 * ± 16.229	1471.25 * ± 26.822	1096.25 *# ± 34.27

*: Significant (P<0.05) difference as compared to its control group.
#: Significant (P<0.01) differences as compared to progesterone and/or hCG groups.

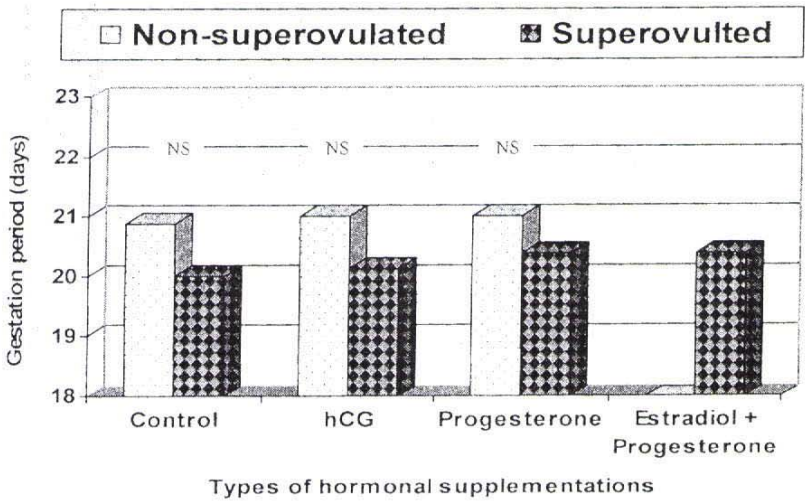

Figure 1: The effect of different hormonal supplementations during first week on gestation period in non-superovulated and superovulated mice

NS: Non significant differences as compared to corresponding group.

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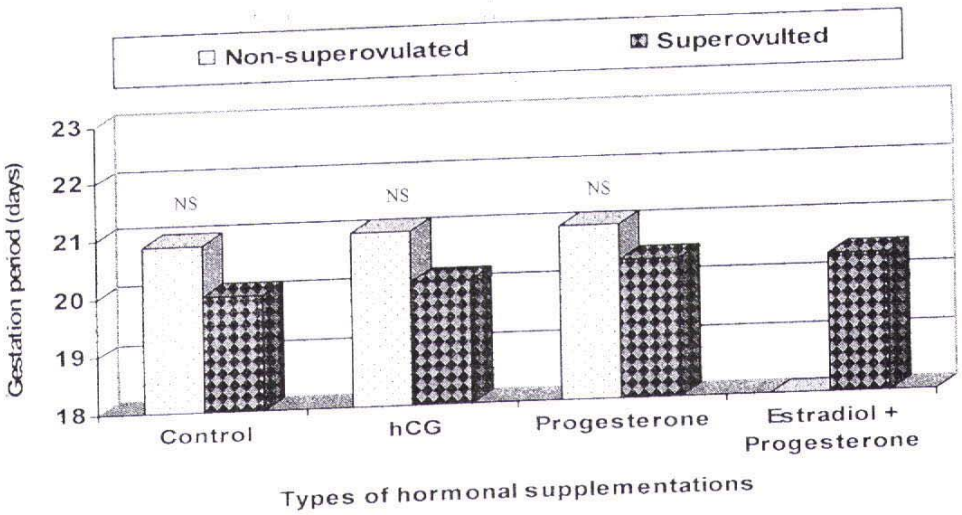

Figure 2: The effect of different hormonal supplementations during first and second weeks on gestation period in non-superovulated and superovulated mice

NS: Non significant differences as compared to corresponding group.

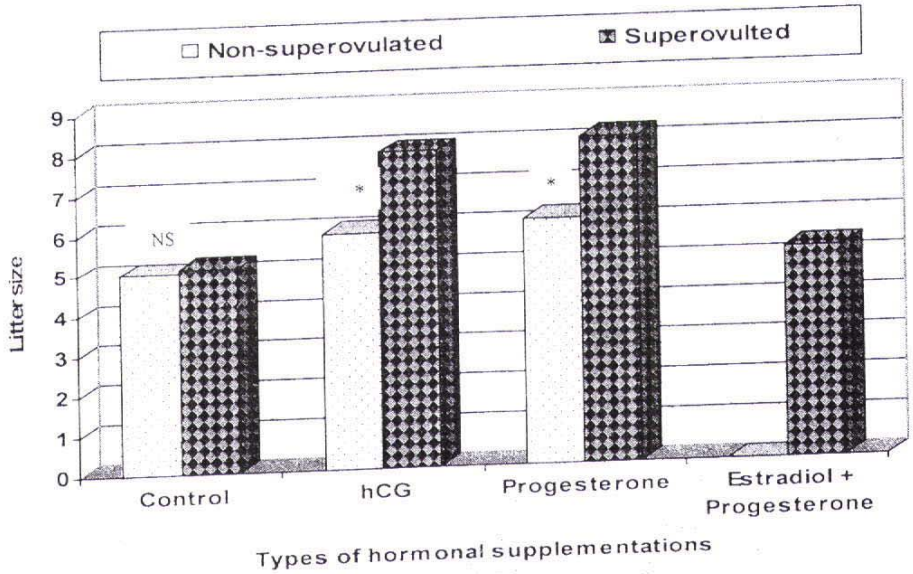

Figure 3: The effect of different hormonal supplementations during first week on litter size in non-superovulated and superovulated mice

NS: Non significant differences as compared to corresponding group.

* : Significant ($P<0.01$) differences as compared to corresponding group.

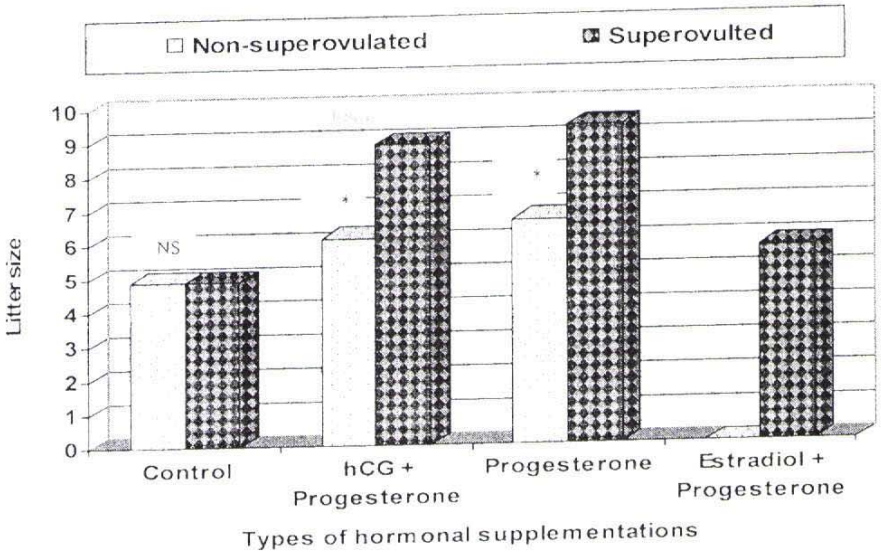

Figure 4: The effect of different hormonal supplementations during first and second weeks on litter size in non-superovulated and superovulated mice

NS: Non significant differences as compared to corresponding group.
* : Significant (P<0.01) differences as compared to corresponding group.

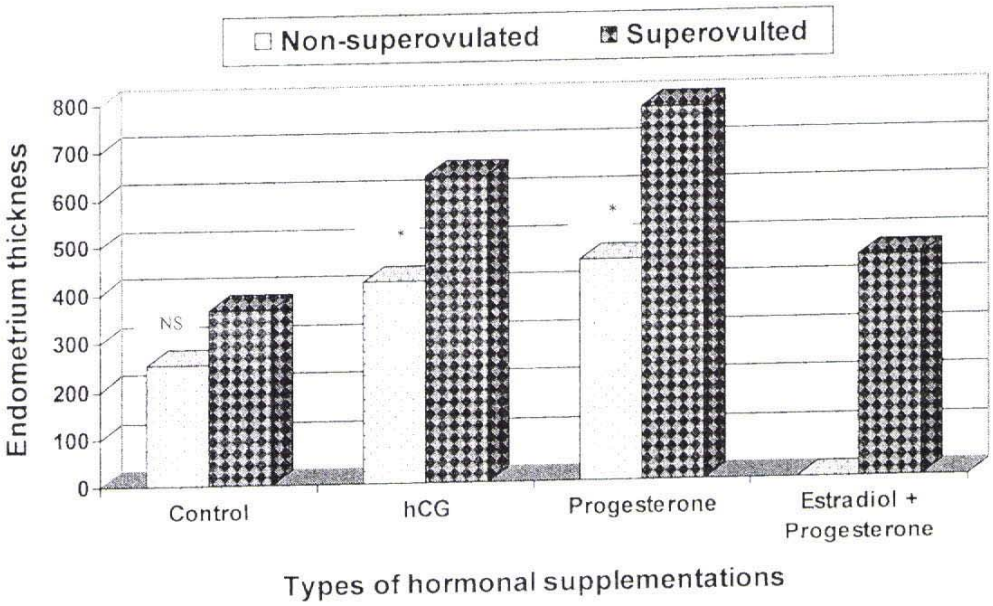

Figure 5: The effect of different hormonal supplementations during first week on endometrium thickness (μm) in non-superovulated and superovulated mice

NS: Non significant differences as compared to corresponding group.
* : Significant (P<0.01) differences as compared to corresponding group.

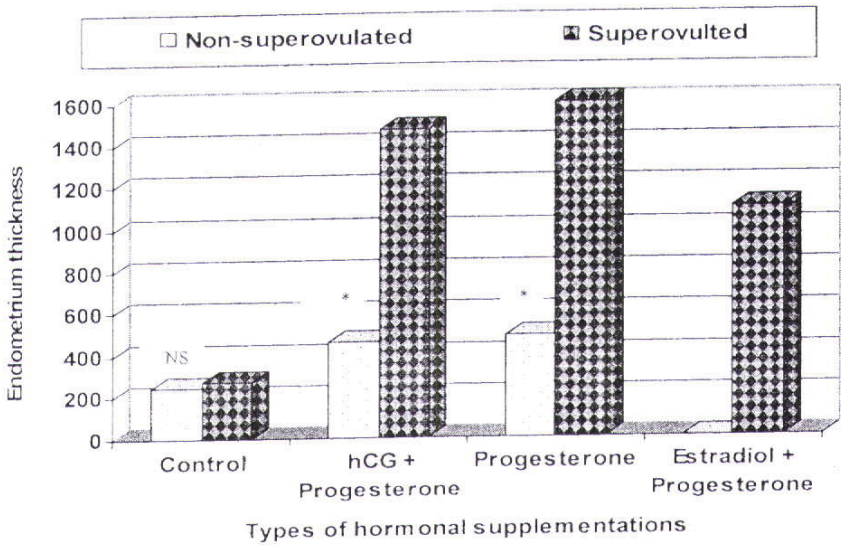

Figure 6: The effect of different hormonal supplementations during first and second weeks on endometrium thickness (□m) in non-superovulated and superovulated mice

NS: Non significant differences as compared to corresponding group.
* : Significant ($P<0.01$) differences as compared to corresponding group.

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