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**SUPEROVULATORY RESPONSE TO eCG
INJECTIONS AND PLASMA PROGESTERONE
LEVEL IN RELATION TO OVARIAN RESPONSE
AND EMBRYO RECOVERY IN RABBITS**
(With 2 Tables and 2 Figures)

By

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(Received at 29/5/2001)

استجابة آثار الإباضة للحقن بالهرمون المشيمي الخيلي وعلاقة مستوى
البروجيستيرون في البلازما باستجابة المبيض وعدد الاجنة المستردة
في الارانب

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أستخدمت في هذه الدراسة عدد ٣٠ من أنثى أرانب الكاليفورنيا والتي كان عمر كل منها حوالي عام. تم تقسيم هذه الإناث إلى ثلاث مجموعات متساوية في العدد (١٠ أنثى في كل مجموعة). تم حقن أنثى المجموعة الأولى بمحلول الملح الفسيولوجي وأستخدمت كمجموعة ضابطة ، بينما حقنت أنثى كل من المجموعة الثانية والثالثة (المجموعة المعاملة) ب ٦٠ وحدة دولية من الهرمون المشيمي الخيلي لمدة ثلاث أيام (٢٠ وحدة دولية يوميا) أو اربعة أيام (١٥ وحدة دولية يوميا) على التوالي. وبعد ٢٤ ساعة من اخر حقنة [سواء بمحلول الملح الفسيولوجي أو بالهرمون المشيمي الخيلي] لقحت جميع الإناث باستخدام ذكور مختبرة تناسليا ثم حقنت بعد التلقيح مباشرة ب ٨ ميكروجرام من البيزرلين (الهرمون الحث للفصدة النخامية). بعد حوالي ٧٢ ساعة من التلقيح تم ذبح عدد ٤ أنثى من كل مجموعة لفحص الجهاز التناسلي (المبيض وعضول الرحم) لكل أنثى على حده لمعرفة استجابة المبيض للحقن (عدد الاجسام الصفراء - الجريبات الدموية - معدل التبويض) وعدد الاجنة المستردة مسن عضول أرحام هذه الإناث. تم جمع عينات من كل الإناث المستخدمة في هذه الدراسة في يوم التلقيح لتقدير مستوى هرمون الاستروجين وكذلك في اليوم الثالث والعاشر بعد التلقيح لتقدير مستوى هرمون البروجستيرون . وقد أظهرت نتائج هذه الدراسة أن الاستجابة الكلية للمبيض (عدد الاجسام الصفراء - الجريبات الدموية) كانت عالية معنويا ($P \leq 0.05$) في أنثى المجموعة المعالجة بالهرمون المشيمي الخيلي (نظام ٣ أيام) عن أنثى المجموعة الضابطة. وكذلك كان معدل التبويض أعلى في الإناث التي حقنت لمدة ٣ أيام ($50.43 \pm$) والتي حقنت لمدة ٤ أيام (57.78 ± 4.96) عن المجموعة الضابطة ($38.98 \pm$)

٤,٤٧). وأن نسبة عدد الأجنة المستردة في غسول أرحام أناث المجموعة المعالجة أعلى من المتحصل عليه من أناث المجموعة الضابطة. كذلك لوحظ تحسن الاستجابة الكلية للمبيض وزيادة عدد الأجنة المستردة في غسول الأرحام باستخدام نظام الحقن لمدة ٤ أيام بالهرمون المشيمي الخيلي لاثارة الإباضة عن نظام الحقن لمدة ٣ أيام. لوحظ أيضا أن هناك علاقة ارتباط موجبه بين مستوى هرمون البروجسترون والاستجابة الكلية للمبيض ($r = 0.85$) و معدل التويوض ($r = 0.82$) و عدد الأجسام الصفراء ($r = 0.77$) و عدد الأجنة المستردة من غسول الرحم ($r = 0.85$). و قد دلت هذه الدراسة أن نظام اثارة الإباضة بواسطة الحقن بالهرمون المشيمي الخيلي لمدة ٤ أيام قد عزز الكفاءة التناسلية للارانب وأنسه من الممكن توقع الاستجابة المبيضة بعد اثارة المبيض باستخدام هذا الهرمون.

SUMMARY

Thirty non-lactating Californian rabbit does aged about one year old, were randomly allocated into equal three groups ($n = 10$). The does in the first group received an injection of physiological saline (0.9% NaCl) and served as control. However, the does in the treated groups were injected daily by 20 I.U. of eCG for three days (second group) or 15 I.U. of eCG for four days (third group). Twenty four hours after the administration of saline or eCG injections, the does were exposed to fertile males for mating and immediately thereafter were injected with 0.8 μ g of bescerelin acetate. At about 72 hours post-mating, four does from each group were sacrificed and their reproductive organs were assessed for:- total ovarian response (TOR), number of CL (NOCL), number of bloody follicles (BFs), ovulation rate (OR) and number of embryo recovery (NOER). Blood samples were collected from all does for determination the estrogen (E_2) level on the day of mating and on day 3 post-mating (slaughtered or alive does) and day 10 post-mating (alive does) for determination the progesterone (P_4). The results of this study indicated that the ovaries of treated does (3 d. or 4 d. regimens) had significantly ($P \leq 0.05$) higher numbers of CL and BFs (TOR) than did those of the control group. The OR/ doe for 3 d. regimen (50.43 ± 2.23) and for 4 d. regimen (57.78 ± 4.96) were significantly ($P \leq 0.05$) higher than for control group (38.98 ± 4.47). Moreover, higher number of embryos were recovered from does treated with eCG (3 d. or 4 d. regimens) than control ones. In addition, 4 d. eCG superovulated regimen improved ovarian response and gave higher number of recovered embryos when compared with 3 d. regimen. A significant ($P \leq 0.05$) positive correlation was found between plasma P_4 level and TOR ($r = 0.85$), OR ($r = 0.82$), NOCL ($r = 0.77$) and NOER ($r = 0.85$). The

present study showed that 4 d. eCG superovulated regimen enhances the efficiency of rabbit reproduction, and the possibility to predict the ovarian response after superovulation with specific hormonal regimen in rabbit does.

Key words: *Superovulation, eCG, Rabbit, Embryo, CL, Bloody Follicles.*

INTRODUCTION

Several reproductive technologies are already available, or under development which can accelerate the progress in genetic improvement programs in mammals (Simm, 1998). These include artificial insemination (AI), superovulation (SO), embryo transfer (ET), sexing of spermatozoa or embryos and cloning (Gordon and Lu, 1990; Castellini, 1996; Nicholas, 1996). The SO is an important technique in basic and applied study of mammalian reproductive physiology (Thibault, 1985).

Over the last few decades a great deal of effort has gone into developing a reliable procedures for SO (Schmidt *et al.*, 1992; Kanayama *et al.*, 1995; Kauffman *et al.*, 1998 and Cheng *et al.*, 1999). Although, SO of rabbits with a single (Kobayashi *et al.*, 1990; Schmidt *et al.*, 1992) or multiple (Kennelly and Foote, 1965; Gravance 1994; Kauffman *et al.*, 1998) injections of FSH or Equine Choronic Gonadotrophin (eCG) are commonly used to synchronize estrus and to increase the number of embryos for various reproductive and embryo manipulation studies. The response in terms of ovulation rate (OR) is highly variable and the percentage of embryo recovered based on CL was lower (Kim *et al.*, 1988; Carney and Foote, 1990; Taneja *et al.*, 1990; Schmidt *et al.*, 1992; Cheng *et al.*, 1999) for superovulated donors, therefore, it might reduce the benefits or efficiency of SO regimens. Both subtle and marked morphological aberrations (Carney and Foote, 1990) and chromosomal abnormalities (Fujimoto *et al.*, 1974) have been reported in embryos recovered from superovulated rabbits treated with FSH or eCG.

The ability to predict the results of SO prior to embryo collection is of great importance (Saumande *et al.*, 1985; Mehmood *et al.*, 1991), especially in rabbits where surgical or laparoscopic embryo collection methods are used (Garcia- Ximenez *et al.*, 1991). The measurement of Progesterone (P₄) level after SO as a prognostic tool for predication of OR and embryo yield have been met with limited success, although, many authors have found a relationship between ovarian response

(number of CL) and blood P_4 levels after SO in different species. The success to predict the exact number of corpora lutea, ova or viable embryos is limited (Greve *et al.*, 1983; Saumande *et al.*, 1985; Wubishet *et al.*, 1991; Petr *et al.*, 1992). Most of the work on this subject has been carried out in ruminants (Greve *et al.*, 1983; Mehmood *et al.*, 1991; Petr *et al.*, 1992), while limited data are available for rabbits (Polidoro and Black, 1970; Thau and Lanman, 1976). The ovarian follicle is the main source of estradiol-17 β (E_2) secretion, which is LH-dependent (Mills *et al.*, 1971) and the reduction in LH level was responsible for the reduction in estradiol-17 β before mating.

The objectives of the present study were to determine: 1) the effect of daily injections of eCG for 3 or 4 days on ovarian activity. 2) The relationship between plasma P_4 level on day of embryo collection and ovarian response as well as number of embryos collected from superovulated Californian rabbit does.

MATERIAL and METHODS

The present study was conducted at Rabbit Research Farm, Dept. of Animal and Poultry Production, Assiut University, Assiut, Egypt, from November 1999 to January 2000.

Animals and Management:

A total number of 30 non-lactating Californian rabbit does (aged about one year old, average live weight of 3.5 Kg and apparently healthy) were used in the present study. The rabbits were housed individually in galvanized wire cages in well ventilated building. Feed and water were available ad libitum. The animals were fed standard diet containing 16.3% crude protein and 2284.7 Kcal/kg digestible energy. All rabbits were raised under the same managerial, hygienic and environmental conditions. Mating was carried naturally and each doe was transferred to the buck's cage to be mated twice (in the same day) and returned back to its cage after mating. Mating was carried out using fertile bucks. Abdominal palpation of does were carried out 10 days post-mating to determine the pregnancy.

Experimental procedures:

The rabbits were randomly assigned into three equal groups. In the first group, the does ($n = 10$) were injected with saline solution (0.9% NaCl) and served as control. However, in the second and third groups (treated groups), the SO was induced by using eCG (Folligon, Intervet

International B.V. Boxmeer-Netherlands). The eCG was injected subcutaneously in the back of the rabbit's neck. The total dose of eCG (60 I.U.) was given either in three (20 I.U. each) or four (15 I.U. each) successive injections, at 24 hours interval to the second and third groups respectively. Twenty four hours after last injection, all does (either control or eCG treated groups) were transferred to the males' cages for mating and that day was defined as day 0. Immediately, after mating, all does were injected i.m. with 0.8 µg of bescerelin acetate (GnRH analogue, Receptal, Hoechst) either receptive or non receptive (forced mating).

At about 72 hours from GnRH injection, 12 does (4 from each group) were slaughtered and the genital organs were immediately excised for examination. The ovaries were washed by normal saline and extraneous tissue was excised. The number of induced corpora lutea (NOCL, post-mating and GnRH injection), the number of bloody follicles (unovulated follicles, BFs) on both ovaries and total ovarian response (number of CL and Bfs, TOR) were counted. The ovulation rate (OR) was defined as the percent of CL per animal (in relation to TOR) was also determined.

Each reproductive tract anterior to the cervix was removed in two parts and each part was straightened carefully (Brackett and Williams, 1965). Flushing of each horn was carried by a 10 ml syringe with a 24g needle attached and contained 10 ml of saline solution (0.9% NaCl w/v). The flushings of both horns of each tract were mixed and examined. The recovered embryos were counted (NOER) and the recovery rate (NOER/NOCL) was calculated.

To evaluate the E₂ and progesterone P₄ levels associated with SO protocols, blood samples (~ 2 ml) were taken: 1) Pre-mating from all does (slaughtered and alive) for measuring E₂ levels. 2) Post-mating on day 3 from all does (slaughtered and alive) and day 10 from only alive does for measuring P₄ levels. Blood was collected into heparinized vacutainer tube by venipuncture from the marginal ear vein of each doe. Plasma was separated by centrifugation and stored at - 20 °C until hormonal assay. Plasma E₂ and P₄ level were determined by using Coat-A-Count tube RIA Kits provided by Diagnostic Products Corporation (DPC) Los Angeles, USA.

The following data were recorded for all does: interval from last kindling (days), type of treatment (control or treated), mode of administration of eCG (3 or 4 days injections), number of does copulated, receptivity at copulation, number of pregnant does (at

kindling) and litter size. Moreover, for slaughtered does, the number of CL and BFs on both ovaries and number of recovered embryos were recorded.

Statistical Analysis:

The effect of treatment on superovulatory response, pregnancy rate and litter size were analyzed by analysis of variance (ANOVA) using SAS procedures (SAS, 1985). Differences between means were compared by Duncan's New Multiple Range Test. The conception rate was analyzed by using Chi-square. The data of superovulatory response [TOR, NOCL, OR, NOER and plasma P₄ level on day of embryo collection (PPLFC)] from slaughtered does were subjected to the appropriate transformations. This allowed the transformed variables to approximate more closely to a normal distribution and hence to stabilize the variance between groups. Regression and correlation coefficient were used to investigate the relationships between these parameters.

RESULTS

The results of the present study indicated that the administration of eCG had a significant ($P \leq 0.05$) effect on ovarian responses (TOR, NOCL, OR, recovery rate and plasma E₂ and P₄ level) and it appeared that this effect was regimen dependent (Table 1). Hormonally stimulated does (3 d. and 4 d. regimens) produced significantly ($P \leq 0.05$) more BFs and CLs (TOR) upon visual inspection than control as shown in Fig. 1. The mean (\pm S.E.) number of CL / doe was 13.00 ± 1.22 for 3 d. regimen, 19.50 ± 2.29 for 4 d. regimen and 5.75 ± 2.38 for control group. The OR/doe for 3 d. regimen (50.43 ± 2.23) and, for 4 d. regimen (57.78 ± 4.96) were significantly ($P \leq 0.05$) higher than OR for control group (38.98 ± 4.47). The mean (\pm S.E.) NOER for 3 d. regimen (6.75 ± 1.39) and 4 d. regimen (10.25 ± 0.83) per doe was significantly greater ($P \leq 0.05$) than the mean NOER from controls (3.33 ± 1.70). However, the mean NOCL, TOR, NOER/doe from 3 d. regimen were significantly ($P \leq 0.05$) lower than that of 4 d. regimen. The mean number of BFs, OR and recovery rate/ doe treated were not affected by type of eCG regimen (Table 1).

Plasma E₂ and P₄ levels were affected by eCG treatment. However, there was a significant ($P \leq 0.05$) effect of the eCG regimen on the E₂ level at day of mating and P₄ at day 3 post-mating (Table 1). The

plasma level of P_4 at day 10 post-mating was not affected by eCG regimen in the treated groups (Tables 1 and 2).

The linear regression revealed a significant correlations between plasma P_4 level on day of embryo collection and TOR ($r = 0.85$, Fig. 2-A); OR ($r = 0.82$, Fig. 2-B); NOCL ($r = 0.77$, Fig. 2-C) and NOER ($r = 0.85$, Fig. 2-D). The effect of various eCG regimens on conception rates are present in Table 2. In general, the overall conception rate of eCG treated does for 3 or 4 successive days increased by about 33.3% and 50.0% when compared with untreated does (control). This differences was only significant ($P \leq 0.05$) between 4 d. regimen and control ones.

DISCUSSION

This study revealed that administration of eCG supplemented with GnRH at day of mating significantly affect the ovarian activity in rabbit does. Moreover, these eCG regimens yielded significantly more recovered embryo per doe than control one. These results confirm and extend on the previous reports concerning the effect of the standard 3 d. eCG treatment (Carney and Foote, 1990; Taneja *et al.*, 1990; Schmidt *et al.*, 1992; Kauffman *et al.*, 1998) on number of ovulation and embryo recovery.

Although, modifying the standard 3 d. FSH procedure are commonly used for rabbit superovulation (Kauffman *et al.*, 1998). There are few reports that investigated the effects of longer or shorter eCG injections (Taneja *et al.*, 1990; Schmidt *et al.*, 1992; Gravance, 1994). Programming follicular development by injection of gonadotrophins (e.g. FSH or eCG) provide basis for development of an induced and synchronized estrous as well as SO protocol in the rabbit. In the present study, irrespective to eCG regimen (3 or 4 days), the obtained results indicated that TOR and OR/ doe were higher when compared with control. This may be due to the direct effect of eCG treatment on ovarian activity (follicular dynamic, quality of follicles and it's ability to ovulation). Bonanno *et al.* (1990); Schmidt *et al.* (1992) and Castellini (1996) reported that the main action of eCG is promoting follicular growth by increasing number of preovulatory follicules with raising of the estrogen released by these follicles. A similar finding was observed in the present study that a significant increase in E_2 levels in eCG treated does than control ones. This increase in E_2 was responsible for that most of treated does showed receptivity to male at time of mating. Theau-

Clement and Roustan, (1992) and Kermabon *et al.* (1994) reported that receptive does have higher number of preovulatory follicles (estrogen secreting follicles) and ovulation frequency. This could explain the increase NOCL in eCG treated does than control ones. The large number of BFs seen on the ovaries of eCG treated does in the present study is similar to the findings of Stradaoli *et al.* (1993); Gosalres *et al.* (1994) and Kanayama *et al.* (1995). The presence of BFs might suggest excessive ovarian stimulation except that small numbers BFs were also found in the control group where ovulation had occurred naturally. Hence, BFs development is a naturally occurring phenomena in the rabbit. However, the biological basis for the formation of BFs is not known. Yun *et al.* (1989) attributed previous ovulations in the superovulated rats to the LH activity of eCG. The LH activity of GnRH administered concurrently with eCG in these protocols could possibly explain the increased number of BFs in treated animals but not those occurring in the control does. Since follicles are continuously in various stages of development in an induced ovulator such as the rabbit, the LH activity of eCG could in fact luteinize follicles that are not prepared to ovulate, resulting in the formation of BFs.

There was a significant increase in NOER from 4 d. regimen than either the 3 d. regimen or control does. This is in agreement with the recent findings of Kauffman *et al.* (1998) who reported a higher recovery rate of embryo from 4 d. regimen than either 3 d. regimen or untreated control does. Moreover, the later authors estimated the efficiency of producing kits based on normal morulae collected from control and 4 d. regimen donors and reported that nearly two folds higher than the donor treated by 3 d. regimen. This indicated that an additional day of eCG treatment does not adversely affect but on the other hand even enhance the efficiency of ovulation by increasing of NOCL and embryo production compared with previous studies using standard treatment schedule by FSH or eCG (Kennelly and Foote, 1965; Carney and Foote, 1990; Vicente and Garcia-Ximenez, 1991 and Kaufmann *et al.*, 1998). Therefore, an extra day of eCG treatment may lead to recruitment of additional small healthy follicles into more active growth phase.

The ability to predict the superovulatory response of rabbit does to a given hormonal treatment is important for deciding the efficiency of superovulation protocol and for embryo manipulation. One of the methods that have been tested for the predication of superovulatory response is the determination of plasma P₄ concentration prior collection.

In agreement with other studies on rabbits (Lau *et al.*, 1982; Stoufflet and Caillol, 1988; Lamb *et al.*, 1991), the results of this study demonstrated a progressive increase in plasma P₄ concentration after superovulation with type of eCG regimen, parallel to an increase in TOR and OR. The CL represents the only source of P₄ during gestation in rabbits. The significant positive correlation between PPCFC and NOCL in our study is in agreement with results of previous work in rabbits (Lamb *et al.*, 1991; Samartzui *et al.*, 1995). The later authors demonstrated that it is possible roughly to predict the number of CL on the ovine ovaries induced to superovulation with specific hormonal regimen. Although, Polidoro and Black (1970) could not show a correlation between numbers of CL in the ovary and P₄ secretion.

To the best of our knowledge, the relationship between P₄ concentration after SO and the NOER has not been studied thoroughly in rabbit. Therefore, the results of regression in the present study were comparable with results reported by Samartzui *et al.* (1995) in sheep. The later authors found a positive correlation between plasma P₄ concentration and number of embryo recovered per treated ewe with eCG. The differences in correlation coefficients reported in the different studies could be associated with differences in the P₄ assays (RIA vs. ELISA) used (MacDonaell *et al.*, 1988) and / or species differences between ovine and rabbits, since such differences exists even between rabbit breed (Soliman *et al.*, 1997).

The obtained results concluded that 1) The 4 d. eCG superovulated regimen enhances the efficiency of rabbit reproduction. 2) The possibility to predict the ovarian response after superovulation with specific hormonal regimen in rabbit does.

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Table (1): Superovulatory response in Californian rabbit does¹ (X ± S.E.)

	Untreated group (Control)	Treated group (eCG)	
		3 days	4 days
No. of does	4	4	4
Mating response (%) ²	2 (50%)	4 (100%)	4 (100%)
No. of animals responded (%) ³	3 (75%)	4 (100%)	4 (100%)
No. of bloody follicles / doe	8.50 ± 2.06 ^b	12.75 ± 0.83 ^a	14.25 ± 1.92 ^a
No. of CL /doe	5.75 ± 2.38 ^c	13.00 ± 1.22 ^b	19.50 ± 2.29 ^a
Total ovarian response /doe	14.25 ± 4.44 ^c	25.75 ± 1.92 ^b	33.75 ± 2.38 ^a
Ovulation rate (%) /doe ⁴	38.98 ± 4.47 ^b	50.43 ± 2.23 ^a	57.78 ± 4.96 ^a
No. of embryo recovery /doe	3.33 ± 1.70 ^c	6.75 ± 1.39 ^b	10.25 ± 0.83 ^a
Recovery rate/doe ⁵	34.40 ± 23.60 ^a	51.60 ± 7.10 ^a	52.80 ± 3.06 ^a
E ₂ conc. at day of mating	16.98 ± 3.10 ^c	29.83 ± 1.34 ^b	36.33 ± 1.70 ^a
P ₄ conc. at day 3 post-mating ⁶	3.21 ± 0.70 ^c	5.28 ± 0.53 ^b	8.52 ± 0.66 ^a

1) Does slaughtered 70 h. post-mating. 2) % of does mated naturally
 3) % Does having embryo recovered after flushing. 4) No. of CL / total ovarian response.
 5) No. of embryo recovered / NOCL. 6) Day of embryo collection or day of slaughter.
 a,b,c: Mean in the same row with different superscripts are significantly different (P<0.05).

Table (2): Effect of eCG treatment on estrogen (E₂) and progesterone (P₄) levels, pregnancy rate and litter size in Californian rabbit does¹.

	Untreated group (Control)	Treated group (eCG)	
		3 days	4 days
No. of does	6	6	6
Mating response (%) ²	4 (66.7%) ^a	4 (66.7%) ^a	6 (100%) ^a
E ₂ at day of mating	19.07 ± 2.14 ^a	29.60 ± 2.08 ^b	35.88 ± 1.86 ^c
P ₄ -Conc. at day 3 post-mating	2.77 ± 0.69 ^a	5.24 ± 0.55 ^b	7.22 ± 0.69 ^c
P ₄ -Conc. at day 10 post-mating	3.47 ± 0.72 ^a	6.44 ± 0.48 ^b	7.58 ± 0.88 ^b
No. of does conceived ³	3 (50.0%) ^b	5 (83.3%) ^{a,b}	6 (100%) ^a

1) Does not slaughtered 70 h. post-mating (alive does).
 2) % of does mated naturally. 3) Does diagnosed pregnant at 10 days post-mating.
 a,b,c: Means in same row with different superscripts are significantly different (P<0.05).

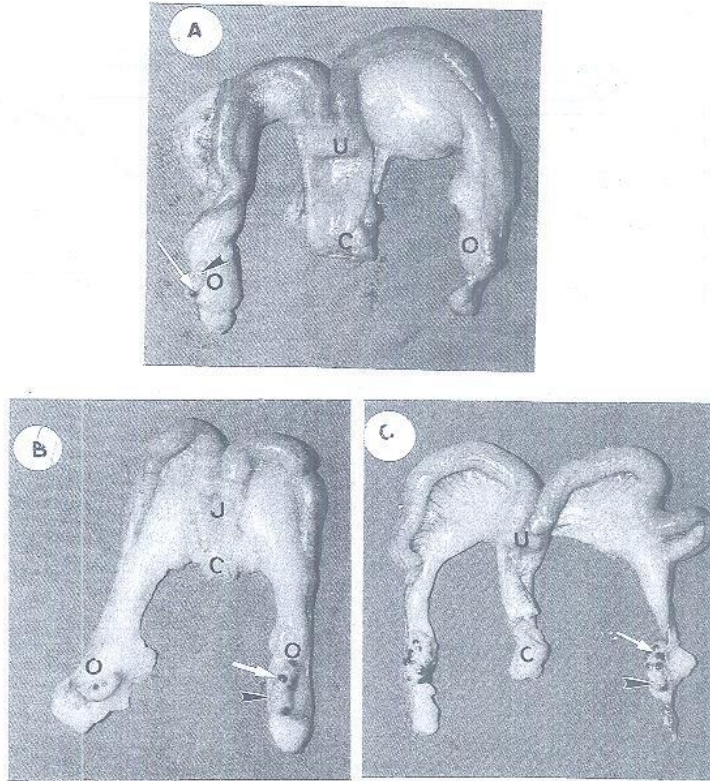


Fig.(1 A-C): Reproductive organs of rabbit does showing the ovarian response to the eCG injection.
A: Control B) 3 d. regimen C) 4 d. regimen
(U) uterus; (C) cervix; (O) ovary; (arrow →) bloody follicle;
(arrow head →) CL

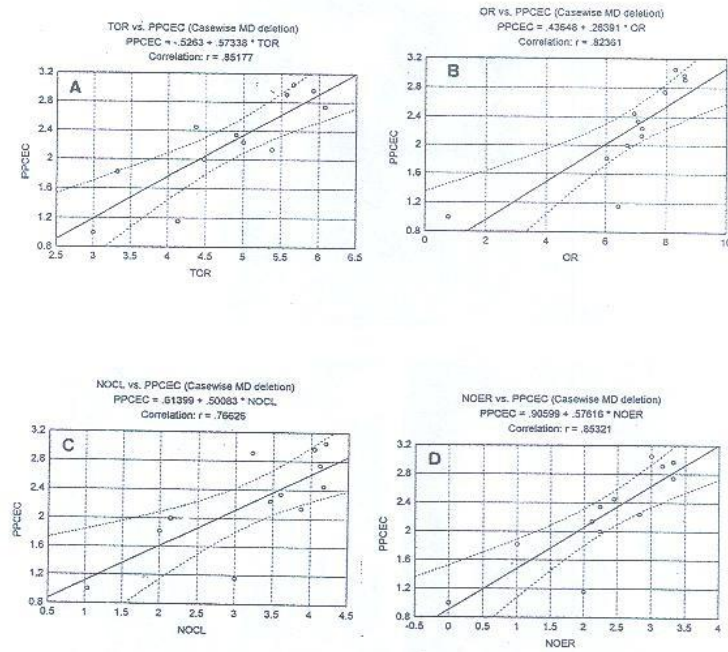


Fig. (2): Regression of plasma P₄ level on day of embryo collection (PPLEC) by:
A) Total ovarian response (TOR). B) Ovulation rate (OR).
C) Number of CL (NOCL). D) Number of embryo recover (NOER)