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IN VITRO AND *IN VIVO* OVULATORY RESPONSE OF RABBIT DOES TREATED WITH GnRH OR PMSG.

Mehrez, A.Z.[1]; M.A. Moustafa[1]; Sh.M. Shamiah[2] and Noha F. Abd El Raouf[1]

[1]Anim. Prod. Dept., Fac. Agric., Mansoura University.

2 Anim. Prod. Res. Inst., Ministry of Agric.

ABSTRACT

Twenty four New Zealand White (NZW) rabbit does (5-7 mo of age, 2.5-4.6 kg LBW and 1-2 parities) were used to study the effect of induced ovulation by GnRH or PMSG on ovarian characteristics, recovery and measurements of embryos at different stages. Also, 3 fertile NZW bucks were used for natural mating. Rabbit does were divided into 3 groups, (8 does in each). Does in the first group (G1) were injected (i.m.) with 0.2 ml saline solution/doe immediately before mating (control). Does in G2 and G3 were i.m. injected with 35 IU PMSG/doe 48 h before mating and 0.2 ml GnRH/doe at mating, respectively. Ovarian and genital characteristics including weight, length and width of the ovaries, oviduct and uterine horn were determined on the right and left side. Embryos were recovered by flushing from each treated doe slaughtered after 72 h of mating for collection of embryos. Embryos were recovered from each uterine horn and oviduct per doe. Results showed that all ovarian characteristics were not affected by treatment, ovarian side and their interaction. Ovulatory response in terms of average number of bleeding and total follicles, corpora lutea (CLs) not significantly affected by treatment. Rabbit does in G3 significantly ($P<0.05$) produced the greatest number of embryos/doe and the highest blastocyst rate *in vitro*, and the highest kindling rate, litter size and viability rate of bunnies.

In conclusion, treatment of NZW doe rabbits with 0.2 ml GnRH/doe (Receptal) at mating may be efficient in *in vivo* embryo production for embryo transfer and also needed for increasing *in vivo* fertility of naturally mated rabbit does.

Keywords: Rabbit, PMSG, GnRH, ovarian characteristics, litter size, *in vivo* fertility.

INTRODUCTION

Major advances in biology research are often achieved through animal experimental models. Rabbits are frequently used for studies in reproductive biotechnologies and genetic modeling, but embryo yield and quality after superovulation protocols are highly variable. Superovulation is normally applied to produce the maximum number of transferable embryos per donor female, and it may significantly affect the efficiency of embryo recovery.

In rabbits, ovulation is a neuro-endocrine reflex that is physiologically induced at the condition of natural breeding. The mechanisms whereby rabbit does become spontaneous ovulators are still unclear, but are likely associated to factors interfering with the control of the gonadal axis involving the hypothalamic centers responsible for GnRH release (Rauw *et al*., 1998). Obtaining successful fertility results in lactating rabbit does inseminated in semi-intensive rhythms requires ovarian stimulation, due to the inhibitory effect of lactation on sexual receptivity (Ubilla and Rebollar, 1995). It has been reported that bio-stimulation induces endocrine changes by stimulating hypothalamus–pituitary–ovary axis activity (Ubilla *et al*., 2000 a&b).

The application of some of the exogenous hormonal substances may cause fertility disorders in does. Therefore, various hormonal treatments were used for induction of ovulation in rabbits. Gonadotrophins such as Equine chorionic gonadotrophin (eCG, Maertens *et al*., 1995), human chorionic gonadotrophin (hCG) and lutinizing hormone (LH) preparations (Foote *et al*., 1963) are used as ovulation-inducing methods. Rabbits are usually injected with eCG to improve receptivity and to increase follicle growth as well as ovulation rate (Maertens *et al*., 1995), but it is known to reduce fertility due to immunogenicity if used routinely (Rebollar *et al*., 2006). This problem is eliminated using gonadotrophin-releasing hormone (GnRH) treatment (Kraemer and Bowen, 1986) and GnRH is also used clinically as adjunctive therapy during ovarian stimulation (Zanagnolo *et al*., 1996).

The administration of GnRH in different species is the most reliable method to induce ovulation, since it enables repeated treatment and does not induce antibody formation (Adams, 1981). Ovulation has to be induced artificially in rabbit does by intramuscular injection of either GnRH, or its synthetic analogues (Moce *et al*., 2003; Rommers *et al*., 2004; Quintela *et al*., 2004; Kalba and Abdel-Khalek, 2011). The GnRH agonist was first tested, at different concentrations, in a small number of experimental rabbit does (Quintela *et al*., 2008).

Therefore, the objective of this study was to evaluate the *in vivo* reproductive performance and *in vitro* ovulatory response of NZW rabbit does injected with 35 IU PMSG/doe 48 h before mating or 0.2 ml GnRH/doe at mating.

MATERIALS AND METHODS

This study was carried out at the Laboratory of Physiology and Biotechnology, Animal Production Department, Faculty of Agriculture, Mansoura University and a private farm, during the period from October 2012 to May 2013.

Animals:

Twenty four New Zealand White (NZW) rabbit does (5-7 mo of age, 2.5-4.6 kg LBW and 1-2 parities) and 3 NZW bucks (7.5- 8.1 months of age and 3.65-4.10 kg LBW) for natural mating in all groups, were used in this study.

All does and bucks were kept under the same feeding, management and environmental conditions in a private farm. All rabbits were individually housed in metal cages (40 x 50 x 60 cm) provided with feeders and water nipple for drinking in each cage. Does and bucks were fed *ad. libitum* on a commercial pelleted total mixed ration (18% CP and 2600 Kcal/Kg of energy).

Experimental animals and treatments:

The experimental rabbit does (n= 24) were divided according to age, body weight and parity into 3 groups, (8 does in each). Does of the first group (G1) were injected (i.m.) with 0.2 ml saline solution/doe immediately before mating with fertile buck and were considered as a control group. Does in the

second group (G2) were injected (i.m.) with 35 IU PMSG/doe (Foligon, Intervet International B.V., Boxmeer, Holland) 48 h before mating, while those in the third group (G3) were injected (i.m.) with 0.2 ml GnRH/doe (Receptal, Intervet, Salamanca, Spain) at mating. Each ml of Receptal contained 0.0042 mg Buserelin acetate equivalent to 0.004 mg Buserelin.

***In vitro* study:**

In this study, three does from each group were slaughtered 72 h post-mating for studying the effect of hormonal treatments on recovery rate and quality of embryos, morphological measurements of the ovaries and reproductive tract of slaughtered does.

Post-slaughter measurements:

Pre-slaughter weight of each doe was recorded and immediately after slaughtering, ovaries and all reproductive tract of each doe were removed, washed by distilled water and dried by cleaning paper. Ovaries and the reproductive tract were excised, submerged in a flacon plastic tissue culture dishes (60 x 15 mm) containing saline solution and carried out at 38.5°C.

Ovarian measurements including ovarian weight (right and left ovaries), number of corpora lutea (CLs), and total number (TNF) of small (less than 1 mm in diameter), large follicles (more than 1 mm in diameter) and hemorrhagic follicles, were recorded on each ovary or doe. In addition, measurements (length and width) of reproductive segments oviduct and uterine horn were also recorded.

Embryo yield:

Preparation of flushing medium:

Phosphate buffer saline (PBS) medium was prepared according to **Gordon (1994).** About 2 mg from bovine serum albumin (BSA) was added to one ml of PBS (2%). The prepared medium was adjusted to pH value of 7.2-7.4 using pH-meter and to osmolarity level of 280-300 mOsmol/kg using osmometer.Then, the medium was filtered by 0.22 µm-millipore filter (milieux GV, millepore, Cooperation Bedford MOA).

Embryo flushing:

Embryos were recovered from each uterine horn and oviduct per doe by flushing using PBS. The flushings were collected in sterile plastic Petri dishes and embryos were washed three times with PBS, counted and evaluated for viable and un-viable embryos under a stereo microscope at a magnification of 20–40 X.

After 72 h of mating 3 rabbit does from each group were slaughtered to collection of embryos from them for determined embryos recovery rate and quality of embryos and reproductive tract measurement.

All other female rabbits were kept till natural delivery. The new born pups were examined litter size, livability, weight at weaning, and sex ratio.

***In vivo* study:**

In this study, the remaining does in each group (five does) were allowed to continue the pregnancy and kindling for studying the effect of

hormonal treatments on reproductive performance of does and performance of their bunnies.

Reproductive traits of does:

Natural mating was carried out with fertile bucks and pregnancy was diagnosed by hand palpation ten to twelve days post mating to detect the pregnancy. After the positive mating the nest boxer were supplied with sawdust in the 25 days of pregnancy to provide a comfortable and warm nest for bunnies.

Within 12 hours after kindling, litters were checked, recorded and stillbirth were removed, afterwards, litters were examined each morning during the sulking period to remove the dead ones from the nest. Young rabbits were sexed and transferred to the progeny cages.

Statistical analysis:

The statistical analysis of the data was performed using a software package (SAS, 2000). The significant differences among means were tested using Duncan's Multiple Range Test (1955).

RESULTS AND DISCUSSION

***In vitro* study:**

Ovarian and genital characteristics:

Data in Table (1) show that weight and measurements of the ovary and genital segments (oviduct and uterine horns) were not significantly affected by hormonal treatments, ovarian side or their interaction.

The present results regarding the ovarian weight are in agreement with several authors showing that ovarian weight of doe rabbits was not affected by treatment with 100-150 IU PMSG (Kennelly and Foot, 1965), 0.5 mg FSH (Yuonglai, 1984) or PMSG and hCG (Daader *et al*., 2003).

Ovulatory response:

Results in Table (2) show that ovulatory response in terms of average number of hemorrhagic follicles and total follicles per ovary (right and left) or per doe was not significantly affected by hormonal treatments. In spit this effect there was a tendency of higher ovulatory response on the left than on the right ovaries, and in G3 than in G2. Rabbit does treated with GnRH (G3) showed the highest ovulatory response by increasing number of bleeding follicles, total follicles and corpora lutea as compared to those treated with PMSG (G2) or the control group (G1).

It is of interest to observe that the tendency of greater number of ovarian structures on the ovaries of G3 was associated with insignificant increase of ovarian weight and dimensions in G3 as compared to G2 and G1. Similar findings were recorded in California (Gosalves *et al*., 1987) and New Zealand White, NZW (Fahim, 2008) rabbit does.

Table (1): Ovarian and genital characteristics of rabbit does as affected by hormonal treatments, ovarian side and their interaction.

Item	Experimental group (G)			Ovarian side (S)		Intera-ction (G x S)
	G1 (Control)	G2 (PMSG)	G3 (GnRH)	Right ovary	Left ovary	
Average weight (g):						
Ovary	0.397 ±0.301	0.289 ±0.052	0.411 ±0.037	0.405 ±0.331	0.363 ±0.330	NS
Oviduct	0.511 ±0.073	0.580 ±0.127	0.354 ±0.090	0.514 ±0.081	0.426 ±0.081	NS
Uterine horn	6.445 ±0.704	5.808 ±1.219	5.117 ±0.862	6.271 ±0.778	5.521 ±0.778	NS
Average length (cm):						
Ovary	1.67 ±0.137	1.65 ±0.237	1.68 ±0.167	1.88 ±0.151	1.45 ±0.151	NS
Oviduct	12.0 ±0.672	13.0 ±1.164	10.3 ±0.823	12.75 ±0.743	10.42 ±0.743	NS
Uterine horn	7.17 ±1.016	9.60 ±1.760	9.25 ±1.244	8.87 ±1.123	7.67 ±1.123	NS
Average width (cm):						
Ovary	0.725 ±0.030	0.601 ±0.053	0.625 ±0.037	0.68 ±0.034	0.67 ±0.034	NS
Oviduct	0.100 ±0.012	0.150 ±0.021	0.130 ±0.015	0.13 ±0.013	0.10 ±0.013	NS
Uterine horn	0.758 ±0.069	0.875 ±0.12	0.650 ±0.085	0.77 ±0.077	0.72 ±0.077	NS

NS: Not significant.

The present total number of total follicles/doe was lower than 62.67 follicles/doe as reported by Dorra *et al.* (2013) on Baladi Red rabbits superovulated by PMSG and GnRH and higher than 30.4/doe in Baladi Black and 20.8/doe in NZW does as reported by Abdel-Khalek *et al* (2010), who found that number of follicles per doe was 30.4/doe in Baladi Black rabbits and 20.8/doe in NZW rabbits superovulated by GnRH .

Incidence of highest bleeding (hemorrhagic) follicles in G3 treated by GnRH treatment may be due to the stimulation of immature and atretic follicles (Adams, 1982 and Bourdage and Halbert, 1988). Similar findings were observed by Mehaisen *et al*. (2005); Salvetti *et al*. (2007) and Dorra *et al.* (2013) following superovulation treatment of rabbit does.

Table (2): Ovulatory response of rabbit does as affected by hormonal treatments.

Item	Experimental group		
	G1 (Control)	G2 (PMSG)	G3 (GnRH)
Right ovary:			
Number of bleeding follicles	0.33±0.333	0.33±0.333	2.33±1.202
Total number of follicles	18.00±5.132	13.67±7.535	23.33±3.712
Number of corpora lutea	3.33±1.453	4.00±2.082	5.33±3.283
Left ovary:			
Number of bleeding follicles	-	0.33±0.333	3.33±2.404
Total number of follicles	28.33±7.424	25.00±7.371	23.67±2.333
Number of corpora lutea	5.67±1.856	5.00±0.577	7.67±2.667
Total/doe:			
Number of bleeding follicles	0.33±0.333	0.66±0.333	5.66±3.412
Total number of follicles	36.33±5.372	38.67±6.854	47.00±2.917
Number of corpora lutea	9.00±1.675	9.00±1.894	13.00±2.473

In agreement with the present results, Ömer and Mehmet Kanter. (2002) recorded that number of corpora lutea was greater in GnRH treated mice than in controls. Similar trend was observed by El-Keraby *et al*. (1991), who found that increasing GnRH dose from 0.2 to 0.4 ml/doe increased number of CL/NZW doe. Generally, number of corpora lutea ranged between 7.4 to 10.3/doe (El-Keraby *et al*., 1991) or averaged 22/doe (Dorra *et al.* 2013) in different rabbit breeds treated with GnRH as compared to 6.6-8.0 or 15.58/doe in the controls, respectively. Moreover, Mehaisen *et al.* (2005) recorded greater number of ovulation sites (15.3 and 15.9) as compared to 13.5 and 13.2 (Vicente *et al*., 2003) for doe rabbits of R and V lines, respectively. On the other hand, higher number of corpora lutea (19.2/doe) for rabbits superovulated with PMSG was reported by Lee *et al*. (1991).

The observed increase in number of corpora lutea in G3 was mainly attributed to the effect of GnRH on increasing LH surge as compared to that occurred in G2 or G1. The rabbit is a reflexively ovulating species in which sensory and neuro-endocrine stimuli act together to induce a LH pre-ovulatory surge (Dufy-Barbe *et al*., 1973) and determine the ovulatory response.

In disagreement with the present results of G2, Taneja *et al*. (1990) found that number of ovulations in the control group significantly decreased than those treated with different protocols of PMSG and/or hCG.

Based on the previous findings, ovulatory response was affected by type and dose of hormonal treatments.

Embryo recovery and fertilization rates:

A total number of embryos (fertilized and unfertilized)/doe collected from oviduct and uterine horns was significantly ($P<0.05$) greater in G3 than in G2, while total number of embryos was lower in G2 than in G1. Also,

embryo recovery rate was similar in G3 and G1, being higher (P<0.05) than in G2 (Table 3).

As affected by hormonal treatment, all ova were fertilized producing 100% fertilization rate in G2 and G3 *versus* 93.9% in G1 (0.33 unfertilized ova/doe). Such trend indicated positive effect of both hormonal treatments (G2 and G3) on fertilization rate of embryos, but adversely affected its recovery in G2 (Table 3).

The effect of hormonal treatments was more pronounced on embryonic stage. In this respect, all produced embryos were at blastocyst stage in both G2 and G3 and only 50% of embryos at blastcyst stages in G1 (control group)..Yield of embryos at blastocyst stage was significantly (P<0.05) higher in G3 than in G2 and G1 (6.67 vs. 1.67 and 2.33/doe, respectively, Table 3).

Table (3): Recovery of embryos at different stages of rabbit does as affected by hormonal treatment.

Parameter	Experimental group		
	G1 (Control)	G2 (PMSG)	G3 (GnRH)
Number of corpora lutea/doe	9.00±1.675	9.00±1.894	13.00±2.473
Total number of embryos/doe	4.67±2.028^{ab}	1.67±1.202^{b}	6.67±1.667^{a}
Embryo recovery rate (%)	51.9	18.6	51.3
Unfertilized ova/doe	0.33±0333	-	-
Fertilization rate (%)	93.9	100	100
Embryonic stage:			
Morula (n)	2.33±1.453	-	-
Morula (%)	50	-	-
Blastocyst (n)	2.33±0.577^{b}	1.67±1.202^{b}	6.67±1.667^{a}
Blastocyst (%)	50	100	100

a and b: Means denoted within the same row with different superscripts are significantly different at P<0.05.

In accordance with the present results, Dorra *et al.* (2013) found no differences in embryo recovery rate of superovulated with GnRH and control rabbit does, but reported higher recovery rate, being 82.87 and 82.24, respectively. However, Fahim (2008) found that number of embryos collected after 72 h of mating was 4.35/ovary and recovery rate was 66.7% in NZW rabbits superovulated by PMSG. In this line, El-Keraby *et al*. (1991) found that the recovery rates of embryos collected from does treated with 0.2 or 0.4 ml GnRH ranged from 88.8 to 90% in treated versus 90% in the control groups.

On the other hand, using different types of hormonal administration to induce ovulation (superovulation), Mehaisen *et al.* (2005) reported lower embryo recovery rate (43.2 and 40.3%) as compared to 63 and 82% in the study of Viudes-de-Castro *et al*. (1995), and 77 and 74%, in the study of Vicente *et al*. (2003) for R and V lines, respectively. These differences may be due to the effect of the superovulation treatment that was used.

In the present study, the percentage of embryos recovered based on CL was lower in G2 than in G1, thus hormonal treatment with PMSG in this study reduced the benefits or efficiency of superovulation regimens (Carney and Foote, 1990).

***In vivo* study:**

Reproductive performance:

Data in Table (4) show that hormonal treatment of does in G2 and G3 improved kindling rate (100 in each), number of matings per conception (1 in each *vs.* 1.2) litter size at birth (6.80 and 7.40 vs. 5.20, P<0.05) and viability rate of bunnies at birth (100% in each vs. 91.7%) when compared with control group. However, gestation period length was not affected by treatments, ranging between 31.0 and 31.67 d).

These results indicated impact of hormonal treatment on *in vitro* study, by increasing number of embryos.

Table (4): Reproductive performance of rabbit does as affected by hormonal treatment.

Parameter	Experimental group		
	G1 (Control)	G2 (PMSG)	G3 (GnRH)
Kindling rate (%)	100	100	100
Number of matings per conception	1.25±0.25	1.00±0.0	1.00±0.0
Gestation period length (d)	31.67±0.33	31.0±0.408	31.5±0.289
Litter size/doe	5.20±1.25^{00}	6.80±0.629^{ab}	7.40±0.45^{a}
Live	4.80±0.700^{b}	6.80±0.629^{ab}	7.40±0.550^{a}
Dead	0.40±0.75	-	-
Viability rate at birth (%)	91.7	100	100

a and b: Means denoted within the same row with different superscripts are significantly different at P<0.05.

Localization of GnRH within the hypothalamus has been well documented in rats, rabbits, sheep, cows and mares (Arimura *et al*., 1973). It has been suggested that GnRH stimulates development of the pre-ovulatory follicle-enclosed oocytes (Srivastava *et al*., 1995). In the rabbit, ovulation is generally non-spontaneous, requiring the stimulus of mating for its induction. Ovulation occurs 10-13 h post-copulation and higher proportions of does (≥25%) fail to ovulate after mating, probably due to a deficiency of LH in their pituitary gland (Adams ,1976).

The significant improvement in kindling rate of rabbit does by GnRH as compared to the controls was mainly due to the effects of GnRH on the ovary include stimulation of oocyte maturation (Dekel *et al*., 1988 and Yang *et al*., 1995). However, a direct gonadal effect of GnRH may interfere with the stimulatory effects of exogenous gonadotropin on follicular development, corpus luteum establishment, and oocyte maturation (Zanagnolo, *et al*., 1996). It has been shown that GnRH mediates the hypothalamic control of pituitary gonadotropin secretion and biosynthesis (Dekel *et al*., 1988).

However, some studies on rabbits have shown that GnRH and its analogues also exert a direct effect on gonadal function, influencing oocyte maturation both *in vivo* and *in vitro* (Yang *et al*., 1995 and Yoshimura *et al*., 1992).

It has been suggested that GnRH induces oocyte maturation via activation of specific GnRH receptors on granulosa cells (Dekel *et al*., 1988 and Koves *et al*., 1989). It has also been suggested that exposure to GnRH stimulates prostaglandin synthesis in preovulatory follicles (Wong and Richards, 1992). Increasing concentration of prostaglandin plays an important role in oocyte maturation (Calder *et al*., 2001). In nearly similarity with the present results, Zapletal *et al*. (1990) reported that the total annual fertility rate was higher (73.02%, $P<0.05$) in rabbit group treated with lecirelin (GnRH). Average numbers of all born and live born young rabbits were 9.82 and 9.70, respectively. The results showed that by application of super analogue GnRH lecirelin a higher fertility rate of does with higher number of all and live borns per litter was achieved in rabbit breeding. However, Zapletal and Pavlik (2008) reported that the conception rates ranged from 10.0 to 89.5% for different GnRH doses.

CONCLUSION

It was concluded that superovulation treatment of NZW doe rabbits with 0.2 ml GnRH/doe (Receptal) at mating increased embryo yield and blastocyst production *in vitro*. Also, the same treatment improved kindling rate, number of matings per conception, litter size at birth and viability rate of bunnies at birth. Therefore this treatment may be efficient in embryo *in vivo* production for embryo transfer and also needed for increasing *in vivo* fertility of naturally mated rabbit does.

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الاستجابه المبيضية في اناث الارانب المعامله بكل من GnRh و PMSG حقليا ومعمليا

أحمد زكي محرز[١] , محمد عبد الرحمن مصطفى[١] , شريف مغاوري شامية[٢] و نها فتحي عبد الرؤف[١].

١- قسم انتاج الحيوان – كلية الزراعة- جامعة المنصورة.

٢- معهد بحوث الإنتاج الحيوانى – وزارة الزراعه

أجريت هذه الدراسة علي ٢٤ أنثي أرنب نيوزيلندي أبيض (NZW), يتراوح عمرها من ٥ الي ٧ شهور ووزنها الحي من ٢,٥ إلي ٤,٦كجم في موسمها الأول أو الثاني لدراسة تأثير تنشيط التبويض باستخدام هرمونات GnRH أو PMSG علي الخصائص المورفولوجية للمبيض وعدد البويضات وقياسات الأجنة في المراحل المختلفة. وكذلك تم استخدام ٣ ذكر نيوزلندي ابيض ناضج للتلقيح الطبيعي. تم تقسيم الإناث إلي ٣ مجموعات (٨ لكل مجموعة), تم حقن كل أنثي في المجموعة الأولي ب٠,٢ مل من محلول الملح في العضل مباشرة قبل التلقيح(كنترول) وإناث المجموعة الثانية والثالثة تم حقنهم في العضل ب ٣٥ وحدة دولية PMSG لكل أنثي قبل التلقيح ب ٤٨ ساعة و٠,٢ مل GnRH لكل أنثي علي التوالي.

تضمنت الخصائص المبيضية والتناسلية الوزن والطول والسمك لكل من المبيض والقناة المبيضة وقرن الرحم وتم القياس لكلا من الجهة اليمني واليسرى. تم عمل غسيل للأجنة وعدها في كل من الحيوانات الخاضعة للدراسة التي تم ذبحها بعد ٧٢ ساعة من التزاوج.

تم جمع الأجنة وعدها في كل من قرني الرحم وقناة المبيض من كل أنثي, وأظهرت النتائج أن خصائص المبيض لم تتأثر سواء بالمعاملة أو اتجاه المبيض أو التداخل بينهم. استجابة التبويض من حيث متوسط عدد الحويصلات والحويصلات الدموية والأجسام الصفراء لم تتأثر تأثر معنوياً نتيجة للمعاملة. إناث المجموعة الثالثة أنتجت زيادة معنوية في عدد المواليد والأجنة لكل أنثى وكذلك أعلى معدل للنمو والوزن عند الفطام والحيوية للنتاج.

ومن هنا نستخلص أن معاملة إناث الأرانب النيوزيلاندي ب ٢مل GnRh/أنثى عند التلقيح قد يكون فعال عند إنتاج الأجنة معمليا لإستخدامها في نقل الأجنة وتستخدم كذلك وهناك حاجة لها أيضا لزيادة الخصوبة في الحيوانات الحية عند التلقيح الطبيعي لإناث الأرانب.