J. Physiol. Biochem., 53 (2), 205-210, 1997
Revista española de Fisiología

Effect of Gonadoreline and Naloxone on induction of ovulation and plasma LH in rabbit

P. G. Rebollar[1], J. M. R. Alvariño[1], J. C. Illera[2] and G. Silván[2]

[1]Depto. Producción Animal, E.T.S.I. Agrónomos, UPM, 28040 Madrid, and
[2]Depto. Fisiología Animal, Facultad de Veterinaria, UCM, 28040 Madrid (Spain)

(Received on October 7, 1996)

P. G. REBOLLAR, J. M. R. ALVARIÑO, J. C. ILLERA and G. SILVÁN. *Effect of Gonadoreline and Naloxone on induction of ovulation and plasma LH in rabbit.* J. Physiol. Biochem. (Rev. esp. Fisiol.), 53 (2), 205-210. 1997.

To study the effect of an opiate antagonist receptor (naloxone) on LH secretion, two groups of seven does were randomly allocated to GnRH (gonadoreline, 20 µg/animal), and GnRH+naloxone (1.8 mg/kg b.w.) treatment, respectively and another group of eight animals was treated with naloxone alone. Blood samples were obtained immediately before treatment (0 minutes) and at 60, 120 and 180 minutes after injection. Plasma levels of LH were determined by a direct, simple and precise sandwich enzyme immunoassay (EIA), adapted for measuring plasma LH in rabbit. Luteinizing hormone reached maximum values 60 minutes after administration of GnRH or GnRH+naloxone ($p < 0.05$) and all the females of these groups ovulated. Only 25 % of naloxone treated rabbit does ovulated and these animals presented a high LH plasma level at 60 minutes post-treatment ($p < 0.05$). In non ovulated animals no variations in plasma LH concentrations were observed at 60 minutes, in relation to blood samples obtained at 0, 120 and 180 minutes. Results show a limited effect of this naloxone dose on the anterior pituitary of non lactating adult rabbit does in relation to induced ovulation. No synergic effect was detected when naloxone was administered in addition to gonadoreline.

Key words: Rabbit LH, Ovulation induction, Enzyme immunoassay, LH EIA.

The female rabbit is a nonspontaneous (i.e., reflex) ovulator and ovulation in rabbit farms using artificial insemination, is usually induced by synthetic analogues of gonadotrophin releasing hormone (GnRH), whose administration induces the anterior pituitary to release LH (1, 3, 9, 14). Opioid peptides may be involved in the normal LH secretion inhibition in adult rabbits but naloxone, an opioid peptide antagonist, causes evident LH secretion increases in prepubertal does and

Correspondence to P. G. Rebollar (Telephone: 34-1-5491880; Fax: 34-1-5499763).

induces ovulation in 100-day-old females (12). On the other hand naloxone administration leads to a significant increase in basal LH levels following suckling in the lactating rabbit (6), without gonadoreline administration or other ovulation inducing hormones.

Radioimmunoassay has been a common method used for LH measurement in predicting ovulation (6, 9) with the disadvantages associated with the use and disposal of radioisotopes, the measurement and study of LH being restricted to specialized laboratories. This study objectives were: to determine the changes in LH plasma levels in adult rabbit does treated with GnRH and/or naloxone by using a simple rapid sensitive EIA sandwich developed and validated with materials readily available to all laboratories, and to examine if naloxone administration can enhance the pituitary response and provoke a similar or larger ovulatory response than gonadoreline in non lactating adult rabbit does.

Materials and Methods

The experiment was performed in 22 mature (8-12 months old), multiparous, non lactating, crossbred female rabbits (*Oryctolagus cuniculus*) (New Zealand White x Californian) raised in the Animal Production Department (mean body weight 3,637 ± 79 g). Animals used in this study were treated according to the CEE council Directive (86/609, 1986) for the Care of Experimental Animals. They were housed in individual metal cages (32 x 52 cm) under controlled light/dark cycles (16 h/8 h). Pelleted commercial diet (Lab Rabbit Chow, Purina Mills Inc.: 16.2 % protein, 2.5 % fat and 13.5 % crude fibre), was restricted to about 125 g day^{-1}, and water was supplied *ad libitum*. Weaning was performed on all animals at least one month before the experiment started.

Drugs.– Naloxone hydrochloride, an opiate antagonist receptor (Sigma-Aldrich Química S.A., Madrid), was dissolved in physiological saline (0.9 % w/v NaCl) and was administered i.v. at a dose level of 1.8 mg/kg (7). Gonadoreline, a synthetic analogue of GnRH (Ovejero Lab., León), was given i.m. at a dose of 20 µg/animal (8).

Sacrifice of animals was performed after administration of a lethal dose i.v. of penthobarbital sodium (Dolethal, Vetoquinol Lab., Spain).

Experimental procedures.– Two groups of seven rabbits each were randomly allocated to GnRH and GnRH+naloxone treatment respectively (Group 1 and 2), and another group of eight animals was treated with naloxone (Group 3). To study the effect of these treatments on LH plasma levels, four blood samples were obtained from the ear artery using sterile heparinized syringes and tubes, immediately prior to naloxone and/or GnRH administration (0 minutes) and at 60, 120 and 180 min after treatment. The blood samples were centrifuged (3500 r.p.m., 10 minutes), and plasma was stored at -20 °C until analyzed.

To determine if ovulation had occurred, a laparotomy was performed within 5-6 days after treatment and ovulation rate was quantified by counting the number of corpora lutea present in each ovary.

EIA procedure.– Ninety-six well Nunc Maxisorp microtiter plates were coated with 100 ml of the primary antibody (250 ng/ml of monoclonal antibody anti-bovine LH (518B7) dissolved in coating buffer: 0.05 M sodium bicarbonate buffer, pH: 9.6) per well, except for the first column used as a plate/assay blank. Plates

were sealed and incubated overnight at 4 °C. Before use the coated plates, non-bound antiserum was removed from the wells by washing the plates five times with wash solution (0.15 M NaCl, 0.05 % tween-20).

Standards and serum samples were prepared by dissolving them in MOPS assay buffer (0.04 M 3-(N-Morpholino) propanesulfonic acid, 0.015 M NaCl, 0.01 M EDTA, 0.05% tween-20, 0.005 % chlorohexidine digluconate, 0.1 % BSA, pH: 7.2). The standard curve covered a range between 0 ng/ml to 20 ng/ml, and was constructed by using 11 standard solutions: 20, 10, 5, 2.5, 1, 0.5, 0.25, 0.12, 0.06, 0.03, 0 ng/ml. At the time of assay, 50 ml of MOPS assay buffer was pipetted across the entire plate, followed immediately by 50 ml of serum samples or appropriate standards.

The plates were then sealed and incubated for 2 hours at room temperature. Non bound LH was then removed from the wells by washing the plates as described previously. The next step was the addition of the second antibody (anti-oFSH:HRP; G4-215B) across the entire plate, at a working dilution of 1:1000 in MOPS assay buffer (100 ml). Plates were sealed and the second antibody conjugate reaction was allowed to proceed for another 2 hours at room temperature. Non-bound second antibody was removed by washing the plates five times with wash solution.

The last step was the addition of 100 ml of substrate solution (3,3',5,5'-tetramethylbenzidine dihydrochloride, pH: 5.0) to all wells. Conversion of the substrate by the enzyme, manifested as a color change, was stopped after approximately 15 minutes by the addition of 100 ml of stop solution (0.1 M phosphoric acid). Absorbance was measured at 450 nm and data were transferred to an interfaced computer (IBM) for analysis.

Enzyme immunoassay validation.– EIA precision was tested by calculating the intra- and interassay CV (%), the former being less than 6.2 % and the latter less than 7.2 %. EIA accuracy was tested by determining the recovery rates of known amounts of rabbit LH (0, 100, 500, 1000 and 1500 pg/ml) added to a pool of serum samples. The average recovery rate was 93.5 ± 1.7 %.

EIA sensitivity was tested by the low detection limit (calculated from the error in the zero and the slope of the standard curve minus 2 SD in ten consecutive assays), being 16.1 ± 1.42 pg/ml.

Statistical analysis.– The treatment effect on the LH plasma levels and on the number of corpora lutea was analyzed with the General Linear Model Procedure (GLM) and means were compared by using the Duncan test. Ovulation rate was analyzed with a non-parametric procedure (Categorical Data Modeling) (10).

Results

The mean LH plasma levels following drug administration are shown in figures 1 and 2.

Before treatments, the mean basal plasma LH concentration was 0.62 ± 0.08 ng/ml, without differences among the studied groups. Sixty minutes after treatment with GnRH and/or naloxone (fig. 1), an increase over the basal values was observed in all groups. Treatment with GnRH caused a significative rise in mean plasma LH levels, statistically different from the animals treated with naloxone alone (9.98 ± 2.3 vs 2.01 ± 0.8 ng/ml). The increase in those treated with GnRH + naloxone was intermediate and not statistically different from the other groups (6.41 ± 0.6 ng/ml).

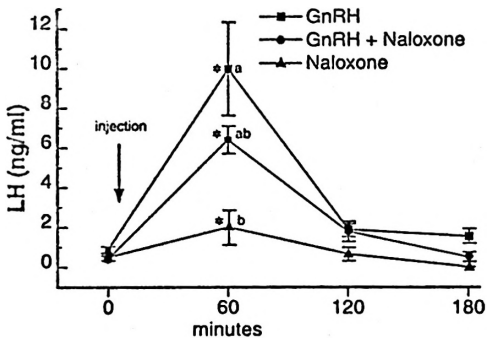

Fig. 1. *Plasma LH levels (means ± SEM) in rabbits treated with 20 mg of GnRH (n = 7), 20 mg of GnRH+1.8 mg/kg b.w. of naloxone (n = 7) and 1.8 mg/kg b.w. of naloxone (n = 8).*
* Significantly different from 0, 120 and 180 minute levels, ($p < 0.05$). Means marked with different letters (a, b) are statistically different ($p < 0.008$).

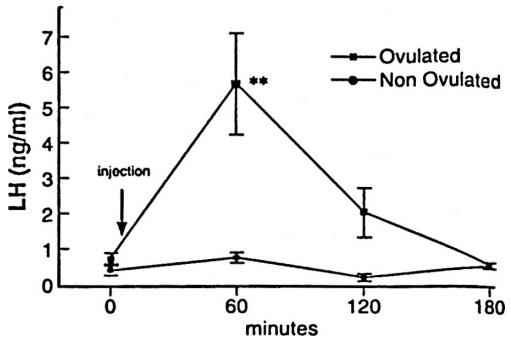

Fig. 2. *Plasma LH levels (means ± SEM) in ovulated and non ovulated rabbit treated with 1.8 mg/kg of naloxone (n = 7) i.v.*
** Significantly different from 60 min LH levels in non ovulated rabbits ($p < 0.01$).

In the naloxone treated group (fig. 2), significantly higher LH plasma levels were observed in rabbit does with ovulation at 60 minutes as compared to the ones without it (5.68 ± 1.43 vs 0.79 ± 0.15 ng/ml).

All the rabbit does treated with GnRH or GnRH+Naloxone ovulated (100 %), although only 25 % of those treated with naloxone presented corpora lutea at sacrifice ($p < 0.001$). The number showing them after laparotomy in ovulated rabbits, was similar among the three groups studied (8.14 ± 0.95, 7.57 ± 1.08, 11 ± 2.5 corpora lutea of groups 1, 2 and 3, respectively).

Discussion

Previous observations that mating or administration of synthetic GnRH analogues causes a rapid increase of plasma LH concentrations in female rabbits (2, 5, 9, 14) have been confirmed. Plasma LH has been measured by a non-isotopic EIA developed and validated with materials readily available to all laboratories (4), the method being in about four hours and the plate-blocking step and over-night incubations being avoided.

Before treatments, basal plasma LH concentrations were similar among the three studied groups. The large LH release in response to ovulation stimulus is always related to it in such a way that all the ovulated animals presented high LH concentrations at 60 minutes post-induction of ovulation.

Previous studies showed that LH plasma levels rose to different intervals in relation to mating or induction treatments. A rapid LH increase at 1.5 hours after mating was observed in rabbits with normal oestrus (2), but it peaked sooner in those pretreated with oestradiol benzoate. In immature rabbits a large increase in LH release 15 minutes after GnRH injection was obtained (14). Other authors (9) also observed an increase in LH basal values at 15 minutes after GnRH administration which apparently exhibits a clear dose response in sexually receptive does. Maximal levels between 60 and 150 minutes and at 2 hours after mating, were observed by different authors (7, 11). Furthermore, the magnitude of LH peak is variable depending on authors and ranges between 14 ng/ml (11) and 60-70 ng/ml (2).

The suitability of naloxone to stimulate the pituitary in the lactating and 100 day-old rabbit does is supported by some authors (6, 12, 13). In our study, females treated with naloxone alone showed a slight increase in plasma LH concentrations at 60 minutes, but it was not significant as five out of seven animals did not respond.

Although previous studies (13), showed that naloxone (2.5 mg/kg b.w.) caused increases in LH secretion, which were occasionally high enough to induce ovulation (6 out of 11 animals ovulated), our results indicate that an i.v. injection of 1.8 mg/kg body weight of naloxone is not totally suitable as ovulation inducer in the mature rabbit, the dose per animal for this purpose having to be higher. On the other hand, naloxone has not proven to be a GnRH intensifier to increase LH release in rabbits at the dose employed.

P. G. REBOLLAR, J. M. R. ALVARIÑO, J. C. ILLERA y G. SILVÁN. *Efecto de la gonadorelina y de la naloxona sobre la inducción de la ovulación y las concentraciones plasmáticas de LH en coneja.* J. Physiol. Biochem. (Rev. esp. Fisiol.), 53 (2), 205-210. 1997.

Se estudia si la naloxona, un antagonista de los receptores opiáceos, incrementa o produce el mismo efecto que la gonadorelina, un análogo sintético de la hormona liberadora de gonadotrofinas (GnRH), sobre la inducción de la ovulación y niveles plasmáticos de LH en la coneja doméstica. Dos grupos de 7 conejas adultas elegidas al azar, se tratan con GnRH (gonadorelina, 20 µg/animal) y con GnRH+ naloxona (1.8 mg/kg p.v.) respectivamente. Un tercer grupo de 8 animales reciben sólo naloxona, a la misma dosis. Las muestras de sangre se obtienen inmediatamente antes del tratamiento (0 min) y a los 60, 120 y 180 minutos post-inyección. Los niveles de LH se determinan por EIA-sandwich, rápido, sencillo, práctico y adaptado para determinar LH en plasma de conejo. La hormona luteinizante alcanza valores máximos a los 60 min del tratamiento con GnRH o GnRH+naloxona ($p < 0,05$); todas las conejas de estos grupos ovulan. De las conejas tratadas con naloxona, sólo el 25 % ovula presentando altos niveles plasmáticos de LH, 60 minutos después del tratamiento ($p < 0,05$); las conejas de este grupo que no ovulan no presentan variaciones en las concentraciones plasmáticas de LH a los 60 minutos, comparadas con las muestras tomadas antes, a los 120 y a los 180 minutos del tratamiento. Los resultados obtenidos muestran un efecto limitado de esta dosis de naloxona en la hipófisis anterior de conejas adultas no lactantes con respecto a la inducción de ovulación. No se observa ningún efecto sinérgico cuando se administra la naloxona junto con la gonadorelina.

Palabras clave: LH, Inducción de la ovulación, Enzimoinmunoensayo.

References

1. Alvariño, J. M. R. and Rebollar, P. G. (1995): *Bol. cunicultura*, 18, 42093-46.
2. Carlson, J. C., Wong, A. P. and Perrin, D. G. (1977): J. Reprod.Fert., 51, 87-92.
3. Foote, R. H. and Simkin, M. E. (1993): *Lab. Anim. Sc.*, 43, 383-385.
4. Illera, J. C., Munro, C. J., Silván, G., BonDurant, R. H. and Illera, M. (1996): *J. Physiol. Biochem.*, 52, 95-102.
5. Lamb, I. C., Strachan, W., Henderson, G., Atkinson, T., Lawson, W., Partridge, G. G., Fuller, M. F. and Racey, P. A. (1991): *J. Reprod. Fert.*, 92, 281-289.
6. Lamb, I. C., Stracham, W., Henderson, G., Partridge, G. G., Fuller, M. F. and Racey, P. A. (1993): *Theriogenology*, 39, 1193-1200.
7. Mills, T. M., Copland, J. A., Coy, D. H. and Schally, A. V. (1983): *Endocrinology*, 113, 1020-1024.
8. Rebollar, P. G., Ubilla, E. and Alvariño, J. M. R. (1995): *World Rab. Sc.*, 2, 87-91.
9. Rodríguez, J. M. R., Agrasal, C. and Esquifino, A. (1989): *Anim. Reprod. Sc.*, 20, 57-65.
10. SAS, 1988: SAS/STAT, Userís guide. SAS Inst. Inc., Cary NC, USA.
11. Tsou, R. C., Dailey, R. A., McLanahan, C. S., Parent, A. D., Tindall, G. T. and Neill, J. D. (1977): *Endocrinology*, 101, 534-539.
12. YoungLai, E. V., Wilkinson, M., Thompson, N. and Byrne, A. (1988): *Can. J. Physiol. Pharmacol.*, 66, 38-42.

13. Younglai, E. V., Wilkinson, M., Thompson, N, and Byrne, A. (1988): *Biol. Reprod.*, 39, 630-637.

14. Younglai, E. V., Thompson, N., and Foster, W. (1989): *J. Reprod. Fert.*, 85, 325-329.