Version of Record: https://www.sciencedirect.com/science/article/pii/S0093691X19300457
Manuscript_24d9ed8a0231360fb6a60456f144f5a5

Choice of protocol for the *in vivo* bioassay of equine Chorionic Gonadotropin (eCG / PMSG) in immature female rats*

Yves COMBARNOUS[1#], Julie MARIOT[1], Lauriane RELAV[1&], Thi Mong Diep NGUYEN[1-2] and Danièle KLETT[1]

1/ CNRS, INRA, Physiologie de la Reproduction et des Comportements (PRC), 37380 Nouzilly (France) ; 2/ Quy Nhon University, Quy Nhon, Binh Dinh Province (Viet Nam)

yves.combarnous@inra.fr

& present address : Centre de Recherche en Reproduction et Fertilité (CRRF), Université de Montréal, Saint-Hyacinte, Province de Québec (Canada)

* This paper is dedicated to the memory of our long-time close colleague and friend Claire CAHOREAU, who passed away on July 7th, 2017.

© 2019 published by Elsevier. This manuscript is made available under the CC BY NC user license
https://creativecommons.org/licenses/by-nc/4.0/

Abstract

Equine Chorionic Gonadotropin (eCG) previously known as Pregnant Mare Serum Gonadotropin (PMSG) has been used for decades in regulating reproduction in various domestic animal species. Its use necessitates a good measurement of its bioactivity in commercial preparations. The EUROPEAN PHARMACOPEIA (EP 7.0) recommends 5-6 subcutaneous injections in immature female rats for the *in vivo* bioassay for eCG as in the case for measurement of FSH bioactivity in the Steelman & Pohley assay (1953). This recommendation is in marked contrast with the classical and long-time used PMSG assay of Cole & Erway (1941) that includes only one subcutaneous injection, 3 days before measurement of ovarian weight. As this difference introduces much confusion in the determination of eCG/PMSG bioactivity in commercial sources, we have performed parallel assays of several PMSG preparations, with both protocols. The single-injection protocol takes into account the long half-life of eCG in bloodstream and provokes an ovarian stimulation at lower concentrations than the multiple-injection protocol. As the single-injection protocol also mimicks the protocol used in cattle, it is preferable to the multiple-injection protocol of the current EP.

Introduction

Like all gonadotropins, equine Chorionic Gonadotropin (eCG), previously known as Pregnant Mare Serum Gonadotropin (PMSG), is a glycoprotein hormone comprising two disimilar glycosylated subunits, named α and β. These subunits are non-covalently associated (2,9,12,22,30) but exhibit a slow rate of dissociation because of the β-subunit seatbelt embracing its α-subunit partner as shown for the human CG (hCG) (14,31).

Equine CG has been used for decades in superovulation and ovulation induction protocols in various mammalian species (21,32). Its *in vivo* potency must be evaluated accurately to allow batch-to-batch consistencey and reproducibility of treatments.

The high potency of eCG is due to its dual FSH/LH activity in non-equid species (5,8,17,19,27-29) and more importantly, to its long half-life in the blood (1,18) and treatment reproducibility. The classical *in vivo* bioassay for eCG which was designed more than 75 years ago by Cole and Erway (6) in which the dose-response effect of eCG on ovarian weight in prepuberal rats is measured. This assay,

requiring one single subcutaneous injection per animal, has been used routinely by academic laboratories and manufacturers for assaying crude and purified eCG preparations.

Nevertheless, a different eCG assay resembling that of Steelman & Pohley for FSH (26) is recommended by the EUROPEAN PHARMACOPEIA (EP 7.0). Its main difference with the Cole & Erway assay is the recommendation to divide the PMSG dose into 5-6 injections instead of administrating a single injection. This difference introduces much confusion among quality control services of PMSG manufacturers and controversies in PMSG activity determinations. Moreover, since this hormone is generally given as a single injection in farm animals (10,11), it is important to determine which of these two assay protocol in immature female rat, is best suited to anticipate eCG superovulatory activity in these species.

In order to see how these two protocols compare in terms of measured PMSG bioactivity, we performed both protocols in parallel using three different eCG/PMSG preparations.

Materials and Methods

Hormones. The eCG/PMSG preparations used were 1/ the WHO IRP2 reference preparation (3), 2/ the NZY-01 reference preparation from our laboratory (16) used in academic studies as well as in quality controls by various manufacturers, and 3/ a commercial PMSG preparation (Folligon, Intervet, Oss, The Netherlands).

In vivo assays. The *in vivo* assays used are based on the one described by Cole and Erway (6) using immature 21-day old female rats at t=0. The first protocol is identical to the 3-day assay described in the princeps paper of Cole & Erway, consisting of a single injection of 10, 20 or 40 IU at t=0. The second one is based on the EP7.0 recommendations with the same eCG doses divided into six separate injections at t= 0h, 8h, 22h, 29h, 46h, and 54h with sacrifice at t=68h (i.e. 68, 60, 46, 39, 22 and 14h before sacrifice). The two assays were performed using lots of 6 animals for each treatment. The animals were housed at controlled temperature (21 ± 1 °C) and lighting (16L:8D) and had an enriched environment. They were sacrificed by CO2 gas exposure at gradually increasing concentration as recommended by directive 2010/63/EU, using a ATEM1 automat (TemSega; Pessac, France). Both

ovaries from each animal, were recovered and thoroughly freed of fat. The pairs of ovaries were then weighed on a Quintix 224-1S analytical balance (Sartorius, Goettingen, Germany) at 0.1 mg precision. The dose-dependent ovarian weight curves for both protocols were fitted by linear regression using all the data for the three eCG preparations. These curves were then compared by a 3+2 parallell-line assay (7) using the Prism 5 package (Graphpad software, San Diego, CA).

In order to test the time-dependence of the assay, we also studied the response to the same three eCG doses, given in single s.c. injections, 18 to 68h before sacrifice.

These protocols were carried out in accordance with the U.K. Animals Act (1986) and associated guidelines, EU Directive 2010/63/EU for animal experiments, and were validated by the local ethical comity of the Région Centre-Val de Loire (France).

Results

Two eCG reference preparations, WHO and NZY, along with one commercial PMSG preparation were tested using the two protocols : either each dose injected as a single subcutaneous injection, 68 hours before sacrifice (t=0h) or each dose divided into 6 injections given at t= 0h, 8h, 22h, 29h, 46h, and 54h with sacrifice at t=68h.

The data in Fig.1A clearly show the ovarian weight gain responses to the same eCG total dose of the three different preparations. There is no significant difference between the data obtained with the three preparations at the same IU concentrations. The data from the three preparations were thus fitted by linear regression for each protocol. Since there is an increase only for two doses in the multi-injection protocol, the two curves were compared by 3+2 parallel-line assay (7) and the potency with the multi-injection protocol was 0.71 ± 0.15 that of the single-injection protocol. The responses are thus significantly higher in the single-injection protocol than in the six-injection protocol.

In order to better understand the origin of this difference, we measured the ovarian weight gain responses to a single injection of 10, 20 or 40 IU eCG NZY-01, at different times, 18 to 68h before sacrifice. It can be seen in Fig.1B, that there is a marked dependence of the ovarian weight response to the duration between injection and recording of ovaries weights. It is clear that the increase in ovarian

weight is marginal for times less than 26h between injection and sacrifice, and is time-dependent over the two following days.

Discussion

The data in the present report clearly show a response to eCG at a lower threshold after 68h when the entire dose is administrated as a single injection at t=0 rather than divided into six separate injections over a 50h period.

A very schematic graphical explanation for this difference between the two protocols is depicted in Fig.2. The hormonal stimulation is expected to be proportional to the area under curve (AUC) of its circulating concentration. Because of the long half-life of eCG (2-3 days) (13), it can be seen that the AUC for the total hormonal dose given in one single injection at t0 (red line) is larger than that of the same dose given in several equal injections from t=0 to t=54h (blue line).

Not only is the AUC for a single injection larger, but in this protocol, the ovary is exposed to higher hormonal concentrations for a longer duration. Indeed this parameter is important since the dose-response curves are much higher when the single injection is performed sooner (Fig.1B). In fact, no increase in ovarian weight is observed over the first 26h after injection, therefore, is time-dependent over the following 42 hours.

It can be seen in the scheme in Fig.2, that eCG concentration in blood would be high sooner following a single injection, than with the multi-injection protocol and thus for a longer period of time. In the multi-injection protocol, the last hormone injections would be made too late to efficiently stimulate ovarian weight gain. It is also likely that a minimum blood eCG concentration has to be reached to stimulate ovarian weight gain. It is obvious that this concentration will be reached sooner and for a longer duration in the one-injection protocol. In addition, since more than a 26h duration is needed to stimulate the ovaries, the eCG concentration will be high for too short a time to maximally stimulate ovarian weight gain.

It is also interesting to point out that the use of uterine weight gain as an end-point instead of that of ovaries, leads to an assay with a much lower threshold (~30 folds), easier dissection and reduced

variation (6,15,23). This could permit reduction in the number of animals per assay for both ethical and economical reasons.

Efforts have been made for years to replace *in vivo* assays by immunoassays (20), radio-receptor assays and cell culture assays (4) but this is precluded for the time being, by the fact that differences between these *in vitro* assays and the *in vivo* test exist because of PMSG polymorphism (4). In addition to natural polymorphism, differences due to purification-induced alterations must also be considered. This renders the evaluation even more difficult when the reference preparation itself is concerned (4).

In the near future, for ethical reasons, it is likely that natural eCG (PMSG) will have to be replaced by recombinant eCG (called rec eLH/CG since the same gene encodes both eLHβ and eCGβ (24) which bear different saccharide moieties (25)). *In vivo* tests will be indispensable to estimate biological activities of these molecules which are dependent on their half-lives. These tests will have to be performed according to the single-injection protocol (6) 1/ to get higher responses, 2/ to take their half-lives in consideration and 3/ to take into consideration the single-injection protocols used in farm animals, in which eCG half-lives are also long (20).

Acknowledgements

The authors are grateful to Claude Cahier and Deborah Crespin (UPEAO, INRA, Nouzilly) for animal care.

Contributions

All authors participated in the design of the study. YC, JM, LR & TMDG performed the experiments. YC, TMDN and DK wrote the paper and all authors approved the final text.

Funding

This research received no external funding

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Aggarwal BB, Papkoff H. Relationship of sialic acid residues to in vitro biological and immunological activities of equine gonadotropins. Biol Reprod 1981;24: 1082-1087.

2. Apparailly F, Laurent-Cadoret V, Lecompte F, Chopineau M, Maurel MC, Guillou F, Combarnous Y. Structure-function relationships and mechanism of action of pituitary and placental gonadotrophins. Reprod Fertil Dev 1994;6: 157-163.

3. Bangham DR, Woodward PM. The second international standard for serum gonadotrophin. Bull World Health Organ 1966;35: 761-773.

4. Cahoreau C, Combarnous Y. Comparison of two reference preparations for horse chorionic gonadotrophin in four in-vivo and in-vitro assays. J Reprod Fertil 1987;79: 281-287.

5. Chopineau M, Maurel MC, Combarnous Y, Durand P. Topography of equine chorionic gonadotropin epitopes relative to the luteinizing hormone and follicle-stimulating hormone receptor interaction sites. Mol Cell Endocrinol 1993;92: 229-239.

6. Cole H, Erway J. 48-hour assay test for equine gonadotropin with results expressed in international units. Endocrinology 1941;29: 514-519.

7. Colquhoun D. Lectures on Biostatistics. Oxford UK: Clarendon Press, 1971;425.

8. Combarnous Y, Guillou F, Martinat N. Comparison of in vitro follicle-stimulating hormone (FSH) activity of equine gonadotropins (luteinizing hormone, FSH, and chorionic gonadotropin) in male and female rats. Endocrinology 1984;115: 1821-1827.

9. Combarnous Y, Salesse R, Garnier J. Physico-chemical properties of pregnant mare serum gonadotropin. Biochim Biophys Acta 1981;667: 267-276.

10. Driancourt MA, Thatcher WW, Terqui M, Andrieu D. Dynamics of ovarian follicular development in cattle during the estrous cycle, early pregnancy and in response to PMSG. Domest Anim Endocrinol 1991;8: 209-221.

11. Gonzalez A, Wang H, Carruthers TD, Murphy BD, Mapletoft RJ. Superovulation in the cow with pregnant mare serum gonadotrophin: effects of dose and antipregnant mare serum gonadotrophin serum. Can Vet J 1994;35: 158-162.

12. Hoppen HO. The equine placenta and equine chorionic gonadotrophin--an overview. Exp Clin Endocrinol 1994;102: 235-243.

13. Katagiri S, Takahashi Y, Hishinuma M, Kanagawa H, Dochi O, Takakura H. PMSG profiles in superovulated and anti-PMSG antiserum treated mice and heifers with enzymeimmunoassay. Jpn J Vet Res 1991;39: 11-21.

14. Lapthorn AJ, Harris DC, Littlejohn A, Lustbader JW, Canfield RE, Machin KJ, Morgan FJ, Isaacs NW. Crystal structure of human chorionic gonadotropin. Nature 1994;369: 455-461.

15. Lecompte F, Harbeby E, Cahoreau C, Klett D, Combarnous Y. Use of the immature rat uterotrophic assay for specific measurements of chorionic gonadotropins and follicle-stimulating hormones in vivo bioactivities. Theriogenology 2010;74: 756-764.

16. Lecompte F, Roy F, Combarnous Y. International collaborative calibration of a preparation of equine chorionic gonadotrophin (eCG NZY-01) proposed as a new standard. J Reprod Fertil 1998;113: 145-150.

17. Legardinier S, Duonor-Cerutti M, Devauchelle G, Combarnous Y, Cahoreau C. Biological activities of recombinant equine luteinizing hormone/chorionic gonadotropin (eLH/CG) expressed in Sf9 and Mimic insect cell lines. J Mol Endocrinol 2005;34: 47-60.

18. Legardinier S, Klett D, Poirier JC, Combarnous Y, Cahoreau C. Mammalian-like nonsialyl complex-type N-glycosylation of equine gonadotropins in Mimic insect cells. Glycobiology 2005;15: 776-790.

19. Licht P, Gallo AB, Aggarwal BB, Farmer SW, Castelino JB, Papkoff H. Biological and binding activities of equine pituitary gonadotrophins and pregnant mare serum gonadotrophin. J Endocrinol 1979;83: 311-322.

20. Menzer C, Schams D. Radioimmunoassay for PMSG and its application to in-vivo studies. J Reprod Fertil 1979;55: 339-345.

21. Murphy BD, Martinuk SD. Equine chorionic gonadotropin. Endocr Rev 1991;12: 27-44.

22. Pierce JG, Parsons TF. Glycoprotein hormones: structure and function. Annu Rev Biochem 1981;50: 465-495.

23. Rafert S, Mariot J, Klett D, Combarnous Y. Involvement of Ovarian Estradiol Biosynthesis and Pituitary FSH Expression in the Mechanism of Human Chorionic Gonadotropin Stimulation of Uterine Growth in Immature Female Rats. J of Hormones 2016;2016: 1-7.

24. Sherman GB, Wolfe MW, Farmerie TA, Clay CM, Threadgill DS, Sharp DC, Nilson JH. A single gene encodes the beta-subunits of equine luteinizing hormone and chorionic gonadotropin. Molecular Endocrinology 1992;6: 951-959.

25. Smith PL, Bousfield GR, Kumar S, Fiete D, Baenziger JU. Equine lutropin and chorionic gonadotropin bear oligosaccharides terminating with SO4-4-GalNAc and Sia alpha 2,3Gal, respectively. J Biol Chem 1993;268: 795-802.

26. Steelman SL, Pohley FM. Assay of the follicle stimulating hormone based on the augmentation with human chorionic gonadotropin. Endocrinology 1953;53: 604-616.

27. Stewart F, Allen WR. The binding of FSH, LH and PMSG to equine gonadal tissues. J Reprod Fertil Suppl 1979: 431-440.

28. Stewart F, Allen WR. Biological functions and receptor binding activities of equine chorionic gonadotrophins. J Reprod Fertil 1981;62: 527-536.

29. Stewart F, Allen WR, Moor RM. Pregnant mare serum gonadotrophin: ratio of follicle-stimulating hormone and luteinizing hormone activities measured by radioreceptor assay. J Endocrinol 1976;71: 471-482.

30. Ward DN, Moore WT, Burleigh BD. Structural studies on equine Chorionic Gonadotropin. Journal of Protein Chemistry 1982;1: 263-280.

31. Wu H, Lustbader JW, Liu Y, Canfield RE, Hendrickson WA. Structure of human chorionic gonadotropin at 2.6 A resolution from MAD analysis of the selenomethionyl protein. Structure 1994;2: 545-558.

32. Yu XF, Cho SJ, Bang JI, Lee HS, Lee YS, Kwon TH, Deb GK, Kong IK. Effect of equine chorionic gonadotropin on the efficiency of superovulation induction for in vivo and in vitro embryo production in the cat. Theriogenology 2010;73: 413-420.

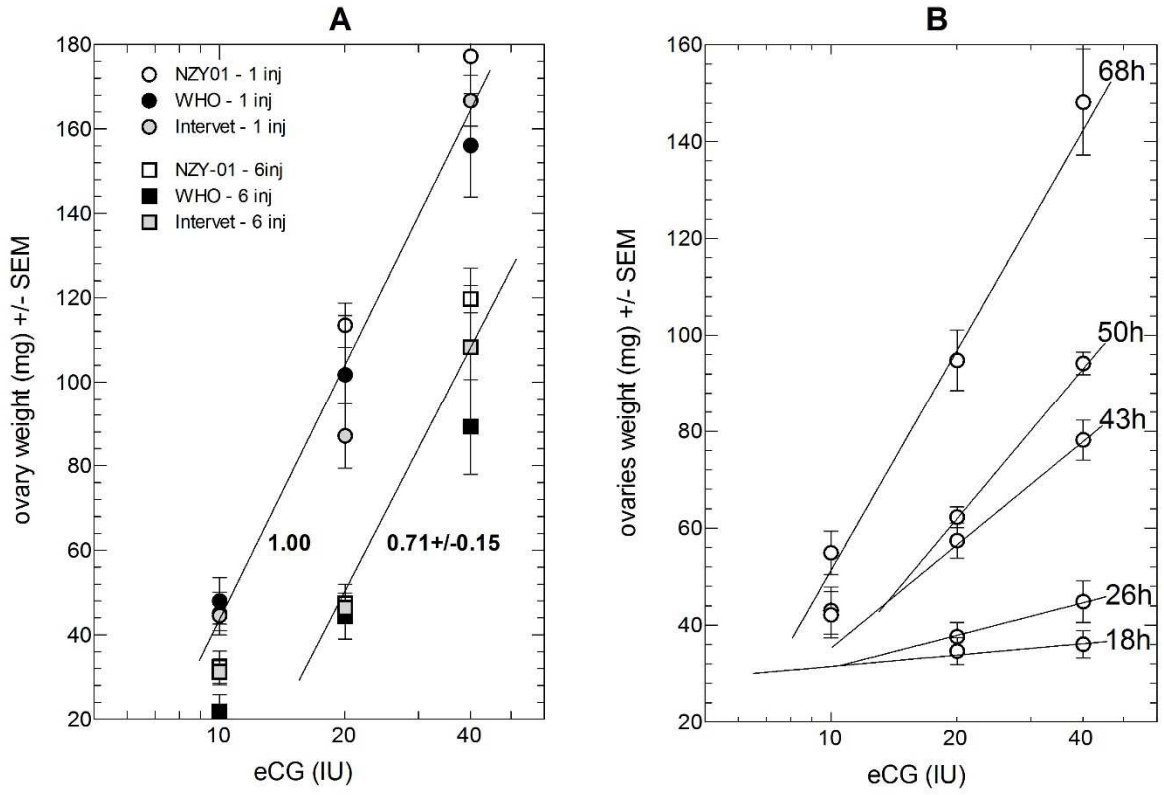


Figure 1. A: **Dose- and injection number-dependence of the ovarian weight response to eCG**. The three eCG preparations were injected subcutaneously at doses of 10, 20 and 40 IU into immature female rats (N=6). The doses were injected either as a single 100µL bolus 68h before sacrifice (circles), or as 6 injections, each containing 1/6 of the total dose in 100µL at 68, 60, 46, 39, 22 and 14 h prior to sacrifice (squares). The relative potencies in the two protocols are shown.

B: **Time-dependence of the ovarian weight response to eCG**

eCG NZY-01 at 10, 20 and 40 IU was given as a single subcutaneous injection in 100µL at the five times shown, 18 to 68h before sacrifice.

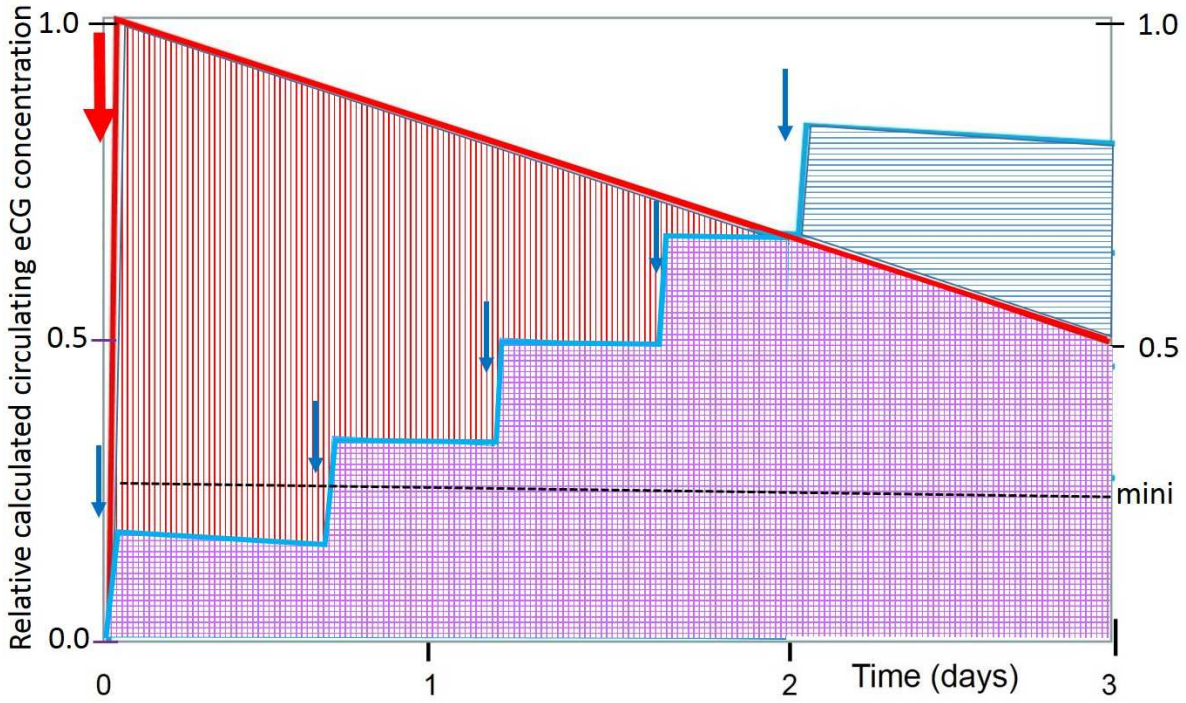


Figure 2. Schematic view of relative calculated eCG concentrations in blood, after single-injection (red) and multiple injection (blue) protocols. In this scheme, the half-life for eCG was taken as 3 days. The dashed black line represents a conventional minimum value, for the eCG concentration in blood, sufficient to permit binding to FSH and LH receptors, and to promote ovarian weight gain.