Journal of Advances in Medical and Pharmaceutical Sciences
12(2): 1-9, 2017; Article no.JAMPS.31956
ISSN: 2394-1111

SCIENCEDOMAIN *international*
www.sciencedomain.org

Biological Activity of Different Batches of Equine Chorionic Gonadotropin as Determined by Reversed-phase High-performance Liquid Chromatography and *in vivo* Assay

Rafael Herrera Alvarez[1*], Fabio L. Nogueira Natal[1], Beatriz E. Almeida[2], João E. Oliveira[2], Alfredo J. Ferreira Melo[1], Maria T. Carvalho Ribela[2] and Paolo Bartolini[2]

[1]Agency for Agribusiness Technology of São Paulo, Postbox 28, 13400-970 Piracicaba-SP, Brazil.
[2]Department of Biotechnology, IPEN-CNEN, Av. Lineu Prestes 2242, Cidade Universitária 05508-000, São Paulo, Brazil.

Authors' contributions

This work was carried out in collaboration between all authors. Authors RHA, MTCR and PB designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors FLNN, BEA, JEO and AJFM managed the analyses of the study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMPS/2017/31956
Editor(s):
(1) Palmiro Poltronieri, National Research Council of Italy, CNR-ISPA, Italy And Institute of Sciences of Food Productions, Operative Unit in Lecce, Italy.
Reviewers:
(1) Dimal Shah, Babaria Institute of Pharmacy, Vadodara, Gujarat, India.
(2) Feyyaz Onur, Ankara University, Turkey.
Complete Peer review History: http://www.sciencedomain.org/review-history/18132

Original Research Article

Received 31st January 2017
Accepted 3rd March 2017
Published 9th March 2017

ABSTRACT

Aims: To evaluate the physicochemical profile of commercial batches of eCG, in order to find if differences can be related to their biological activity.
Study Design: Commercial eCG was analyzed by reversed-phase high-performance liquid chromatography **(**RP-HPLC) and *in vivo* bioassay.
Place and Duration of Study: Department of Biotechnology (IPEN-CNEN) and Animal antibody production Laboratory (Animal Science Institute), between June 2013 and April 2014.

**Corresponding author: E-mail: rherrera@apta.sp.gov.br;*

Methodology: Two commercial eCG batches for veterinary use (I and II) and an eCG official International Standard from the World Health Organization (WHO) were analyzed by RP-HPLC. Additionally, two experiments were designed to validate the biological activity. In experiment 1, groups of prepubertal 21–25 day old Wistar female rats (n = 6/group) received the equivalent to 0 UI (saline) and 10 IU of eCG of each one of these preparations. Autopsy was performed 48 h later and ovaries were removed and weighed. The experiment 2 was designed to determine whether increasing the dose of less active eCG batches could increase the ovarian response. Therefore, groups of prepubertal rats (n = 6/group) were treated with 10 and 30 IU eCG from batch II, while eCG from WHO (10 IU) and saline were control. The evaluation of ovarian response was done similar to Experiment 1. Differences among treatments were analyzed by one-way ANOVA.
Results: Results of RP-HPLC showed differences in the main t_R peak profile (t_R 26.7) of the standard WHO compared with eCG batches I and II. In experiment 1, the average ovarian weight of rats treated with eCG from WHO (60.0 ± 12.1 mg) was higher ($P < .01$) than saline (23.1 ± 1.6 mg) and batches I (37.6 ± 1.4 mg) and II (31.0 ± 4.3 mg). In experiment 2, the ovarian weight of rats treated with 30 IU of eCG of batch II (45.7 ± 4.1 mg) was higher ($P < .01$) than saline (32.6 ± 1.4 mg) and significantly lower ($P = .05$) than 10 UI of the standard WHO (63.3 ± 8.1 mg).
Conclusion: The low ovarian response to eCG treatments can be related to differences in the physicochemical profile of eCG batches and RP-HPLC is a fast and reliable tool for detecting these differences.

Keywords: eCG; RP-HPLC; rat; ovary; physicochemical profile.

1. INTRODUCTION

Gonadotropins play an important role in the control of endocrine and reproductive process in mammals. Equine chorionic gonadotropin (eCG) is a glycoprotein hormone produced by trophoblast cells in the mare between the 36th and 120th days of pregnancy [1]. Similar to other glycoprotein hormones, eCG is composed of two different subunits, α and β, whose non-covalent association is required to exercise its biological activity [2]. The α subunit is common to all the glycoprotein hormones (LH, FSH, TSH, hCG), while the β-subunit is specific and responsible for receptor binding specificity. In mares, the dimeric eCG binds only to the LH receptor [3], while in other species displays a marked follicle-stimulating activity, in addition to luteinizing activity [4,5]). Still, eCG is the heaviest (~ 45 to 60 kD) glycosylated glycoprotein, due to the high amounts of carbohydrates, about 45%, of which 10% correspond to sialic acid [5-7]. This high content of sialic acid would be responsible for the long half-life of eCG, of 21 hours in sheep [7] and 45.6 hours in cows [8]. Thus, the double activity (FSH-LH), its long half life and its availability in large quantities make of eCG an extremely convenient hormone to be used in the animal production industry.

In pigs, for example, eCG has been used to induce early puberty of replacement females and to induce and synchronize estrus of sows after weaning [9]. In ruminants, the use of eCG at doses lower than 1000 IU has been indicated to stimulate ovarian activity in anoestrus animals submited to hormonal synchronization protocols for fixed-timed artificial insemination [10-12], whereas doses higher than 2000 IU have been established to increase the number of ovulations (superovulation) of females involved in embryo transfer programs [13]. However, sometimes the results of eCG treatment are inconsistent, with some animals exhibiting excessive stimulation, while others do not change their ovarian condition [13,14]. This variability of ovarian response has been related to the animal (breed, species, body condition, ovarian condition at the time of application, etc.) and dose of eCG [13-15]. It is also possible that ovarian variability can be also explained by the use of different batches of commercial eCG, as shown in cows treated with commercial pituitary FSH of different sources [16].

Currently, the standardization of the biological potency, in IU, of commercial eCG products or batches is carried out by tests measuring the increase in the genital tract of prepubertal female rats to increasing doses of the hormone [17]. However, limitations in the quality control process or inadequate conservation conditions could determine a loss in potency of commercial eCG batches. Recently, our group developed a chromatographic method (RP-HPLC) able to detect structural changes responsible for the loss of biological activity of eCG [18]. This study aimed to establish the physicochemical profile in

commercial batches of eCG by RP-HPLC method and associate these structural differences with his ovarian activity in prepubertal rats.

2. MATERIALS AND METHODS

2.1 eCG Source

Two batches of a commercial eCG were purchased at a local veterinary pharmacy. In order to preserve the identity of the manufactured eCG, batches were coded as I and II. As reference standard was used the WHO International Standard of eCG (NIBSC code 62/001), purchased from the National Institute for Biological Standards and Control (NIBSC, South Mimms, UK).

2.2 Reversed-phase High-performance Liquid Chromatography (RP-HPLC)

The identity and purity of the commercial and reference preparations of eCG were investigated by RP-HPLC for human and mare gonadotropins according methodology set up in our laboratory [18]. Briefly, aliquots of 100 µL (100 IU) of eCG were processed in a Shimadzu Model SCL-10AHPLC apparatus equipped with a SPD-10AV UV detector and a C4-Grace Vydac (Separation group, Hesperia, CA, USA) 214 TP 54 column (25 cm x 4.6 mm I.D., pore diameter of 300 Å and particle diameter of 5 µm) coupled to a guard column Grace Vydac 214 FSK 54, used for separation. Mobile phase with a flow rate of 0.5 mL/min and column temperature at 25 ℃ were used. Detection was by UV at 220nm and quantification was achieved by peak area determination reported to the International Standard of eCG-WHO 612/001. Gradient solutions A and B were utilized, solution A being ammonium phosphate buffer (pH 8.6; 0.05M) and solution B acetonitrile. The elution was performed with a linear gradient from A:B (85:15, v/v) to A:B (50:50, v/v) over 40 min, at a flow-rate of 0.5 ml/min. The water used was obtained from a Milli-Q Plus water-purification system (Millipore, Bedford, MA, USA). Acetonitrile (HPLC-grade, Mallinckrodt Baker) was purchased from Hexis (São Paulo, Brazil). All other chemicals were analytical reagent grade, purchased from Merck (São Paulo, Brazil). The precision of this specific quantitative methodology based on direct eCG determinations provided CVs of 1.3% and 3.1% for intra- and inter-assay, respectively. From a qualitative point of view, we calculated retention times (tR) with CVs $<$ 0.4% and $<$ 3% for intra-day and inter-day determinations, respectively. For what concerns a complete validation of the linear dose-response curve we can refer to the data obtained in an analogous RP-HPLC assay based on the same methodology for human hormones (hCG and hLH) [19]. In that case, the equation of the curve was: Yarea = 959.4 Xµg − 27.6 (r = .9999; $P < .001$; n = 20), the sensitivity was 34 ng, and the average recovery was 99.7 ± 3.04%.

2.3 *In vivo* Biological Assay

2.3.1 Experiment 1

The biological activity of different eCG batches was assessed using the *in vivo* method of Cole and Erway [16] recognized by the international pharmacopoeia. Briefly, Wistar albino rats (*Rattus Norvegicus)* with 21-23 days of age weighing 65.5 ± 1.2 g were purchased from the Multidisciplinary Center for Biological Investigation of Laboratory Animal Science (CEMIB-UNICAMP, Campinas, Brazil). Animals were housed in a temperature-controlled room (24 ± 2℃) in clean polypropylene cages under standard condition and were given standard pellet diet and water ad libitum. Rats were randomly separated in groups with six animals each and received an ip injection of 10 IU/animal of eCG from batch I (group 1), batch II (group 2) and International Standard WHO (group 3), reconstituted in 100 µL of saline buffer solution, while the control (group 4) only received saline solution. Forty-eight hours after injection of treatments, rats were euthanized via ip injection of a mixture of 80 mg of ketamine with 15 mg of xylazine and removed the genital tract. The ovaries were dissected free of surrounding tissue and weighed separately on an analytical balance.

2.3.2 Experiment 2

The objective of this experiment was to determine whether increasing the dose of the less efficient eCG batch could improve the ovarian response. Therefore, groups of prepubertal rats (n=6/group) were treated with 10 IU (group 1) or 30 IU (group 2) of well-known low efficiency eCG (batch II). As control, were used groups of animals treated with 10 IU of eCG International Standard WHO (group 3) or saline solution (group 4), respectively. The evaluation of ovarian response was done as described in Experiment 1.

2.4 Statistical Analysis

The statistical analysis was carried out by utilizing the software StatPlus [20]. Differences of ovarian weight among treatments were analyzed by one-way ANOVA and, when a significant difference was found, the averages were compared via the Duncan test. Data results were represented as Mean ± Standard Error of Mean.

3. RESULTS

3.1 Reversed-phase High-performance Liquid Chromatography (RP-HPLC)

The result of the chromatographic profile (RP-HPLC) of samples of eCG is shown in Fig. 1. The International Standard WHO showed an isolated major peak of retention time (t_R ~ 26.7 min), with area of 3,346 mAU and magnitude of 41627 mAU. Batches I and II, however, presented a very small pic at this t_R (area of 2,801 mAU and magnitude of 24272 mAU) showing also two much higher peaks at quite different t_R (Fig. 1).

3.2 *In vivo* Biological Assay

3.2.1 Experiment 1

With exception of one rat treated with eCG from the batch I and another from the standard WHO, respectively, all rats treated with eCG had an ovarian weight higher than the mean ± two standard deviations of the control group. The average weight of the ovaries of rats treated with the standard WHO (60.0 ± 12.1 mg) was significantly higher than saline (23.1 ± 1.6 mg) and eCG batches I (37.6 ± 1.4 mg) and II (31.0 ± 4.3 mg). Batch I was superior (P = .04) to saline, while batch II was not different of saline and batch I (Fig. 2).

3.2.2 Experiment 2

In Table 1 are shown the results of the ovarian response to increased dosage of batch II eCG. The ovarian weight of rats treated with 30 IU of eCG of the batch II (45.7 ± 4.1 mg) was higher (P < .01) than saline group (32.6 ± 1.4 mg) and significantly lower (P < .05) than 10 UI of the standard WHO (63.3 ± 8.1 mg). The ovarian weight of rats treated with 10 IU eCG of the batch II (38.6 ± 4.1 mg) was not different from saline control.

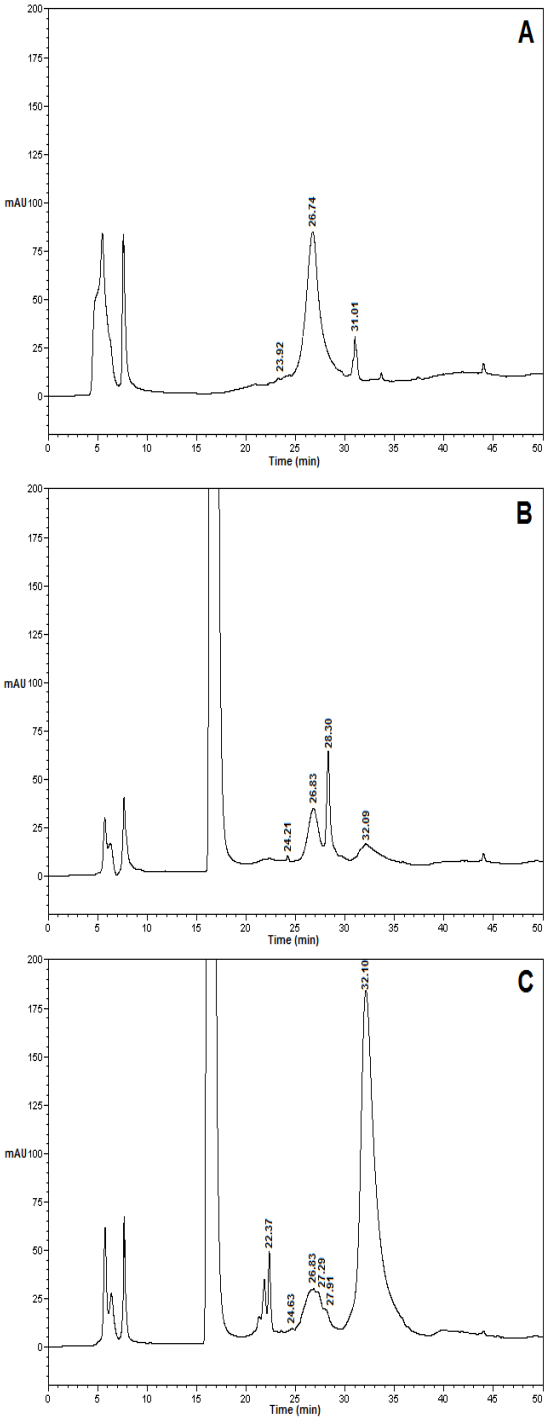

Fig. 1. Reversed-phase high-performance liquid chromatography analyses showing retention time (t_R) of 100 µl (equivalent to 100 IU) from WHO (A), batch I (B) and batch II (C) eCG

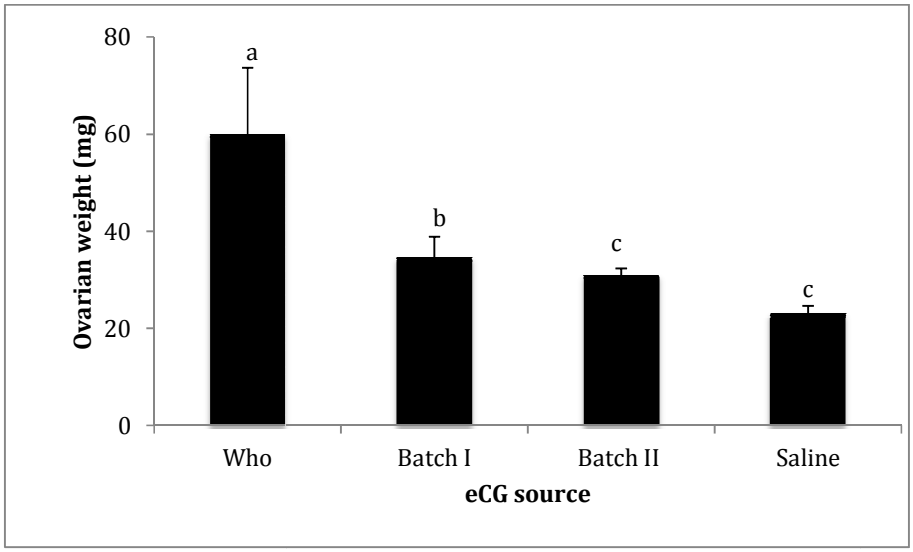

Fig. 2. Ovarian weight (mean ± SEM) of Wistar rats treated with 10 IU eCG from WHO, batches I and II or saline (control). Different letters indicate significant differences between treatments (P < .05)

Table 1. Ovarian weight of Wistar rats treated with eCG from WHO (10 IU), batch II (10 IU or 30 IU) and saline (control)

eCG source	Dose (IU)	Ovarian weight (mg)
WHO	10	63.3 ± 8.1^{ab}
Batch II	10	38.6 ± 4.2^{bc}
Batch II	30	45.7 ± 4.1^{b}
Saline	0	32.6 ± 1.4^{c}

Different superscript letters indicate significant differences between treatments (P < .05). Data are presented as mean ± SEM

4. DISCUSSION

In recent decades, many tests have been developed to monitor the quality of commercial preparations of gonadotropins, with emphasis on bioassays, immunoassays and physicochemical tests. Each one has advantages and disadvantages that should be considered in order to select the most appropriate. Bioassays are typically conducted to evaluating the response of a biological system (*in vivo* or *in vitro*) to gonadotropin stimulation. The *in vivo* assay considering the increase of genital tract (ovary and uterine weight) in prepubertal female rats [17] is still the only approved test for the evaluation of eCG potency, however, there is a growing ethical restriction to the use of animals for those tests. The *in vitro* assays have the limitation of not taking into account possible differences in the half-life of hormone preparations [21]. The immunoassays (RIA and ELISA) are able to precisely quantify the content of immunologically active preparation, but may be unable to bind to specific receptors and therefore unable to stimulate the target cell response [22]. Finally, physicochemical analysis tests, such as size exclusion chromatography [23], isoelectric focusing [24] and more recently, reverse phase HPLC [25,26] have made possible the quantitative analysis of gonadotropins with speed, sensitivity and precision. Indeed, various HPLC separation modes have been applied to gonadotropin preparations, including reversed-phase (RP-HPLC), hydrophobic interaction (HI-HPLC), high-performance size-exclusion chromatography (HPSEC) and ion exchange (IE-HPLC) chromatography, based on different molecular properties such as hydrophobicity, size and charge [26]. Although chemical and physical alterations and post-translational modifications in the structure of the proteins that compromise their integrity can in fact be evaluated by some of these HPLC modes, RP-HPLC is practically the only tool available for identification of altered forms generated by methionine oxidation or disulphide exchange [26]. Their ability of exploiting differences in hydrophobicity and polarity of target molecules makes of the RP-HPLC the most used HPLC separation mode in a number of analytical fields, including quality control of biopharmaceuticals, such as commercial gonadotropins of chorionic and

pituitary origin [18,25]. Moreover, recently it was shown that RP-HPLC can be highly specific for the analyte and under certain circumstances it reflects the biological activity as determined in bioassays [18,25].

Results of experiment 1 suggest that, compared with the standard eCG from WHO, the biological potency of the two batches of commercial eCG was significantly lower. Moreover, batch II has a less amount of eCG than batch I, as shown by the peak area differences of supposedly active eCG peak (t_R 26.7). Peaks with very different t_R, as the observed in batches I and II, can be due to protein impurities, to different excipients added to the drug formulation or degradation during storage [25]. However, drugs adequately freeze-dried (lyophilized) remain stable for several years even when stored without refrigeration [27], therefore, degradation may have occurred during purification or lyophilization processes [28]. The eCG manufacturing process generally consists of fractional precipitation, removal of impurities, pH adjustment, final precipitation in the presence of increasing concentrations of acetone or alcohol and lyophilization before packaging [29]. As evidenced in processed urinary hMG, perhaps mare sera not only contain eCG, but also relatively high percentages of other protein impurities including leucocyte elastase inhibitor, protein C inhibitor, and zinc-α_2-glycoprotein, that are not reported to induce follicular development and which may influence the efficacy and possible safety of gonadotropin products [30]. None of these contaminants appear to confer any proven advantage in terms of clinical outcome and, moreover, the concentration and type of impurities varies considerably between batches [31]. It is also possible that contaminants such as residual sanitizing agents, materials leaching from product contact surfaces, and contaminants from the environment of the production area can be introduced during the manufacturing process, resulting in structural alterations related to the carbohydrate moiety, with a consequent alteration in the hydrophobicity and possibly in the bioactivity of the molecule [28]. Moreover, eCG is usually collected from pregnant mares and since bioactivity varies between individual mares and different days of gestation [2], it is likely that different batches could show similar variation. Using a competitive eCG ELISA test, Ciller et al. [32] compared various commercial preparations of eCG relative to isoform composition. Liquid phase iso-electric focusing was used to fractionate plasma and the commercial preparations were grouped into acidic (pH 3.0–5.1), intermediate (pH 5.2–7.9), or basic (pH 8.0–10.0) isoform categories. Immunoactivity between commercial eCG products ranged from 44% to 362% of stated bioactivity. Iso-electric focusing showed that the majority of the immunoactivity (92%) of the commercial preparations was found in the acidic fractions (pH 3.0–5.1), and in particular in the pH range 3.0–3.8. Testes *in vivo* with female mice showed differences in the ovaries weight associated with subtle differences in the pH of these eCG isoforms [33].

On the other hand, there is evidence that biological efficiency of eCG could be related with differences in the titer of eCG of serum collected of pregnant mares [34]. The eCG isolated from the serum of mares with low eCG titer had lower amounts of carbohydrates and sialic acid and resulted in significantly lower biological response compared with the serum of high titer. The importance of sialic acid content in the biological activity *in vivo* would be a result of the eCG residence time in the bloodstream [7]. Martinuk et al. [7] noted that the experimental removal of 80% of the sialic acid from the eCG molecule resulted in the eCG disappearance of blood within 1 h, whereas the intact eCG was still present after 120 h. Consequently, if the batches I and II were affected by a partial desilylation or alteration of isoforms, their length of stay in the systemic circulation would be insufficient to cause significant changes in the condition of the ovaries.

The threefold increase in the dose of batch II (experiment 2) resulted in an improvement in ovarian response. In rats, Martinuk et al. [7] found that dosages of 10-100 IU of intact eCG increased the ovulation rate and ovaries weight compared to treated with saline. The application of the same dose of eCG without 20% of the sialic acid also caused superovulation and increase in ovarian weight, but it was necessary to increase the dose (100-500 IU) to achieve the same result as that produced by the intact eCG. Finally, removing 53% and 80% of the sialic acid from eCG preparations induced a slight superovulatory response but no response in ovarian weight [7]. The smaller amount of bioactive eCG in some batches of commercial eCG would be responsible for the variability of ovarian response in domestic animals, as evidenced by Newcomb et al. [35]. Comparing three different batches of commercial eCG to superovulate heifers, the authors found that one

of the batches had, in average, 16.7% (max. 41%) less total gonadotrophic potency. Heifers treated with this batch had the lowest ovarian response (6,2 ovulations) compared to the others batches (9.4 ovulations).

This study demonstrates the extreme care that must be exercised in the quality control of eCG preparations and confirm that changes in physicochemical profile of different eCG batches may be related to the large variation in ovarian response observed in domestic animals treated with eCG. Obviously, a single type of physicochemical analysis cannot mirror all aspects of the *in vivo* biological activity of a hormone. Even so, there is a growing tendency to substitute *in vivo* bioassays by analytical chemistry (notably RP-HPLC) to validate methods or batches of manufactured hormones that could complement the classical *in vivo* bioassay [36].

5. CONCLUSION

The biological activity of the eCG hormone seem to be related to its physicochemical profile, thus, considering the ability of RP-HPLC to detect alterations in this profile it can be an interesting tool for using in the quality control of commercial eCG products.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the ethics committee of the Animal Science Institute (resolution IZ-159).

ACKNOWLEDGEMENTS

The authors wish to thank to FAPESP for financial support (grant 2011/13096-0).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Allen WR, Moor RM. The origin of the equine endometrial cups. I. Production of PMSG by fetal trophoblast cells. J Reprod Fertil. 1972;29:313-316.
2. Murphy BD. Equine chorionic gonadotropin: An enigmatic but essential tool. Anim Reprod. 2012;9:223-30.
3. Sugino H, Bousfield GR, Moore WT Jr, Ward DN. Structural studies on equine glycoprotein hormones. Amino acid sequence of equine chorionic gonadotropin beta-subunit. J Biol Chem. 1987;262:8603-9.
4. Moore Jr WT, Ward DN. Pregnant mare serum gonadotropin. An *in vitro* biological characterization of the lutropin-follitropin dual activity. J Biol Chem. 1980;255:6930-6.
5. Combarnous Y, Salesse R, Garnier J. Physico-chemical properties of pregnant mare serum gonadotropin. Biochim Biophys Acta. 1981;667:267-76.
6. Cole HH, Bigelow M, Finkel J, Rupp GR. Biological half-life of endogenous PMSG following hysterectomy and studies on losses in urine and milk. Endocrinology. 1967;81:927-30.
7. Martinuk S, Manning A, Black W, Murphy B. Effects of carbohydrates on the pharmacokinetics and biological activity of equine chorionic gonadotrophin *in vivo*. Biol Reprod. 1991;45:598-604.
8. Schams D, Mentzer C, Schallenberger E, Hahn J, Hahn R. Some studies of pregnant mare serum gonadotropin and on endocrine responses after application for superovulation in cattle. In: Sreenan J, editors. Control of Reproduction in the Cow. The Hague: Martinus Nijhoff; 1978.
9. Manjarin R, Cassar G, Sprecher DJ, Friendship RM, Dominguez JC, Kirkwood RN. Effect of eCG or eCG plus hCG on oestrus expression and ovulation in prepubertal gilts. Reprod Dom Anim. 2009; 44:411-13.
10. Baruselli PS, Reis EL, Marques MO, Nasser LF, Bo GA. The use of hormonal treatments to improve reproductive performance of anestrous beef cattle in tropical climates. Anim Reprod Sci. 2004; 82-83:479-86.
11. Souza AH, Viechnieski S, Lima FA, Silva FF, Araújo R, Bó GA, et al. Effects of equine chorionic gonadotropin and type of ovulatory stimulus in a timed-AI protocol on reproductive responses in dairy cows. Theriogenology. 2009;72:10-21.
12. Sá Filho MF, Torres Junior JRS, Penteado L, Gimenes LU, Ferreira RM, Ayres H, et

al. Equine chorionic gonadotropin improves the efficacy of a progestin-based fixed-time artificial insemination protocol in Nelore (Bos indicus) heifers. Anim Reprod Sci. 2010;118:182-7.

13. Bo GA, Mapletoft, RJ. Historical perspectives and recent research on superovulation in cattle. Theriogenology. 2014;81:38-48.
14. Gonzalez A, Wang H, Carruthers TD, Murphy BD, Mapletoft RJ. Superovulation in the cow with pregnant mare serum gonadotrophin: Effects of dose and anti pregnant mare serum gonadotrophin serum. Can Vet J. 1994;35:158-62.
15. Ptak G, Tischner M, Bernabo N, Loi P. Donor-dependent developmental competence of oocytes from lambs subjected to repeated hormonal stimulation. Biol Reprod. 2003;69:278-285.
16. Henderson KM, Weaver A, Wards RL, Lun S, McNatty KP. Comparison of commercial gonadotrophins using bioassays. Proceedings of the New Zealand Society of Animal Production. 1990;50:161-5.
17. Cole H, Erway J. 48-hour assay test for equine gonadotropin with results expressed in international units. Endocrinology. 1941;29:514-19.
18. Alvarez RH, Natal FLN, Ribela MTCP, Almeida BE, Oliveira JE, Bartolini P. Physical-chemical and biological characterization of different preparations of equine chorionic gonadotropin. J Vet Sci. 2016;17:459-65.
19. Almeida BE, Oliveira JE, Carvalho CM, Dalmora SL, Bartolini P, Ribela MTCP. Analysis of human luteinizing hormone and human chorionic gonadotropin preparations of different origins by reversed-phase high-performance liquid chromatography. J Pharm Biomed Anal. 2010;53:90-7.
20. StatPlus:mac of AnalystSoft Inc. –Analyze statistics program. Version 2009. Available:www.analystsoft.com/br/
21. Rose MP, Gaines Das RE, Balen AH. Definition and measurement of Follicle Stimulating Hormone. Endocrine Reviews. 2000;21:5-22.
22. Braileanu GT, Albanese C, Card C, Chedrese PJ. FSH bioactivity in commercial preparations of gonadotropins. Theriogenology. 1998;49:1031-7.
23. Driebergen R, Baer G. Quantification of follicle stimulating hormone (follitropin alfa): Is *in vivo* bioassay still relevant in the recombinant age? Curr Med Res Opin. 2003;19:41-6.
24. Mulders JWM, Derksen M, Swolfs A, Maris F. Prediction of the *in vivo* biological activity of human recombinant follicle stimulating hormone using quantitative isoelectric focusing. Biologicals. 1997;25: 69-281.
25. Almeida BE, Oliveira JE, Damiania R, Dalmora SL, Bartolini P, Ribela MTCP. A pilot study on potency determination of human follicle-stimulating hormone: A comparison between reversed-phase high-performance liquid chromatography method and the *in vivo* bioassay. J Pharm Biomed Anal. 2011;54:681-6.
26. Ribela MTCP, Gout PW, Oliveira JE, Bartolini P. HPLC analysis of human pituitary hormones for pharmaceutical applications. Current Pharmaceutical Analysis. 2006;2:103-26.
27. Remmele RL, Krishnan S, Callahan WJ. Development of stable lyophilized protein drug products. Curr Pharm Biotechnol. 2012;13:471-96.
28. Wang W, Ignatius AA, Thakkar SV. Impact of residual impurities and contaminants on protein stability. J Pharm Sci; 2014.
DOI: 10.1002/jps.23931
29. Lunenfeld B. Historical perspectives in gonadotrophin therapy. Hum Reprod Update. 2004;10:453-467.
30. Ezcurra D, Humaidan P. A review of luteinizing hormone and human chorionic gonadotropin when used in assisted reproductive technology. Reprod Biol Endocrinol. 2014;12:95.
DOI: 10.1186/1477-7827-12-95
31. Giudice E, Crisci C, Altarocca V, O'Brien M. Characterization of a partially purified human menopausal gonadotropin preparation. J Clin Res. 2001;4:27-33.
32. Ciller UA, Ciller IM, McFarlane JR. Equine chorionic gonadotrophin isoform composition in commercial products compared to isoform composition in pregnant mare plasma. Reprod Fertil Develop. 2008;20:77(Supplement).
33. Ciller UA, McFarlane JR. Commercial equine chorionic gonadotrophin isoform composition, immunoactivity, and bioactivity in a murine model using ovarian augmentation and testicular interstitial cell bioassays. Reprod Fertil Develop. 2009; 21:111(Supplement).

34. Aggarwal BB, Farmer SW, Papkoff H, Seidel GE Jr. Biochemical properties of equine chorionic gonadotrophin from two different pools of pregnant mare sera. Biol Reprod. 1980;23:570-6.
35. Newcomb R, Christie WB, Rowson LEA, Walters DE, Bousfield WED. Influence of dose, repeated treatment and batch of hormone on ovarian response in heifers treated with PMSG. J Reprod Fertil. 1979; 56:113-18.
36. Garthoff B, Hendriksen CFM, Bayol A, Goncalves D, Grauer A, Deleeuw R, et al. Safety and efficacy testing of hormones and related products – The report and recommendations of Ecvam workshop-9, ATLA-Altern Lab Anim. 1995;23:699-712.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/18132