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Measurement of Equine Placental Gonadotropin with the Antiserum Specific to Equine Pituitary Gonadotropin from Pregnant Mare Serum

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Abstract: ***Rabbit antiserum to equine pituitary Luteinizing Hormone (eLH) was generated and characterized in terms of titer, specificity and cross reaction with probable antigens with potential shared epitopes. The rabbit antiserum to equine pituitary Luteinizing Hormone (eLH) is intriguing as it demonstrates its ability to cross - react with other related hormones such as Pregnant Mare Serum Gonadotropin (PMSG) to varying degrees presents an interesting tool for hormone measurement and these hormones share some common epitopes or structural similarities with eLH. While cross - reactivity offers opportunities for multiplexed hormone measurements, it also requires careful consideration during assay development and data interpretation to ensure accurate and specific results. Further, a competitive ELISA based on the rabbit antiserum to eLH was standardized for the measurement of Pregnant Mare Serum Gonadotropin (PMSG) from the PMS. By calibrating this assay, it becomes possible to quantify the amount of PMSG present in an unknown sample based on the degree of competition with the labelled PMSG.***

Keywords: PMSG; eLH; FSH, CG; Gonadotropin.

1. Introduction

PMSG is a glycoprotein hormone of equine origin and exhibits both lutropin and follitropin biological activity in heterologous species likes rat, sheep, cattle etc. (Combarnous *et al.*, 1978; Donrov *et al*., 1998) but with predominantly LH like activity in their respective species i. e., horse/mare (Roser et al., 1986). The structural basis for this dual activity remains incompletely understood. The presumptive reason is that the dual biological activity could be dependent on the structural determinant (either protein part or carbohydrate part) of PMSG or it depends on the promiscuity of the FSH and LH receptor of heterologous animals. The last few decades had witnessed a swift development of a variety of new techniques and probes to comprehend the regulatory functions of hormones. One such tool is antibodies against hormones. Antibodies against gonadotropins, Lutropin (LH) and Follitropin (FSH), in addition to showing hormonal specificity, react with LH and FSH of a variety of other species (Moudgal *et al*., 1978, Alexandra M.2021). Gonadotropin specific antibodies were employed to understand several aspects of structure of hormones with respect to their immunological properties and functions (Berger *et al*., 1988; Moyle *et al*., 2004). Further, specific gonadotropin antibodies had also been employed successfully in devising procedures like follicular maturation, ovulation, and implantation and also as a vaccine to prevent or terminate pregnancy. This aspect has been reviewed by Moudgal and Rao in 1984. The gonads of both the male and female of a variety of species (e. g., rat, mouse, hamster, rabbit and monkey) are extremely susceptible to dispossession of LH support. Maurel *et al* in 1992 established an immunochemical mapping of PMSG using three monoclonal antibodies raised against the native PMSG. Single injection of PMSG (15 IU) was able to induce 3 - fold increase in functional FSH receptor and 12 - fold increase in functional LH receptor concentration of granulosa cells of immature rat up to 72hrs after which a sharp decline could be observed.

Even though numerous roles have been proposed for PMSG in the FEMALE horse and other heterologous species (Martinuk et al, 1990, 1991) but most of them have not been ascertained with validated reports However, the equine LH receptor displays enhanced homology with other mammalian LH receptors, as well as its potential to interact with its affinity towards the source of LH from other species (Saint - Dizier et al., 2011).

As mentioned above, even though a great amount of work had been performed using antibody probes for LH, FSH and hCG, barring a few reports there are not many reports for PMSG hence we decided to generate an immunological probe which can help us in elucidating the enigmatic dual role of PMSG. In order to understand the structure - function relationship of PMSG, immunological probes against PMSG were generated and characterized. A competitive ELISA to measure PMSG from PMS was developed and their sensitivities were also analysed.

2. Materials and Methods

2.1 Materials

Equine LH antiserumwase raised in our Hormone Research lab. Crude buLH 50% LH pellet (Ammonium sulphate) was prepared in hormone research lab as per published procedures. eLH was purchased from Sigma Aldrich Company, USA. PMSG 1000 IU/mg was purchased from Sigma Aldrich Company, USA and PMS was procured from Gemini Bio products, USA. hCG 5000 IU/mg and Ortho Phenylene Diamine was purchased from Sigma Aldrich

Company, USA. ELISA Plates were procured from Greiner bio one, Microloan, Made in Germany. Goat anti - rabbit IgG HRP Conjugate and Goat anti - rat IgG HRP conjugate was purchased from Bangalore Genei Ltd, Bangalore, India. Ammonium bicarbonate, Disodium hydrogen phosphate, Sodium chloride, Potassium dihydrogen phosphate, Oxalic acid, Hydrogen peroxide, and buffer tablets of pH 7 were purchased from Merck (India) Limited, Mumbai and were of GR quality. Sodium phosphate monobasic (Anhydrous), Citric Acid (monohydrate) purchased from Sisco Research Laboratories Mumbai and Sodium azide purchased from Central Drug House. Mumbai, India was of AR quality. Trisodium Citrate, di - Sodium hydrogen ortho phosphate and Potassium chloride of ExcelaR quality was purchased from Fine Chemicals, Mumbai. Casein was purchased from SRL. Mumbai and Tween - 20 was purchased from MP Biomedical, France. Ammonium bicarbonate, Disodium hydrogen phosphate, Sodium chloride, Potassium dihydrogen phosphate, Oxalic acid, Hydrogen peroxide, and buffer tablets of pH 7 were purchased from Merck (India) Limited, Mumbai and were of GR quality. Sodium phosphate monobasic (Anhydrous), Citric Acid (monohydrate) purchased from Sisco Research Laboratories Mumbai and Sodium azide purchased from Central Drug House. Mumbai, India was of AR quality. Trisodium Citrate, di - Sodium hydrogen ortho phosphate and Potassium chloride of ExcelaR quality were purchased from Fine Chemicals, Mumbai. Casein was purchased from SRL. Mumbai and Tween - 20 was purchased from MP Biomedical, France. Agarose was purchased from Hi Media laboratories, Mumbai. Goat anti - rabbit IgG HRP Conjugate was purchased from Bangalore Genei Ltd, Bangalore, India. ELISA Plates from Greiner bio one, Micro Lon, were 96 wells, Flat Bottom, High Binding (LOT E100602N, REF 655061). ECIL, India makes ELISA Reader (MICROSCAN MS5605), Weighing balance (Shimadzu make), pH meter (Elico Limited, LI 120/ LI 610), Incubator, Magnetic Stirrer and Vortex mixer of Genei, Bangalore, India make, and Refrigerated Table top Centrifuge of M/s Sigma make were used.

2.2 Direct Binding ELISA

The Samples (Antigen) such as PMSG, eLH, hCG etc. were coated on the wells using ammonium bicarbonate buffer (0.1M pH - 9.5), each well was coated with 100 µL of antigen, with the blank being coated through addition of 100 µL of coating buffer only. Plate was then incubated for one hour at 37 ^{o} C and then left overnight at 4^{0}C. After keeping the plate for overnight incubation at 4 ^{o}C. Further, the plate was washed with Phosphate buffer thrice. The non - specific sites of the wells were blocked by coating each well with 1% casein in 0.1 M Phosphate buffer and incubated the plate for one hour in an incubator at 37 ^{o} C. After incubation, casein was later removed by washing the plate again with Phosphate buffer with PBT for three times.100 µL of rabbit and rat antiserum of appropriate dilution was made in 0.1% casein in 0.1M Phosphate buffer and added in each well. Then, the plate was incubated for three hours in incubator at 37^{o}C. Dilution of antibody was done slowly so that there was a minimum loss of antibody action. After incubation, washed the plate with PBT for five times each to ensure that there was no trace of unbound primary antibody left over. Then 100 µL of Secondary antibody, Goat anti - rabbit IgGG HRP Conjugate of 1: 1000 dilution was made in same buffer like the primary antibody, was added into each well and incubated for one hour at 37 ^{o}C. Then the plate was washed with PBT for five times each to remove any unbound antibody.100µLof Ortho - phenylenediamine (OPD) solution (1mg/mL in 0.05M Citrate Buffer containing 0.06% H_2 O_2) was added into each well, the reaction was allowed to occur for 10 - 15 minutes in the dark for the color to appear. After proper development of colour, the reaction was stopped by adding 40 µL of 1 M Oxalic acid. Then the plate was read at 490 nm in ELISA Reader.

2.3 Competitive ELISA for assessment of PMSG activity in PMS

The ELISA plates were coated with 1IU of PMSG/eLH in competitive ELISA with anti eLH antiserum and anti buLH antiserum but 0.5 IU of PMSG was coated in competitive ELISA with anti PMSG antiserum. All the dilutions were made in ammonium Bicarbonate buffer (0.1 M, pH 9.5) while blank was set by adding 100 µL of the buffer only. Plates were then incubated at 37ºC for one hour and then left at 4ºC overnight. After overnight incubation at 4ºC, the plate was washed twice using PBT (10mM phosphate buffer pH - 7.4 containing 0.05 % Tween 20). The nonspecific sites of the wells were then blocked by 300 µL of 1 % casein in 0.1M phosphate buffer pH - 7.4, incubated for one hour at 37^{o}C. Casein was later removed and the plate was washed 2 to 3 times with wash buffer PBT.50 µL of standards of PMSG, PMS and NHS (50 µL, 25 µL, 10 µL, 5 µL) of all the respective doses were taken in 0.5 mL centrifuge tube and then added 50 µL of 1: 25, 0000 dilution of absorbed anti eLH a/s anti PMSG antiserum in 0.1 % casein in 0.1 M PB to micro centrifuge tubes. Then micro centrifuge tubes were incubated at 37ºC for one hour. After one hour of incubation plate washed 2 to 3 times with wash buffer and then added 100 µL of pre incubated antigen and antibody solution. Wells where no competition was done, where added only buffer with antibody of respective dilutions, then plate was incubated at 37 ºC for three hours.50 µL of standards of PMSG, eLH PMS and NHS (50 µL, 25 µL, 10 µL, 5 µL) of all the respective doses were added in the respective wells and then added 50 µL of 1: 25000 dilution of absorbed anti eLH, 1: 10, 000 dilution of absorbed anti PMSG antiserum in 0.1 % casein in 0.1 M PB to the respective wells. Wells with no competition of PMSG were added only buffer with antibody of respective dilutions and then plate was incubated at 37 ºC for three hours. After incubation of the plate with primary antibody for three hours the plate was washed thoroughly 3 to 4 times with the washing buffer to ensure that there was no trace of unbound primary antibody left over. Then 100 µL of secondary antibody 1: 1000 dilution was made in the same buffer as that of primary antibody was also added to each well and incubated at 37ºC for 1 hour. The plates were washed 3 to 4 times with wash buffer to remove any unbound antibody.100 µL of Ortho - phenylenediamine (OPD) solution made in 1 mg /mL of 50mM Citrate buffer, pH 5.5 containing 0.06 % H_2O_2 was added to each well. The reaction was allowed for 10 to 15 min in the dark for the colour to appear. After the proper development of the colour the reaction was terminated by adding 40 µL of 1 M oxalic acid. Absorbance

was taken at 490 nm using an ELISA reader. The % binding was calculated by using the formula (Vasudha et al 2013):

Percentage binding= B - NSB/B0 - NSB X100
Where, B = wells with competitor
B0 = wells without competitor
NSB= Normal sample blank

Competitive ELISA of hCG was also done with buLH a/s by coating of 1 IU of hCG and competition by different concentration of hCG (in mIU). Then, standard curve was by plotted by plotting % binding with doses of PMSG on semi log plot and values of unknown were calculated from the standard curve.

3. Results and Discussion

eLH serum were absorbed with 1: 10 diluted normal horse serum respectively toeliminateany non - specific antibodies. The immunoreactivity of eLH antiserum was confirmed by conducting a checker board analysis experiments for its titer in its different booster antisera as well as evaluated for its cross reactivity with PMSG antigen (Fig 1) and it was observed that the antiserum against eLH was potent enough to probe 1IU of eLH up to dilutions of 1: 100, 000 with ELISA. A Direct Binding curve was plotted using serial dilution of PMSG and eLH with final boosted antiserum, considerable immunoreactivity was observed with eLH and PMSG (Fig 2). Thereby, indicating that PMSG cross reacts with eLH a/s as eCG (PMSG) shares identical amino acid sequence like eLH and there was 40 - 50% cross reactivity of eLH a/s with PMSG (Fig 2). The Competitive ELISA was performed to measure the concentration of PMSG present in PMS. Which is standardized both by coating of PMSG/eLH and competition by PMSG/eLH to measure PMSG and eLH in PMS and NHS. When 1IU of PMSG was coated and competition was done 10, 000 mIU of PMSG onwards with preincubation of standards along with PMS and NHS, it was observed that there was inhibition and sensitivity of this assay lies in the range between 10, 000mIU to 10mIU (Fig 3). When, 1 IU of PMSG was coated and inhibition was done 10, 000 mIU of eLH down, the assay range was between 10, 000 mIU to 78mIU (Fig 4). When 1 IU of PMSG was coated and inhibited by 10 IU of PMSG both preincubation of competitor and antiserum and without preincubation, with preincubation assay range was 10, 000mIU to 10mIU and without preincubation the sensitivity of assay lies in 10, 000mIU to 20mIU (Fig 5). Competitive assay was developed to measure the amount of PMSG present in PMS. By comparing the % binding of PMS and subtracting % binding of NHS from it, amount of PMSG present in 1 mL of PMS was calculated for the assays. Further, the inhibition curve of PMS was found to be parallel to that of PMSG representing that PMSG in PMS. Similar experiment with normal horse serum at the same dilutions gave us values for eLH present and this could be subtracted from the values obtained with PMS to give accurate values for PMSG. By comparing the % binding of PMS and subtracting % binding of same volume of NHS from it. It was calculated that around 483mIU of PMSG was present in 50µL of PMS giving a value of 42.12 of PMSG in 1 mL of PMS when competition was done by coating of PMSG and competition by PMSG itself. While, it is 44.7IU/mL of PMS and 67.76 IU/mL of PMSG in PMS, 71.7IU/mL of PMSG in PMS and 47.84 IU/mL of eLH in Normal Horse Serum respectively, when there was variation made in antigen coating and competition between PMSG and eLH (table 1). That cross - react to varying degrees with eLH (equine luteinizing hormone) is indeed fascinating. The observation regarding PMSG's existence in different confirmations with unique epitopes, suggests that PMSG exhibits structural micro heterogeneity, means it possesses various structural forms or arrangements at a microscopic level. This conclusion has important implications because it indicates that PMSG is not a uniform or monolithic molecule but rather a complex entity with diverse structural features. The presence of different conformations and epitopes may have implications for its biological activity, interactions with other hormones, and overall function. In order to validate and confirm this finding, further structural work on PMSG is essentially required. Structural work typically involves techniques such as X - ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, or cryo - electron microscopy (cryo - EM). These methods can provide detailed insights into the three - dimensional shape and arrangement of the molecule's atoms, helping to identify different conformations and epitopes present in PMSG. Confirming the existence of structural micro heterogeneity in PMSG could open up new avenues of research, shedding light on its molecular behaviour, potential modifications, and possible applications in fields such as reproductive biology, animal husbandry, and pharmaceuticals. Moreover, understanding the structural complexity of PMSG could also lead to the development of more targeted and effective therapies or interventions based on its unique properties.

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Authors' contributions: NK and TA conceived and designed the experiments. NK performed the experiments. NK and TA analysed and compiled the data. NK wrote the paper. Both the authors read and approved the final manuscript

Table 1

S. N	Antigen coating	Competition by	Antiserum used	Assay range	PMSG/eLH measured
1.	1 IU PMSG	10IU PMSG DOWN	Anti eLH at the dilution of 1: 50, 000	10mIU - 10, 000mIU	42.12IU/mL of PMS
2.	1 IU PMSG	10IU eLH DOWN	Same	78mIU - 10, 000mIU	44.7IU/mL of PMS
3.	1 IU eLH	10IU eLH DOWN	Same	78mIU - 10, 000mIU	67.76 IU/mL of PMSG in PMS and 47.84 IU/mL of eLH in NHS
4.	1 IU eLH	10IU PMSG DOWN	Same	156mIU - 10, 000mIU	71.7IU/mL of PMS

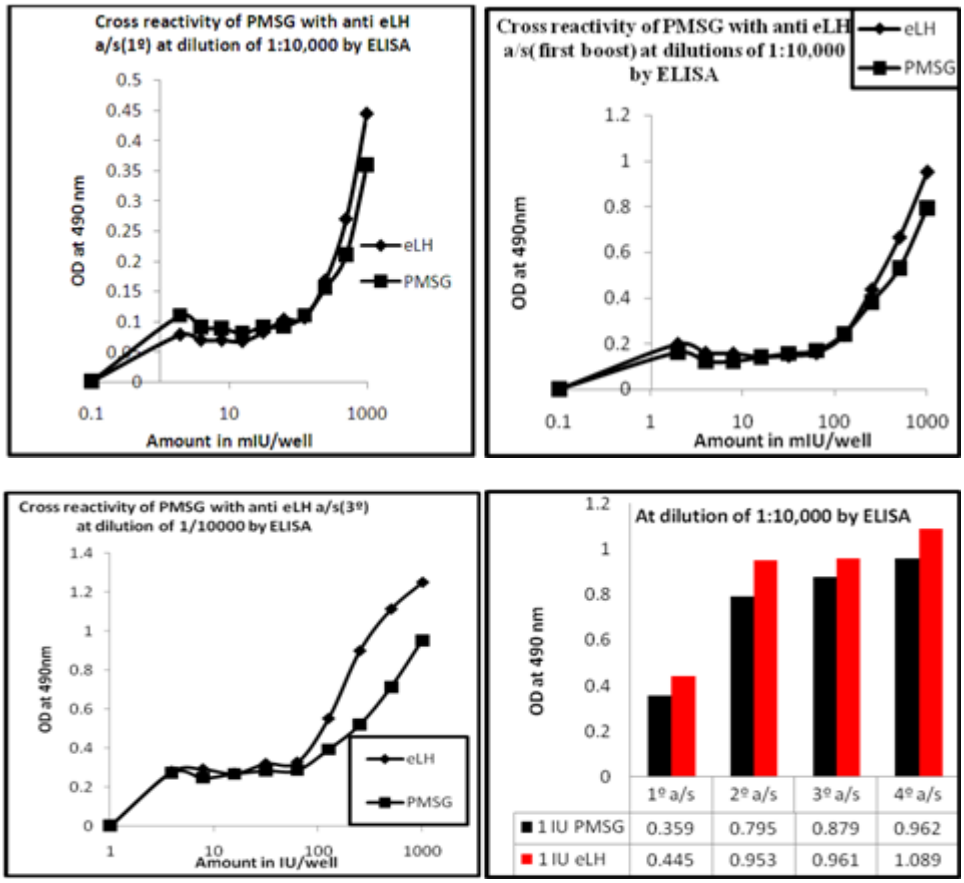

Figure 1: Comparative cross reactivity profile of various boosters of anti eLH antisera between eLH and PMSG.

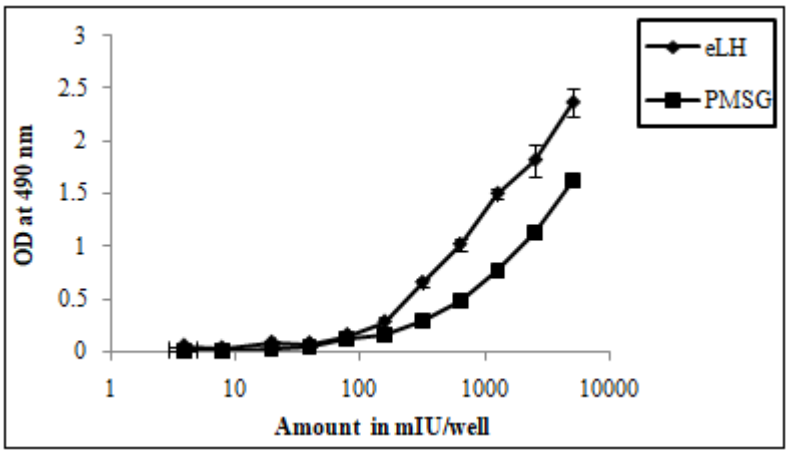

Figure 2: A Calibration curve of eLH and PMSG with last boosted anti eLH antiserum. ELISA plate's wells were coated with 1 to 5000 mIU of PMSG and eLH and detected by eLH antiserum at the dilution of 1: 50, 000 by direct ELISA.

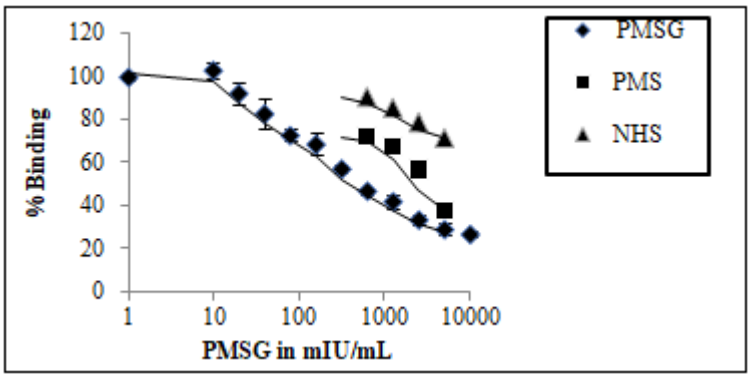

Figure 3: A standard curve of competitive ELISA with anti eLH antiserum. ELISA plate's wells were coated with 1 IU of PMSG and competition was done by serial dilutions of 10 IU of PMSG onwards and detected by eLH antiserum at the dilution of 1: 50, 000. A parallelism between PMS and Sigma PMSG was noticed.

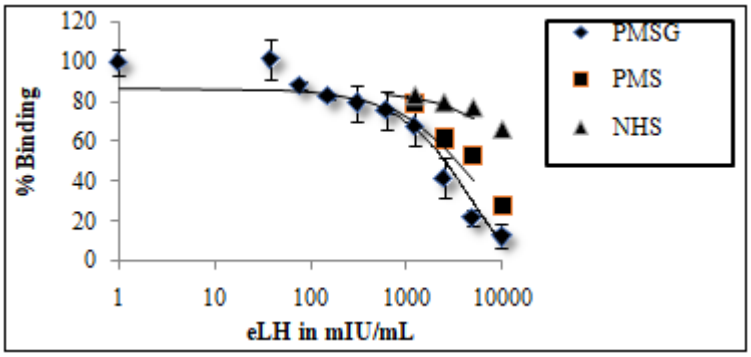

Figure 4: A standard curve of competitive ELISA with anti eLH antiserum. ELISA plate's wells were coated with 1 IU of PMSG and competition was done by serial dilutions of 10 IU of eLH onwards and detected by eLH antiserum at the dilution of 1: 50, 000. A parallelism between PMS and Sigma PMSG was noticed.

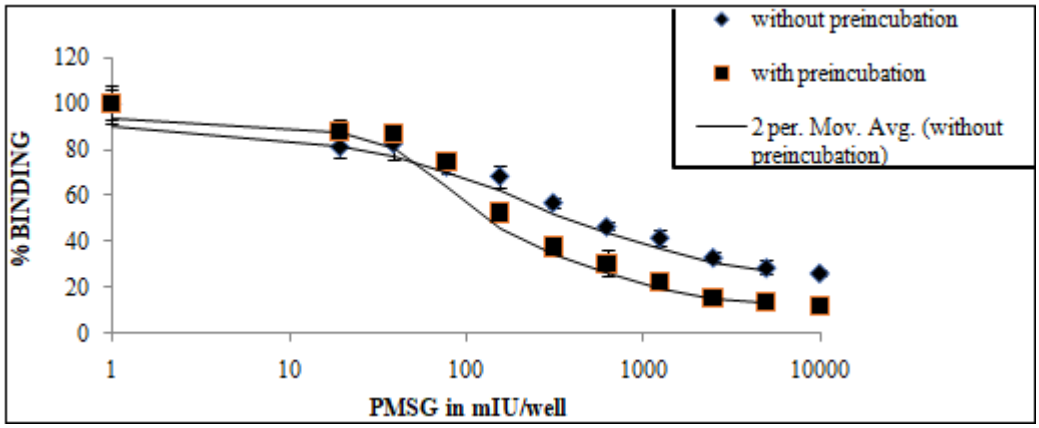

Figure 5: Comparison of standard curve of Competitive ELISA with anti eLH antiserum. ELISA plate's wells were coated with 1 IU of PMSG and competition was done by serial dilutions of 10 IU of PMSG onwards and detected by eLH antiserum at the dilution of 1: 50, 000 by both preincubation and without preincubation of competitor and antiserum.

References

[1] **Alexandra M. (2021).** Determining the Effects of Exogenous Human Chorionic Gonadotropin and Pregnant Mare Serum Gonadotropin on Plasma Testosterone, Testicular Volume, and Semen Production in Leopard Geckos (*Eublepharis macularius*. *LSU Master's Theses*.5309. https: //digitalcommons. lsu. edu/gradschool_theses/5309.

[2] **Berger, P., Panmoung, W., Khaschabi, D., Mayregger, B., Wick, G.** (1988). Antigenic Features of Human Follicle Stimulating Hormone Delineated by Monoclonal Antibodies and Construction of an Immunoradiometric Assay. *Endocrinology*.*1***23,** 2351 - 2359.

[3] **Combarnous, Y., Hennen, G., Ketelsleger, J. M.** (1978) Pregnant mare serum gonadotropin exhibits higher affinity forlutropin than follitropin receptors of porcine testis. *FEBS Letters*.*9***0**, 65 - 68.

[4] **Donrov, Ts., Batsaihan, D., Ley, W. B.** (1998) Gonadotropin extraction from pregnant mares' serum and effectof PMSG preparation on the fertility of Mongolian native ewes, Small Ruminant Research **28**, 61–66.

[5] **Martinuk SD, Bristol F, Murphy BD. (1990).** Effects of the dam on equine chorionic gonadotropin concentrations during pregnancy. Domest Anim Endocrinol, 7: 551 - 557. Martinuk SD, Manning AW, Black WD, Murphy BD.1991. Effects of carbohydrates on the pharmacokinetics and biological activity of equine chorionic gonadotropin in vivo. Biol Reprod, 45: 598 - 604.

[6] **Maurel, M. C., Ban, E., Bidare, J. M., Combarnous, Y. (1992).** Immunochemical study of chorionic gonadotropin (eCG/PMSG): antigenic determinants on α - and β - subunits. *Biochimica et Biophysica Acta*.*1***159**, 74 - 80.

[7] **Moudgal, N. R., Mukku, V. R., Prahalada, S., Murty, G. S. R. C., Li, C. H.** (1978) Passive immunization with an antibody to the beta - subunit of ovine luteinizing hormone as method of early abortion - a feasibility study in monkeys (Macaca radiate). *Fert. Ster*.*3***0**, 223.

[8] **Moyle, W. R., Xing, Y., Lin, W., Cao, D., Myers, R. V., Kerrigan, J. E., Bernard, M. P. (**2004) Model of glycoprotein hormone receptor ligand binding and signalling. *J Biol Chem*.2**79,** 44442 - 44459.

[9] **Roser, J. F., Carrick, F. N., Papkoff, H.** (1986) Properties of equine luteinizing hormone alpha subunit alone and in combination with various beta subunits. *Biol Reprod*.3**5**, 493.

[10] **Roser JF, Papkoff H, Murthy HMS, Chang YS, Chloupek RC, Potes JAC. (**1984) Chemical, Biological and immunological properties of pituitary gonadotropins from donkey: Comparison with the horse. Biol. Reprod.30, 1253 - 1262.

[11] **Moudgal, N. R., Rao, A. J.** (1984) United Nations Fund for Population Activities, New York. *in: Human Fertility, Health and Food (Puett D, ed)*, 73.

[12] **Saint - Dizier M, Foulon - Gauze F, Lecompte F, Combarnous Y, Chopineau M. (2011).** The additional N - glycosylation site of the equine LH/CG receptor is not responsible for the limited cyclic AMP pathway activation by equine chorionic gonadotropin relative to luteinizing hormone. Reprod Biol, 11: 157 - 164.

[13] **Vasudha, R, N, Kumari and K. (2013).** Muralidhar. Antiserum to buffalo pituitary Luteinizing hormone enables differential measurement of placental gonadotropins. Int. J. Bioassays.2 (2) 341 - 345.