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Expression Analysis of Recombinant Equine Chorionic Gonadotropin in Three Host Systems: *E. coli BL21C, Sf insect cell lysate and COS-1 mammalian cells*

Anuradha Bhardwaj[1], Varij Nayan[2], Sanjay Kumar[1], Parvati Sharma[1], Sanjeev Kumar[1], Neha Chakarvarty[3], Sudarshan Kumar[3], Yash Pal[1], S.C. Yadav[1], A.K. Mohanty[3], B.N. Tripathi[1]

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ABSTRACT

Mammalian cells are the recommended host for recombinant eukaryotic protein production aimed at incorporation of post-translational modifications for downstream applications. The bacterial system and insect cells are widely used because of ease of technical methodology, economics of production, purification and yield of final protein. The present research objective was expression of recombinant reproductive hormones of animal origin and study of their immunogenic potential for reproductive applications. The equine Chorionic Gonadotropin (eCG) is one of the most heavily glycosylated protein amongst all glycoprotein hormone family. Hence, experiments were carried out to observe its expression in the three most popular host systems and it led to comparative studies for their post-translational modifications. The Pregnant Mare Serum Gonadotropin (PMSG, also called as eCG) gene was cloned in TOPO-TA vector, pIX 4.0 and pTARGET vectors accordingly and expression analysis in *E. coli* BL21C, *Sf* insect cell lysate and *COS-1* cells was carried out. We observed diverse sizes of recombinant proteins in SDS-PAGE analysis which indicated post-translational modification in mammalian expression system towards the linking of tags as well as side chains in respective host cells. Basic diagnostic immunogenicity tests showed encouraging results, however, no significant *in vivo* and *in vitro* activity was observed for the expressed reCG in all the employed host systems.

Key words: Equine, Gonadotropin, Hormone, Host, Protein, Recombinant.

INTRODUCTION

The recombinant protein production is a proven technology for generating alternatives of biological products in *in vitro* systems, as well as the potential of recombinant products for serving as diagnostics and bioproducts is well established. The Pregnant Mare Serum Gonadotropin (PMSG) which is also known as equine Chorionic Gonadotropin (eCG), is a glycoprotein hormone of the gonadotropin TGF-β family. This hormone is well known for its role in animal reproduction and maintenance of pregnancy. It is produced by the trophoblast cells of endometrial cups in mares and is primarily responsible for the early gestation in the pregnant animals (Allen and Moor, 1972). Like the other members of TGF-β superfamily the PMSG/eCG is also composed of two dissimilar and non-covalently connected α and β subunits; α-subunit is encoded by a single gene within same species whereas β-subunit is encoded by diverse genes and mainly responsible for receptor binding specificity (Combarnous, 1992). The functional properties are specifically reported for β-subunit, however in a recent study on molecular docking and in silico analysis of α-subunit of eCG with ganirelix paved an insight into functional properties of alpha eCG also (Bhardwaj *et al.,* 2017). Many researchers across the world reported work on reproductive hormones and their receptors for their role in reproductive functions, ovulation, pregnancy establishment and reproductive disorders in human and animals. Other important factors including Inhibin (Bhardwaj *et al.,* 2012), Insulin-like growth factor-I (Faraj *et al.,* 2017) and PAPP-A (Nayan *et al.,* 2013) were also studied during pregnancy and other reproductive conditions.

Akin to the reason of its well reported and experimented biological properties, eCG has been used in the fertilization and fecundity augmentation programs since time immemorial specially for superovulation in assisted reproductive technologies (ART). Conversely, the available preparations of partly purified eCG (PMSG) from pregnant mare serum could restrain contaminants insisting for

[1]ICAR-National Research Centre on Equines, Hisar-125 001, Haryana, India.
[2]ICAR-Central Institute for Research on Buffaloes, Hisar-125 001, Haryana, India.
[3]ICAR-National Dairy Research Institute, Karnal-132 001, Haryana, India.

Corresponding Author: Anuradha Bhardwaj, ICAR-National Research Centre on Equines, Hisar-125 001, Haryana, India.
Email: dranu.biotech@gmail.com; Anuradha.Bhardwaj@icar.gov.in

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production of more purified forms (Legardinier *et al.*, 2005). Therefore, it is of colossal concern to produce a bioactive substitute for eCG in large quantities and other gonadotropins to be used as therapeutic agents and in ARTs (Leao and Esteves, 2014). We have published reports on expression of recombinant reproductive hormones of animal origin and carried out studies on their potential for reproductive applications. Earlier we reported expression of bINH-α at room and reduced temperatures (Bhardwaj *et al.*, 2012, 2013) and recently we reported expression of eCG in *E. coli* host (Bhardwaj *et al*, 2019), in continuation we also tried to express the recombinant hormone in eukaryotic systems and analyze the protein molecular weight for its expression and post-translational modification in comparison to the one produced in bacterial host cells and insect cells. Here we present the study on expression of recombinant equine Chorionic Gonadotropin, which is one of the most heavily glycosylated protein amongst all glycoprotein hormone family and comparative study for post-translational modifications in recombinant hormone was done.

MATERIALS AND METHODS

Ethics statement

All the animal procedures were performed according to the approval by Institute Animal Ethics Committee (IAEC) of ICAR-National Research Centre on Equines.

Vectors and host cells for transformation

For bacterial expression, pET 32a vector and *E. coli* strain *DH5alpha* and *E. coli BL21C* were used for the heterologous expression as reported earlier (Bhardwaj *et al*, 2019). For insect cell lysate expression, vector pIX 4.0 and EasyXpress Protein Synthesis Insect Kit (Qiagen Ltd) and for mammalian cell expression studies pTARGET vector (Promega) and *COS-1* cells were used as vectors and host systems. The synthetic eCG gene was cloned into pUC57 vector according to the manufacturer's instructions and propagated in *E. coli DH5 alpha* cells. The recombinant plasmids were isolated from the *E*. coli DH5 alpha cells (harboring the plasmid pUC57-eCG) by alkaline lysis method and the βαeCG gene was amplified by PCR using gene specific primers containing *BseR1* and *Bgl II* sites for cloning in pIX 4.0 vector. The pUC 57-βα and pIX4.0 vector were digested with *BseR1* and *Bgl II* and the insert was ligated into pIX 4.0 vector. The recombinant pIX plasmids were grown in *E.coli DH5 alpha* cells and then purified and checked by PCR for the presence of insert. Before carrying out the expression in *Sf* cell lysate the plasmid was made linear with *Sma* 1. For expression in mammalian cells, the βα-eCG gene was amplified with gene specific primers containing *Eco RI* and *Sma I* sites for cloning in pTARGET vector. The pUC 57-βα and pTARGET vector were digested with *Eco RI* and *Sma I* and the insert was ligated into pTARGET vector. The recombinant pTARGET/ βα-eCG plasmids were made ready for transfection into *COS-1* cells by Lipofectamine.

In vitro transcription and translation reaction for protein synthesis in *Sf* cells

For expression of eCG in insect cell lysate the EasyXpress Protein Synthesis Insect Kit (Qiagen ltd) was used and all the steps were followed according to the protocol given and the manufacturer's instruction. Briefly, for *in vitro* transcription reaction, the 5x transcription buffer, 5x NTP mix, 20x enzyme mix, RNase-free water and EasyXpress insect positive Control DNA were thawed and stored on ice. Before use, each tube was gently vortexed and briefly centrifuged to ensure homogeneity of solutions. The components of the transcription reactions were pipeted together in three DNase- and RNase-free 1.5 ml microcentrifuge tubes at room temperature (15-25°C). All components were mixed by vortexing and briefly centrifuged to collect the reactions at the bottom of the tubes. The *in vitro* transcription reactions were incubated for 120 min at 37°C. The reaction mixture was centrifuged for 1 min at 12,000 rpm at room temperature (15-25°C). The reaction supernatants were pipetted into clean DNase- and RNase-free 1.5 ml microcentrifuge tubes and kept at room temperature (15-25°C). Each tube was labeled clearly. For translation, mRNA was cleaned up using a DyeEx gel-filtration spin column before addition to the translation reaction. The DyeEx spin column was vortexed gently to resuspend the resin. The cap of the column was loosened a quarter turn to avoid vacuum development inside the spin column. The bottom closure of the spin column was snaped off and placed into a DNase- and RNase-free 2 ml microcentrifuge tube which was then centrifuged for 3 min. The spin column was transferred to a clean centrifuge tube and *in vitro* transcription reaction mixture was applied slowly onto the center of the slanted gel-bed surface. The sample was pipetted slowly so that the drops are absorbed into the gel. Centrifuged for 3 min at 3000 rpm and then the spin column was removed from the microcentrifuge tube. The eluate was kept at room temperature and processed immediately with the *in vitro* translation reaction. The eluate contains the purified RNA. For protein expression, the EasyXpress insect reaction buffer, EasyXpress insect extract, EasyXpress energy mix and RNase-free water were thawed and stored on ice. The components of the three translation reactions were pipetted together in three DNase- and RNase-free 1.5 ml microcentrifuge tubes. Each tube was labeled clearly. All the components were mixed by vortexing and briefly centrifuged to collect the reactions at the bottom of the tubes. The *in vitro* translation reactions were incubated in a Thermomixer for 90 min at 27°C and 500 rpm. After translation reaction is over, the *Sf* lysate was immediately processed for SDS-PAGE to check the expression of rβαeCG.

Transfection in *COS-1* Cells and expression analysis

The *COS-1* cells were cultured in Dulbecco's modified Eagle's medium (D MEM; Gibco) supplemented with 10% fetal bovine serum, penicillin/streptomycin (10000 U/ml penicillin and 10 mg/mL streptomycin (in 0.9% saline) and

L- glutamine in an atmosphere of 5% CO_2 and 95% air at 37°C. The *COS-1* cells were plated at 1×10^4 cells/plate. The next day, 10µg of vector DNA (pTARGET/βα-eCG) was transfected into the cells using Lipofectamine transfection reagent. For this, about six plates were seeded with *COS-1* cells (approximately 1X 10^4 cells/ well). Next day the cells were visually inspected under microscope for confluency. About 90-95% confluence at the time of transfection is recommended to obtain high efficiency and expression levels and to minimize decreased cell growth associated with high transfection activity. Then, to a 96 well plate, added DNA (at 2 µg concentration) and DMEM (0% FBS, no antibiotic) 100 µl in well A and lipofectamine (approx. 8 µL) and DMEM (0% FBS, no antibiotic) 100 µl in well B. Then both components (A, B) were mixed gently. Likewise vector and, positive control were added. The A/B complexes were incubated at RT for 30 min. Following incubation, aspirated medium off cells and 1ml/well DPBS was added to wash and aspirated off. Then, 2 ml of DMEM was added in each well of plate. Dripped 200 µl A/B complex mixture onto cells respectively and gently mixed media on cells with back and forth motions. It was then incubated at 37°C with 5% CO2 in incubator for atleast 4-5 hours. After that, DMEM media containing A/B complexes was removed and discarded. The fresh 2 ml/well of 10% FBS/DMEM were added and the cells were passaged into fresh growth medium 24 hours after transfection. The selective medium (G418 antibiotic containing medium) was added after 1-2 passages and cultured cells were checked for transfection and for expression of rβαeCG in SDS-PAGE.

Quantification of reCG proteins

The expressed eCG proteins secreted by the transfected *COS-1* cells and the stable cell lines into serum-free media were collected and rβαeCG was quantified using the PMSG-ELISA (Pregmare kit), according to the standardized protocol (ICAR-NRCE, Hisar). Briefly, the collected medium was dispensed into wells coated with an antibody against eCG molecule. The wells were incubated for an hour at room temperature and about 100µL of conjugate and afterwards the substrate solution was added and plates were incubated for 30 min at room temperature. The reaction was stopped with 100 µL stop solution. The absorbance was noted at 450 nm with ELISA plate reader.

Analysis of expression in SDS-PAGE

For bacterial cells expression, the cell pellet was lysed with cell lysis buffer and centrifuged at 4000xg for 5 min and the supernatant was collected and analyzed for protein expression. The Sf cell lysate after translation reaction and transfected *COS-1* cells homogenates were also checked for expression of recombinant eCG in SDS PAGE (12% polyacrylamide) for comparative analysis for recombinant protein production in all the recommended host systems.

Characterization of the *in vitro* diagnostic capacity of rβαeCG by immunoassays

The crude cell lysate expressing the rβαeCG as well as purified protein was analyzed in SDS-PAGE (12% polyacrylamide) along with non-induced and induced host bacterial lysate, expressed Sf cell lysate and transfected *COS-1* cells homogenates. The gels were stained with Coomassie brilliant blue and the bands were analyzed for determination of the molecular weight. The protein concentrations were determined through BCA and Lowry assay and confirmed by spectrophotometry (ND-1000 Spectrophotometer, Nanodrop Technologies Inc. USA). The expressed and purified recombinant proteins were characterized by sandwich enzyme linked immunosorbent assay (sELISA), by Pregmare kit (NRCE) and Western blotting. For sELISA, the presence of rβαeCG was confirmed based on the color development and intensity. The absorbance was also recorded. The un-induced cell lysate and antigen negative samples were kept as negative control for qualitative characterization of recombinant rβαeCG. For Western blot analysis, the band of purified protein was separated on SDS-PAGE (12% polyacrylamide), electro-blotted to nitrocellulose membrane, probed with specific polyclonal antibodies [the primary antibody (anti-eCG raised in poultry; dilution 1:2000) and secondary antibody HRPO conjugate (anti-goat IgG raised in donkey; dilution 1:1000)]. Diaminobenzidine (DAB) with H_2O_2 was used for detection of rβαeCG band on nitrocellulose membrane corresponding to the band on SDS-PAGE.

RESULTS AND DISCUSSION

Equine luteinizing hormone (eLH) and chorionic gonadotropin (eCG) hormone are composed of identical alpha and beta polypeptide chains, but eCG subunits are known to be glycosylated to a heavy extent. Accordingly, eCG exhibits a much longer half-life than eLH and makes it applications to a wider extent in assisted reproductive technologies including superovulation. Recombinant Chorionic Gonadotropin hormone (rβαeCG), was expressed in three types of host systems in this study, that are mostly used as cell factories for recombinant protein productions *viz.,* bacterial, insect and mammalian systems and the expressed proteins were compared for molecular weight and yield in all the employed systems for further research applications.

Amplification, cloning and expression of rβαeCG in *E.coli BL21C, Sf insect cell lysate and COS-1 mammalian cells*

The selected region for βαeCG/LH was specifically designed and custom synthesized with codon optimization for expression in prokaryotic host while retaining the original amino acid sequence (Bhardwaj *et al.,* 2019). The synthesized DNA was amplified by PCR with specific primers and the ~700bp product was purified and double digested with respective restriction enzyme for ligation with required expression vector. The amplification, cloning and expression in bacterial cells have already been reported by our lab (Bhardwaj *et al*, 2019). Further the amplified eCG gene was

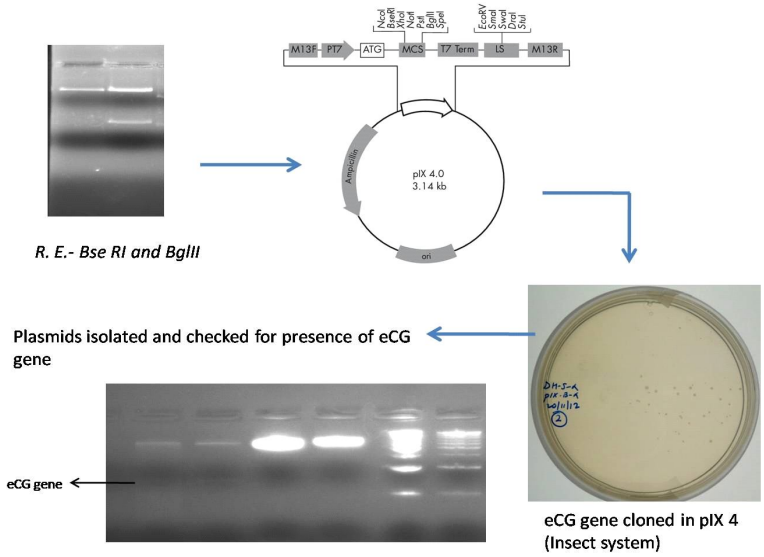

Fig 1: Amplification and cloning of *βα-eCG* gene in insect lysate system.

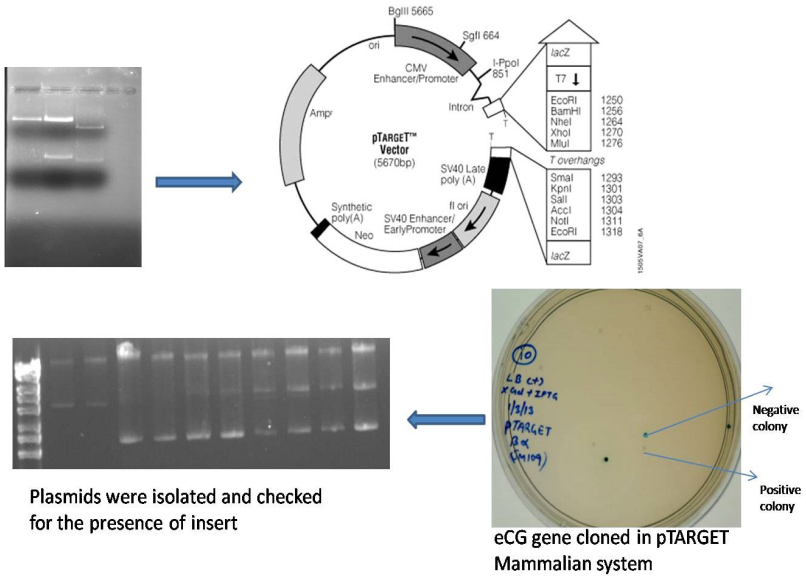

Fig 2: Amplification and cloning of *βα-eCG* gene in mammalian system.

also subcloned into insect cell system compatible vector (Fig 1) and similarly for mammalian host vector system (Fig 2).

Stable expression rec-eCGβ/α in *COS-1* cells

The pTARGET/β-αeCG vectors were transfected into *COS-1* cells (Fig 3) and the cells stably expressing proteins were then selected with G418 treatment. The rβαeCG secreted by the stably transfected cells into the serum-free medium were isolated and concentrated. The rβαeCG were quantified using sELISA. The quantities of rβαeCG obtained from stably transfected cells were about 100-150 mIU/mL. The size of the rβαeCG protein was found to be approximately 72 kDa, which was almost consistent with previous studies (Park *et al.,* 2010, 2017, Jeoung *et al.,* 2010). However, the size of the rβαeCG expressed in bacterial cells and insect cell lysate was about 42 kDa and 27 kDa owing to post-translational modifications in mammalian cells and accounted for higher molecular weight as its biological counterpart in comparison to bacterial and insect systems.

SDS-PAGE analysis and immunological assays

The expressed rβαeCG in different vector-host systems were comparatively analyzed in SDS-PAGE (Fig 4) for molecular weight and also to observe the post-translational modifications as eCG has been reported to be the most heavily glycosylated hormone in the TGF-beta superfamily of gonadotropins. We have observed a ~42 kDa rβαeCG in bacteria because of his and trx tag, 27 kDa rβαeCG in insect as expected since no tag was present, however, we did not observed any post-translational modifications in it since no

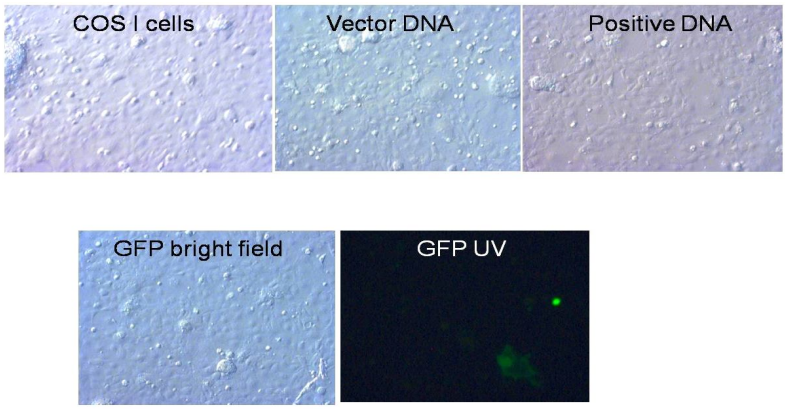

Fig 3: Transfection and Expression in mammalian cells (*COS-1*): Vector DNA- pTARGET without eCG, Positive DNA - pTARGET with eCG, GFP- Positive control with GFP vector, Cells were grown to different passages (DMEM medium with 10% FBS) and 5% CO_2.

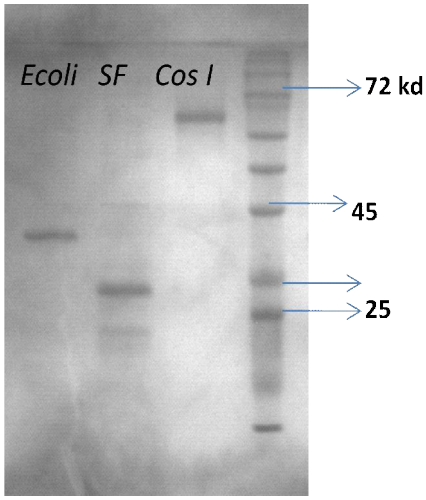

Fig 4: SDS-PAGE analysis for expression of rβαeCG in three host systems.

increase in molecular weight of expressed rβαeCG was observed as calculated from the amino acid sequences. The size of the reCGβα protein was found to be approximately 72 kDa in mammalian cells clearly indicates presence of side chains on account of post-translational modifications. The immunogenic property of rβαeCG protein was investigated by sELISA and Western blot analysis (Bhardwaj *et al.,* 2019). Briefly, the host cell lysates (control and recombinants) were used as antigens. The absorbance obtained was quite comparable to the pregnant mare serum (PMS) which confirmed the presence of rβαeCG protein. In Western blot analysis, the total expressed proteins were separated on SDS-PAGE and transferred to nitrocellulose membrane. The membrane showed the presence of band for rβαeCG at the respective positions as reported, thus confirming the presence of rβαeCG in the cell lysate. These results confirmed the presence of immunologically active rβαeCG in the expressed cell cultures. The ovarian hyperaemia reaction (OHR) was carried out for testing the biological efficacy of rβαeCG for its effectiveness in increasing the size and weight of gonads (uterus, ovary and oviduct) in immature mice (Bhardwaj *et al*, 2019), however, no increase in gonadal weight was observed for any of the control and experimental groups.

CONCLUSION

In the present study, comparative expression analysis of equine Chorionic Gonadotropin, which is one of the most heavily glycosylated protein amongst all glycoprotein hormone family was carried out to observe its expression in the three different host systems, for post translational modifications in recombinant hormone, yield of expressed protein and immunogenic effect was also observed. We observed different sized recombinant proteins in SDS-PAGE analysis which indicated post-translational modification in eukaryotic expression systems towards the linking of tags as well as side chains in respective host cells. The lowest yield was observed in mammalian cells in comparison to bacteria and insect cell lysate as expected which affected the *in vitro* diagnostic tests.

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Data Availability Statement

The data of all experiments are available with the corresponding author and can be made available on request.

Disclosure

This manuscript is based on the Institute project “Cloning, Expression and Characterization of equine Chorionic Gonadotropin (eCG) (project code: IXX02769) funded by ICAR.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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