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Production and Purification of Equine Chorionic Gonadotropin Hormone Using Polyclonal Antibody

Mohammad Ali Sharifi [1]; Hamid Kohram [1,*]; Ahmad Zare Shahneh [1]; Hossein Zolfagharian [2]; Bahman Abedi Kiasari [2]; Mehdi Hedayati [3]

[1]Department of Animal Science, Faculty College of Agriculture and Natural Resources, University of Tehran, Karaj, I.R. Iran
[2]Razi Vaccine & Serum Research Institute, Karaj, I.R. Iran
[3]Cellular & Molecular Research Center, Research Institute for Endocrine Research Center, Shahid Beheshti University of Medical Sciences, Tehran, I.R. Iran

*Corresponding author: Hamid Kohram, Department of Animal Science, Faculty of Agricultural Science and engineering, College of Agriculture and Natural Resources, University of Tehran, Karaj, I.R. Iran. Tel.: +98-2632248082, Fax: +98-2632246752, E-mail: kohram@can.ut.ac.ir

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Background: Equine chorionic gonadotropin (eCG) is commonly used in association with a progestagen treatment to synchronize estrus of goats and ewes during breeding and non-breeding seasons. Classical purification of the eCG from serum includes pH fractionation with metaphosphoric acid two ethanol precipitation steps as well as dialysis followed by fixed-bed chromatography.
Objectives: The aim of the current study was to develop an accurate and fast method for production and purification of eCG using a polyclonal antibody assay.
Materials and Methods: The blood samples (300 mL) were taken from the jugular vein of 17 mares on days 50, 70 and 90 of pregnancy. Plasma of the samples was siphoned and phenol solution was added to the plasma and stored in the refrigerator until eCG extraction. To prepare the polyclonal antibody against eCG, four male rabbits, about four months old with 2 kg weight, were chosen. A basic immunization was done by injecting 25 IU of eCG to the rabbits. Ouchterlony assay or double immunodiffusion test was used to assess the immunization and the titer of antiserum against eCG. eCG was purified from the plasma via the solid-phase extraction (SPE) method.
Results: Results based on agarose-gel double immunodiffusion test showed that rabbits were completely immunized. SDS-PAGE analysis showed purified eCG is extracted without any significant contamination.
Conclusions: The extraction of eCG with polyclonal antibody using the SPE method and production of anti- eCG antiserum in rabbits is suitable and may be a cost effective method for large scale production of eCG and anti-eCG antiserum in Iran.

Keywords: eCG; Ouchterlony; Polyoclonal Antibody; Solid-Phase Extraction (SPE)

1. Background

The low reproductive performance of the ewe may affect the efficiency and benefit cost of ewe production systems in Iran (1). Generally in Iran, sheep raised under semi-intensive systems with a high mobility are less fertile and less prolific compared to sheep raised under intensive systems (2). It is well-documented that the ovulation rate and litter size have a major impact on the reproductive efficiency of goats and ewes. Indeed, the goal of ewe production system is to have a high ovulation rate and litter size. Estrus synchronization is the key event in breeding programs (3). In small ruminant, equine chorionic gonadotropin (eCG) injection is commonly used in association with progestagen treatment to synchronize estrus and allow high ovulation during breeding and nonbreeding seasons (4).

There are numerous applications for eCG treatment in domestic species including induction of puberty, reversal of anestrus, superovulation and improvement of fertility and litter size (5). eCG treatment possesses both FSH and LH activities in ruminants. The long half-life (i.e. 5 days) and availability in large quantities make this unique gonadotropin a convenient exogenous hormone to induce estrus synchronization in small ruminants (4, 5). However, eCG treatment may cause postovulatory follicular waves (6) along with a high level of steroid production (3). It has been proposed that an injection of anti-eCG antibody 1-2 days following eCG injection may minimize the adverse effects of eCG by lowering the half-life of eCG in systemic circulation, which may have beneficial effects on the ovulation rate, pregnancy rate and quality of em-

Implication for health policy/practice/research/medical education:
eCG treatment possessing both FSH and LH activities in ruminant. Equine chorionic gonadotropin (eCG) is commonly used in association with a progestagen treatment to synchronize estrus of goats and ewes during breeding and non-breeding seasons.

bryos (7-10).

Equine chorionic gonadotropin previously known as pregnant mare serum gonadotropin (PMSG) is secreted by trophoblastic cells to the blood of pregnant mare between the 36th and 120th days of gestation (11, 12). The structure of eCG is composed of two dissimilar subunits with a non covalent association which is required for the biological activities. Each subunit is composed of a peptidic part connected to a glycan moiety (N- and O-chains) (6, 13). eCG is the most heavily glycosylated glycoprotein hormone, with the majority of biantenna glycans ending mainly in sialic acids that play an important role in the long half-life of the hormone in systemic circulation (5). Glycan chains are also involved in the stability of the heterodimeric structure of the hormone and are necessary for the efficiency of signal transduction activity (14).

There are numerous productions of eCG reported in the literature (14-16) and also available in market (Bioniche Animal health (A/Asia) Pty Ltd, Australia). Generally, eCG may be purified from pregnant mare serum with the classical purification scheme in two main steps (14, 17). First, precipitation and filtration steps remove the main contaminants like albumin to generate an intermediate product. Second, the final extract is generated by chromatographic steps. However in recent publications, isolation of biomolecules directly from crude suspensions using solid-phase immune extraction (SPE) has been used in different areas of biotechnology. Small scale bioseparation experiments applying SPE have been reported to produce acrylamide in food (18), recombinant human myelin basic protein (rhMBP) in milk of transgenic cows (19) and plant sulfolipids (20).

2. Objectives

Therefore, the purpose of the current study was to develop an accurate and fast method for purification of eCG from pregnant mare serum using the SPE method with polyclonal antibody of eCG which was produced in rabbits.

3. Materials and Methods

3.1. Production of Polyclonal Antibodies

3.1.1. Immunization of Rabbit

Four male rabbits, about 4 months old with 2 kg weights, were chosen for preparing antibody against eCG. A basic immunization was done by injecting 25 IU of eCG (Bioniche Animal health (A/Asia) Pty Ltd, Australia) as the immunogen, emulsified with complete Freund's adjuvant, into each rabbit by a multipoint subcutaneous implantation. After 10 days of growth, eight enhancing immunizations were further carried out as described above, but using incomplete Freund's adjuvant, with seven-day intervals between each implantation. Inoculations were performed by high purity eCG of Bioniche Animal Health (A/Asia; Pty Ltd, Australia). At completion of the immunization protocol, rabbit's heart blood was taken using the Ouchterlony assay (Double immunodiffusion test). Volume and number of injections were according to the Canadian Antibody Production Council on Animal Care & UIC Polyclonal Antibody Production, Guidelines/ACC 2012 (21-23).

3.1.2. Agarose-Gel Double Immunodiffusion

Ouchterlony assay or double immunodiffusion test was used to assess the titer of antiserum against eCG. For double immunodiffusion, the antiserum was diluted to 1:4 and 1:16 in 1% agarose gel, followed by incubation with eCG at 37°C for 48 h.

3.1.3. Farr Assay (Ammonium Sulphate Precipitation) and Dialysis

Immunoglobulins were separated from the whole serum by precipitation with ammonium sulphate solution. If ammonium sulphate is added to diluted serum to reach 50% saturation, most of the immunoglobulins are precipitated, while other serum proteins such as albumin remain in the solution.

During the first step, whole blood sample was collected by the invasion method from rabbit hearts and to access the serum from samples, all tubes were kept at room temperature for an hour and immediately centrifuged at 3000 rpm with 15°C temperature for 30 minutes. During the second step, to obtain the immunoglobulin and eliminate protein contaminants, 35% aluminum sulfate was added. Then, the serum was centrifuged at 3000 rpm and 12°C temperature for 30 minutes. Subsequently, the obtained immunoglobulin pellet was washed again using 0.01 M Phosphate buffer saline (PBS). For removing the added aluminum sulfate, it is shake using dialysis bags overnight in 4 °C. Then, immunoglobulin solution was lyophilized and stored at -20 °C.

The Biuret assay was performed for total protein of anti-rabbit antibody using albumin as a standard.

3.2. Blood Collection From MARES

Seventeen mares during initial stages of pregnancy were identified using the ultrasonography diagnosis method. Blood samples (300 mL) were taken from the jugular vein in a tube containing sodium citrate, on days 50, 70 and 90 of mare's pregnancy. The samples were immediately transfer to the laboratory and kept at 4°C overnight. After red cell precipitation, plasma was siphoned and phenol solution was added to the plasma. In the final step, collected plasma was mixed together and made ready for the next step, being the solid-phase extraction of eCG.

3.3. eCG Purification

3.3.1. Solid-Phase Immune Extraction (SPE)

Coating Step: 1:15000 dilution of the antibody in coating buffer (Carbonate Bicarbonate Buffer 50 mM, pH 9.6) was prepared and 300 μL of the solution was added to the wells of a microplate (96 wells). Then, the microplate was incubated overnight at 4°C. After incubation and coating of the wells with the antibodies, content of the wells were poured out and the wells were washed three times using a wash buffer (Phosphate buffer saline with 0.05% tween 20).

Blocking step: for blocking the uncoated surface of the wells, 300 μL of phosphate buffer saline with 0.1% bovine serum albumin was added to allocated wells and incubated for 2 hours at 37°C and finally the wells were washed using the above mentioned wash buffer.

Sample loading: For immune-separation of the hormone, 300 μL of plasma was added to each antibody coated well and incubated for 2 hours at room temperature (RT). Next, the wells contents were poured out and the wells were washed three times using the wash buffer. During this step, the hormone bonded to the coated antibodies on the inner surface of the well.

Hormone separation: For separating and releasing eCG from immobilized antibodies, the wells were washed with the wash buffer with a higher detergent content. Thus initially, 300 μL concentrated wash buffer (phosphate buffer saline with 0.5% tween 20) was added and incubated for 2 hours at RT. The wells contents which contained the immune separated hormone were collected with a sampler, lyophilized and stored at -80°C. The eCG content of SPE end product was determined by the method of Bradford using human serum albumin and gamma globulin as the standard.

3.4. Sodium Dodecyl Sulfate–Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

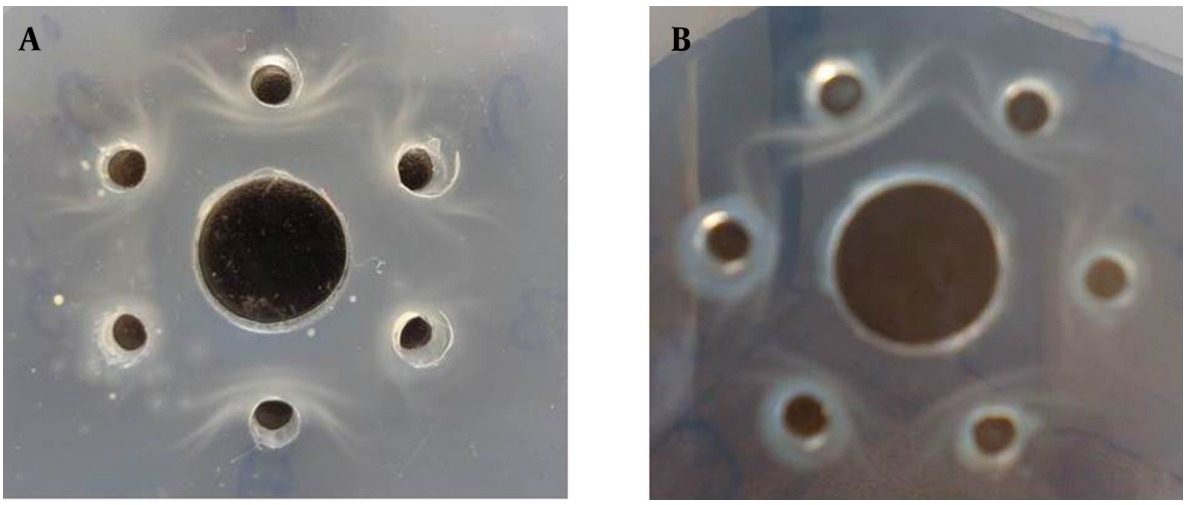
Briefly, the gel concentration was 12%, condition was none reducing, voltage 200V and time was 3 hours, electrophoresis instrument included a tank and the power supply was Bio-Rad made.

4. Results

Results based on the dual diffusion of antigen and antibody in agarose gel (Agarose-gel double immune-diffusion) showed that the rabbits had been completely immunized. Due to the precipitate, lines were produced from rabbit's antibodies with injected antigen (Figure 1).

One of the first observations from the antigen-antibody reaction was their ability to precipitate when combined in proportions at or near equivalence. By performing these reactions in agar gels, it is possible to distinguish separate antigen-antibody reactions produced by different populations of antibodies present in the serum (Figure 1). The precipitin arcs formed between the antibodies and the antigen fuse, indicating that the antibody is precipitating identical epitopes (24). The purity and composition of the various preparations were assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE analysis of commercial and purified equine gonadotropin preparations are shown in Figure 2.

Figure 1. Photographs of Ouchterlony Plates Show the Precipitin Reaction Between Equine Chorionic Gonadotropin and its Homologous Various Rabbit Antisera.



eCG 100 μl/well; the antiserum were in the surrounding wells. Number 1, 1; 2, 2 and 3, 3 diluted at 0:0, 1:4 and 1:16, respectively, after 48 hours of diffusion; after fourth (A) and last (B) inoculation.

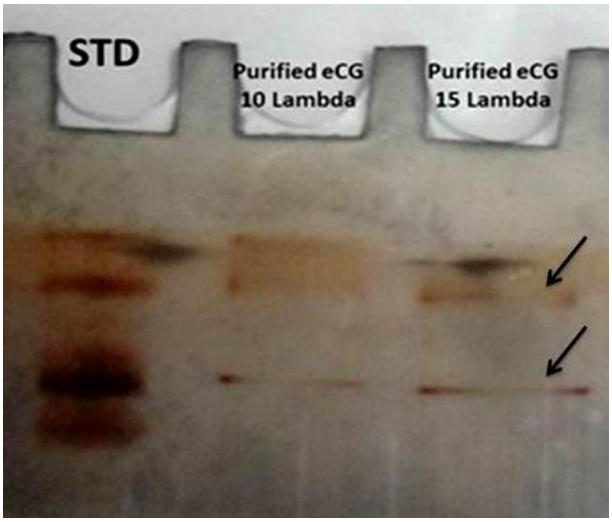


Figure 2. SDS-PAGE Analyses of Commercial (STD) and Purified Equine Gonadotropin Preparations with 10 and 15 Lambda.

Comparison of loaded samples beside the standard commercial hormone can demonstrate concentration and evidence of the isolated hormone in the present study (Figure 2). On the other hand, these results may support the effectiveness of the extracted antibody against eCG and also accuracy of the suggested method for extraction and purification of the hormone.

Total protein of Anti-eCG antibody has been calculated via the Biuret assay (25). Immunoglobulin's concentration has been determined (3g.dl-1).

The eCG content of SPE end product was determined by the method of Bradford using human serum albumin and gamma globulin as the standard (R2=0.996) (25). Therefore, eCG concentration has been evaluated (5250 $mg.ml^{-1}$).

5. Discussion

There are various methods used for the purification of eCG from pregnant mare serum during 40 to 120 days of gestation. Cartland and Nelson (15) collected 16.5 liters of plasma from 49 mares around day 65 of pregnancy. They made fractional precipitation, using ethanol and acetone, and in each step they removed the impurity. Aggarwal et al. (14) separated eCG from two groups of pregnant mares with high and low serum titers. From a biochemical perspective, the structure of eCG in both groups showed different amino acid and carbohydrate content. Owen Reed (16) patented a method using molecular ultrafiltration for separation. With an assumption of 30000 Dalton molecular weight for eCG, they used the membranes cut off for metabolites smaller than 20000 and larger than 40000 Dalton to separate the hormone. It has been reported that it is not possible to directly apply ion exchange adsorbents, due to the high salt (conductivity: 10-13 $mS.cm^{-1}$) and protein (60 $mg.ml^{-1}$) content of mare serum. Accordingly, it has been documented that several precipitation steps are needed in order to reduce the foreign protein content and the conductivity of pregnant mare serum (26). Classical purification of eCG from serum includes pH fractionation with metaphosphoric acid, two ethanol precipitation steps as well as dialysis followed by fixed-bed chromatography. In the present study, a new method for the purification of eCG has been suggested.

High specificity of antibodies is a valuable marker for recognition, purification and quantitative measurement of their own antigens. Since, it is possible to produce *in vivo* antibodies for macromolecules and small chemical materials, methods based on using antibodies were developed (24). Previously, using antibodies in purification methods was dependent on the ability of the antibody and its specific antigens to build a big immune complex (either in solution or in gel) for recognition by optical methods. These methods have been replaced by simpler ones for fixing antibodies and antigens on solid surfaces, like the standard method of SPE (27, 28).

Solid-phase extraction has been known as one of the most important methods of extraction and pre-concentration (28). It is suggested that SPE is preferred over other purification and concentration methods. The advantages of SPE are its fast and easy operation, high preconcentrate factor, various solid phases, small usage of organic solvents, absorbance of interested materials on solid phase with high stability, and low recognition level (29). SPE is a method, which separates and concentrates analytes by absorption during the solid phase. This process continues washing analytes with one or some appropriate solvents to prepare for instrumental analysis (27, 29). Different factors should be optimized in the process of absorption and reabsorption of analytes. Results of SDS-PAGE analysis in the present study showed that eCG was precisely extracted and purified.

In the current study, using anti-eCG antiserum, a simple and rapid technique for the isolation and characterization of eCG, was proposed; this method could be superior to other methods that use multiple precipitation and salting out steps. The current method only uses one-step purification, which results in eCG recovery in one fraction without significant contamination.

In conclusion, the extraction of eCG with a polyclonal antibody using the SPE method and production of anti-eCG antiserum in rabbits is suitable and may be a cost effective method for large scale production of eCG and anti-eCG antiserum in Iran.

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Authors Contributions

All authors participated equally in the manuscript preparation.

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