

Equine Chorionic Gonadotropin (eCG) Polyclonal Anti Body Production in Madura Bulls for Estrus Synchronization and Superovulation Program in Ruminants

Herry A. Hermadi¹, Sunaryo H. Warsito², Erma Safitri³

^{1,3}*Department of Reproduction Veteriner, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia, 60115*

²*Department of Animal Husbandary Veteriner, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia, 60115*

Abstract: *This study aimed to produce polyclonal anti-body (Abpo) equine chorionic gonadotropin (eCG) in Madura bulls. The Abpo-eCG was an alternative hormone for the superovulation technique and estrus synchronization of ruminant. The isolation of sera eCG derived from local Indonesian pregnant mare sera at 1-3 months age had been collected from jugular veins, and then purification with charcoal was done by chromatography Sephadex G100. After that, the produce of Abpo eCG have done in two Madura bulls carried out injected with 3,000 IU eCG from local Indonesian pregnant mare sera 3 times at 10 day intervals. Furthermore, Abpo eCG was isolated from serum taken from the jugular vein of bull, then extraction was done by adding absolute charcoal and ethanol and centrifuged at 3000 rpm for 15 minutes at 4°C. Next step, the identification of Abpo-eCG that produced from Madura bull by SDS-PAGE 12% method, Western Blot and Elisa indirect to find out the highest level at the 7th week of 408.50 mIU /mL. Then purified by CM Sephadex G-100 coloumn chromatography technique. The final product was made as a Frozen Dry dosage form.*

Keywords : *Antibody polyclonal eCG, SDS-Page, Western Blot, Elisa, sephadex G-100.*

1. INTRODUCTION

The difficult to obtain and the expensive FSH-LH preparation causes the Abpo-eCG hormone to be an alternative for superovulation techniques and estrus synchronization in ruminants [1,2]. In ruminants the use of Abpo-eCG in superovulation results in a larger follicular size [3], improvement of pregnancy rate [4] and higher number of embryos [5] and lower costs [4]. Therefore, research was needed to be able to produce Abpo-eCG. The

produce of Abpo eCG can done in bull, this research in Madura bull, through carried out injected with 3,000 IU eCG from lokal Indonesian pregnant mare sera.

2. MATERIALS AND METHODS

Animal handling was carried out ethical approval by Animal Care and Ethical Clearanve Committee of Faculty of Veterinary Medicine, Universitas Airlangga and conform with the Natioanl Research Council's guadelines through the ethical seminar.

In this study used two Madura bulls aged 3.5 years and weighing around 400 kg. The bulls were immunized with 3,000 IU eCG (from local Indonesian pregnant mare sera 3 times at 10 day intervals) which was injected with 200 μ L Complete Freund's Adjuvant (CFA) and booster with 200 μ L Incomplete Freund's Adjuvant (IFA) 3 times at 10 days intervals and with the ratio of eCG : Adjuvant = 1: 1 μ L [6]. Furthermore, Abpo eCG was isolated from bull serum taken from the jugular vein, then extraction was done by adding absolute charcoal and ethanol and centrifuged at 3000 rpm for 15 minutes at 4°C [7]. Next, the identification of Abpo-eCG by the method of SDS-PAGE (Sodium Sulphate Deodecyl Polyacrilamide Gel Electrophoresis) 12%, Western Blot and Indirect Elisa to determine the highest level. Then purified by Chromatography Sephadex G-100 coloumns chromatography technique. The final product was made as a Frozen Dry form.

3. RESULTS

eCG from local Indonesian pregnant mare sera was identificated based on molecular weight obtained through SDS PAGE 12% method. The molecular weight of the eCG protein varies between 55 kDa, 43 kDa and 28 kDa (Figure 1).

The protein that has been identified in SDS-PAGE is a protein that has not been specific, therefore to determine the specificity of the eCG protein produced is identified using Western Blotting (Figure 2).

The eCG protein that has been isolated and identified used as an antigen and immunized into the body of bulls to get the Abpo-eCG titer. The first immunization is done by adding eCG to the Complete Freund's Adjuvant (CFA) in a ratio of 1: 1 which is 200 μ L eCG and 200 μ L CFA. The second immunization (booster 1) and the third immunization (booster 2) was done by adding eCG to the Incomplete Freund's Adjuvant (IFA) with the same ratio of 1 : 1 = 200 μ L eCG : 200 μ L IFA. eCG in CFA or IFA solvents that are immunized into the body of bulls is recognized as a foreign body so that the body will react with the formation of an immune response in the form of antibodies [6], which in this study

formed Abpo- eCG. Furthermore, after purification, the antibody titer that is formed is measured based on the absorbance value through the Indirect Elisa method. In addition, the concentration was measured. The antibody titer and the concentration of Abpo-eCG are indicated by absorbance values in which can be seen in table I.

4. DISCUSSION

The characterization of serum eCG protein in mare blood was carried out by the SDS-PAGE 12% method to determine the protein band from eCG and continued with the Western Blot method to determine the specificity that the protein isolated was truly an eCG protein. During electrophoresis, the protein moves from the negative electrode to the positive electrode to a certain distance on the polyacrylamide gel, depending on its molecular weight. The lower the molecular weight, the further the protein moves, so that the mobility is high. Proteins with large molecular weight will move at shorter distances or have lower mobility. In polyacrylamide gels, the various proteins in a sample separate according to their respective mobility.

The determination of eCG protein specificity through western blot test is based on the presence of a purplish blue band on the nitrocellulose membrane on the protein band with a range of molecular weights of 55 kDa and 28 kDa. The molecular weight can be read using a reference protein marker with molecular weight. Protein band in weight is seen in samples of male bovine serum, this is due to the formation of specific bonds between eCG antibodies and eCG antigens because it can recognize eCG which is isolated from bovine serum.

Production of polyclonal antibodies in cattle is used CFA (Complete Freund's Adjuvant). CFA is an adjuvant containing microbacteria and emulsifying oil acts as a stronger adjuvant in stimulating antibodies for a long time by releasing emulsion drops slowly and stimulating macrophage function [8]. Serum derived from each blood collection of bulls immunized with eCG in adjuvants is purified to obtain ECG antibodies in the serum. Purification aims to separate albumin and globulin in serum, so that the eCG antibodies found in the serum of cow's blood globulin from immunization. The ability of eCG to induce eCG antibodies is measured quantitatively using indirect ELISA. Antibodies in the form of IgG purified, antibody titers were measured using the ELISA method for each bleeding. Antigen in the form of eCG protein in the ELISA test, binds with primary antibodies (anti-eCG) from bovine serum immunized with eCG. Primary antibodies bind to secondary antibodies labeled enzymes. Furthermore, the enzyme binds to the substrate which is visualized in the form of color. eCG antibody titers were measured based on absorbance values at a wavelength of 405 nm using an ELISA reader. Titer measurements are performed on each bleeding, aiming to

determine the bleeding that produces the highest eCG antibodies. The lowest titers occur during the preimmune bleeding, this is because the cow has not been immunized by the eCG antigen so that the cow's body does not produce antibodies against eCG. Bleeding preimmune is used as a control which then compares the absorbance value with bleeding after the first booster and the second booster. Anti eCG that enters the body of a cow is considered as a foreign body, thus triggering an immune response in the form of binding of proteins by B cell receptors.

Post-booster plasma B cells will induce the formation of IgG which is an antibody against eCG [8]. The increase in antibody titers starts after the first booster from the first week to the third week of bleeding then gradually decreases. This is supported by the opinion of Kusnoto *et al.* [9], which states that IgG is detected in the serum approximately 6-7 days after exposure to antigens that have been identified previously (booster). The decrease in antibody titer at the 4th week of bleeding occurs because the concentration of antigens in the body of the cow has decreased so that the number of antibodies produced also decreases. Booster acts as a secondary immune response in the body of a cow, namely by activating B memory cells to recognize antigens that enter the body and respond to these antigens by producing specific antibodies. The secondary immune response has the characteristics of the formation of antibodies reaching the highest point, immunoglobulins progress faster, and immunoglobulins mainly consist of IgG. Based on the results of high titer values, it can be concluded that the eCG antibodies resulting from isolation of bulls are immunogenic because they can stimulate the body's immune system with how to produce polyclonal antibodies against these antigens [10].

5. CONCLUSION :

The eCG protein can induce a humoral immune response from Madura bull, so that Abpo-eCG can be produced, with the highest titer and concentration occurring at the 7th week of bleeding, with a titer value of absorbance (Optical Density) around 0.15 and a concentration of around 400 mIU / mL.

Acknowledgment:

The authors thank to Faculty of Veterinary Medicine and Institute for Research and Innovation of Universitas Airlangga for approving this work.

Fund: The authors declare that this work from supporting charges of the Directorate General of Higher Education, Indonesia (DIKTI) 2019, The National Education Ministry, Republic of Indonesia.

Conflict of interest:

The authors declare that there is no conflict of interest in this study.

6. REFERENCES

- [1] Martemucci, G. and D'Alessandro, A.G. Synchronization of oestrus and ovulation by short time combined FGA, PGF(2alpha), GnRH, eCG treatments for natural service or al fixed-time. *Anim Reprod Sci.* (2011). 123: 32-39.
- [2] Dias, C.C.; Wechsler, F.S.; Day, M.L. and Vasconcelos, J.L. Progesterone concentrations, exogenous equine chorionic gonadotropin, and timing of prostaglandin F2alpha treatment affect fertility in postpuberal Nelore heifers. *Theriogenology.* (2009). 72: 378 -385.
- [3] Bergamaschi, M.A.C.M.; Vicente, W.R.R.; Barbosa, R.T.; Machado, R.; Baruselli, P.S.; Alencar, M. and Binelli, M. Effect of equine gonadotrophin (eCG) on ovarian dynamics in nelore cows. In: International Reproduction Symposium. Proceeding. Wellington. New Zealand. (2006). p6
- [4] Hermadi, H.A.; Adikara, R.T.S.; Warsito, S.H. and Safitri, E. Improvement of Pregnancy Rate in Bali Cows with the Combination of Equine Chorionic Gonadotropine (eCG) from Local Pregnant Mare with PGF2 α . *Indian Vet. J.* (2019). 96 (11): 52 - 55
- [5] Baruselli, P.S.; Marques, M.O.; Nasser, L.F.; Reis, E.L. and Bó, G.A. Effect of eCG on pregnancy rates of lactating zebu beef cows treated with cidr-b devices for timed artificial insemination. *Theriogenology.* (2003). 59: 214-219.
- [6] Samik, A. and Safitri, E. Anti early embryonic protein (EEP) for pregnancy test by microtiter strip in dairy cows. *Indian Vet. J.* (2019). **96(09)**: 40-42.
- [7] Hermadi, H.A.; Santanu, R.T.; Hariadi, M. and Safitri, E. human Chorionic Gonadotropin (hCG) from urine of pregnant women for in vitro maturation of Madura cattle oocytes. *Philipp. J. Vet. Med.* (2018). 55(2): 127-134.
- [8] Abbas, A.K.; Lichtman, A.H. and Pillai, S. Cellular and Molecular Immunology. 7th Ed. Elsevier Saunders, Philadelphia. (2014). p. 99-107.
- [9] Kusnoto; Koesdarto, S.; Mumpuni, S. and Prasetyo, R.H. Immunoglobulin subclass in experimental murine *Toxocara cati* infection. *Vet. World.* (2018). 10(11): 1333-1336.
- [10] Fiqriyana, A.T.; Aulanni'am, A. and Roosdiana. Characterization of polyclonal antibody induced by autoantibody TPO (Thyroidperoxidase) from autoimmune thyroid disease (AITD) serum with ELISA and Western Blotting. *J. Pure app. Chem. Res.* (2013). 2(1):19-26.

Tabel I. Titer Based on Absorbancy (Optical Density = OD) and Concentration (mIU/mL) Antibody eCG on Bull After Immunisation with eCG Isolate from Pregnant Mare

No.	Code of Sample	Sample 1		Sample 2	
		Absorbancy (OD)	Concentration (mIU/mL)	Absorbancy (OD)	Concentration (mIU/mL)
1	Blank	0	43,50	0	41,00
2	Premium	0,055	228,50	0,064	228,50
3	Bleeding 1	0,089	253,50	0,083	238,50
4	Bleeding 2	0,089	253,50	0,090	256,00
5	Bleeding 3	0,089	253,50	0,094	266,00
6	Bleeding 4	0,093	263,50	0,098	276,00
7	Bleeding 5	0,100	281,00	0,097	273,50
8	Bleeding 6	0,106	296,00	0,104	291,00
9	Bleeding 7	0,154	416,00	0,148	401,00
10	Bleeding 8	0,069	203,50	0,064	191,00

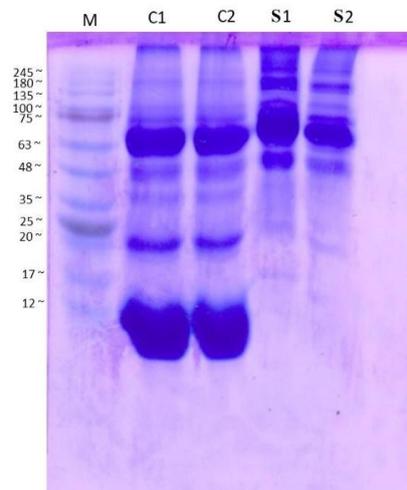


Figure 2. Molecular weight of eCG : 55, 43 and 28 kDa (SDS-PAGE 12% method)

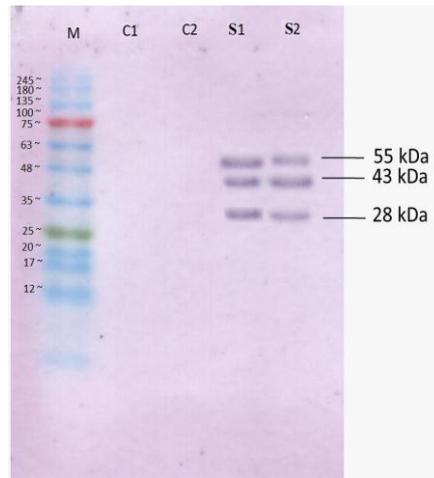


Figure 2. Specificity of eCG: 55 kDa, 43 kDa and 28 kDa (Western Blotting method)