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Isolation and Identification of Transforming Growth Factor β from *In Vitro* Matured Cumulus Oocyte Complexes

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Transforming growth factor-β (TGF-β) is a two-chain polypeptide with molecular weight of 25 kDa which takes significant role in the steroidogenesis process. In the ovarian oocyte in particular, TGF-β has an important role in regulating reproductive function. TGF-β represents a key intrafollicular protein that regulates follicle development and aromatization process. The purpose of this research was to characterize and identify a protein fraction of TGF-β from the bovine isolated oocytes, which is synthesized during *in vitro* oocyte maturation process. Oocytes were collected from follicles with diameter of 3-8 mm. Oocytes were then matured in TCM 199 media supplemented with 5 μg/mg LH, 3% BSA, and 50 μg/ml gentamicin sulfate, and cultured in CO_2 incubator (5%, 38.5 °C) for 20 hours. TGF-β receptors were identified immunohistochemically. Characteristics of the TGF-β protein were determined using SDS PAGE and TGF-β specification was tested using Western Blotting. The results showed that TGF-β receptors were identified and found in cumulus oocyte complexes (COCs). TGF-β protein was isolated from bovine oocytes with molecular weight 25 kDa and it was identified by Western blotting methods in the same molecular weight.

Key words: transforming growth factor-β (TGF-β), cumulus oocyte complexes (COCs), identification

INTRODUCTION

Currently provision of *in vitro* embryos for embryo transfer is still not able to meet the standard of the highly viable embryos. This is based on the results that when embryos produced in vitro are transferred to the recipients, the pregnancy rate is, in fact, fairly low. This low pregnancy rate needs to be investigated in the terms of molecular reproduction since oocyte maturation is not only affected by size of oocyte and hormones, but also by various growth factors, and until recently the synthesis and function of the protein in molecular sense is still not much understood.

Size of follicles surely influences the size of the cumulus cell complex. In the molecular terms, every variation in follicle size represents different protein synthesis, which presumably greatly determines the quality of oocytes after maturation, that in turn determines the quality of the embryos to be produced *in vitro*. Varying sizes of oocytes will bring about disturbances in the final maturation process so that the synthesis of several proteins related to the maturation process will work suboptimally (Hytell *et al.* 1997). Oocytes derived from heterogeneous follicles will not cause achievement of homogeneous growth *in vitro* and this condition significantly influences the subsequent development processes. It is therefore necessary to make homogeneousness when performing *in vitro* culture (Pawshe *et al.* 1996).

The molecular control of oocyte maturation is affected by the MPF activity that consists of 2 components namely cyclin B and p39 cdc2. MPF causes nuclear chromosome condensation. Another protein called as mitogen actived protein kinases (MAP Kinases) serves to undertake phosphorylation of the MPF-modified substrates to prevent oocytes from going through the interphase stage. Major protein that regulates the maturation process is protein $p39^{mos}$, serine threonine kinase that binds to cyclin B to activate and stabilize the maturation promoting factor (MPF). $p39^{mos}$ molecule is very sensitive to calcium and degraded by calcium dependent cystine protease. Secretion of intracellular Ca is associated with the development stage of germinal vesicles break down (GVBD) in the meiotic process. Increased intracellular Ca will cause a reduced cAMP level and lead to the meiotic process. To maximize the production of oocytes *in vitro*, various attempts have been made, among others, by engineering the *in vitro* maturation condition in order to improve the oocyte viability. Several growth factors are added to maturation media and culture media to enhance the ability of oocytes to make progression to the meiosis II stage. Several growth factors commonly added to the culture medium to boost oocyte maturation are including epidermal growth factor (EGF), Insulin-like growth factor (IGF), transforming growth factor (TGF), platelet-derived

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endothelial cell growth factor (PDGF). The addition of hormones and growth factors into the culture medium is intended to increase the number of oocytes reaching meiosis II stage (Roberts & Sporm 1993; Margawati 1999).

It is reported that TGF-β is a two-chain polypeptide with molecular weight of 25 kDa that takes considerable role in the process of steroidogenesis. There are two kinds of TGF, TGFα, and TGF-β that is unrelated structurally or functionally. Some of the specific protein receptors have been found in almost all mammalian cell types and effect of this molecule varies, depending on cell types and growth rate (Brand *et al.* 1998; Gurneet *et al.* 2003).

In the ovarian oocyte, TGF-β has an important role in regulating reproductive function. TGF-β stands for a crucial intra-follicular protein that regulates follicle development. TGF-β is secreted by the large follicles at about 300 pg/fol/day (May *et al*. 1996; Dodson & Schemberg 1996).

In the process of steroidogenesis, androstenedione and testosterone hormones will be formed. These hormones will be carried by blood to the granulosa cells and through stimulation by follicle stimulating hormone (FSH), they are converted to estradiol by aromatization process. Steroids in the form of testosterone serve as a substrate for aromatization and assisted by TGF-β, and influence steroidogenesis. TGF-β plays a role in the process of aromatization. TGF-β expression in granulose cells regulates gonadotrophin receptor expression and enhances steroidogenesis activity through conversion of cholesterol to pregnenolone. The results of this aromatization process is a production of estradiol 17 β receptors that act to increase luteinizing hormone (LH) receptor for oocyte maturation. Mechanisms of both TGF-β and FSH are related to the secretion of extracellular matrix to stimulate an expansion of the cumulus oocyte complexes and produce hyaluronic acids. It be used as an indicator for oocyte maturation (Lobb & Dorrington 1992; Gitay-Goren *et al.* 1996; Dore *et al.* 1996; Godkin & Dore 1998; Breveni *et al.* 1998).

TGF-β generates the effect of lowering the level of cAMP produced by the cumulus oocyte complexes (COCs). Elevated cAMP level will inhibit oocyte maturation and reduction in cAMP level would be the beginning of the meiotic process. Meiotic stage is maintained by the transfer of cAMP via gap junctions from follicles to oocytes and LH secretion will preclude the transfer of cAMP to the oocyte resulting in a decrease in cAMP level (Feng *et al.* 1988; Vitt *et al.* 2000; Saragiieta *et al.* 2002). It is therefore important to isolate and identify TGF-β proteins from the *in vitro* matured cumulus oocyte complexes (COCs) in order to modify the maturation medium for increasing maturation rate.

MATERIALS AND METHODS

Research Sample. Research sample was bovine ovaries that were obtained from slaughterhouse Pegirian Surabaya. Oocytes were collected by aspirating follicle ovaries in 3-8 mm diameter. Oocytes were *in vitro* maturated in $C0_2$ incubator (5%, 38.5 °C). Mature oocytes were characterized on the protein fractionation.

Oocyte Collection. Bovine ovaries were obtained from Slaughterhouse Pegirian Surabaya and stored in 0.89% NaCl which was already supplemented with penicillin - G (1000 IU/ml) and Streptomycin Sulfate (0.2 ug/l) at temperature of 30-35 °C. Oocytes were collected by aspiration using needle 20 G which was connected with 5-ml syringe containing 1 ml PBS that has been supplemented with 0.3% BSA and 50 mg/l Gentamicin. Aspiration was undertaken on 3-8 mm-sized follicles. Oocytes having cumulus complex layers were washed three times consecutively within phosphate buffer saline (PBS) media and just once in tissue culture medium 199 (TCM 199).

Oocyte Maturation. Oocytes were matured using a medium supplemented with 0.01 mg/ml FSH, 3% BSA and 50 ug/ml gentamicin sulfate. Sixty oocytes by their respective sizes were cultured in 100 µl drops medium and covered with mineral oil. Oocyte maturation was carried out at 38.5 °C in 5% CO_2 incubator for 20 hours (Pawshe *et al.* 1996). The matured oocytes were isolated their proteins for characterization and identification tests.

Identification of TGF-β Receptors with Immunohistochemistry. *In vitro* matured oocytes having cumulus complexes were fixed on the glass object. Immunohistochemical staining was then done with avidin-biotin complex for determining a position of the TGF-β receptor.

Isolation of Protein Fractions from Cumulus Oocytes Complexes (COCs). *In vitro* matured oocytes with cumulus complexes were dissolved in PBS solution supplemented with 0.05 mM PMSF (Phenyl methyl sulfonyl fluoride). Afterward the samples were washed with sterile phosphate buffer saline (PBS) temperature 2-4 °C by transferring the sample into petridish containing PBS. Next the sample was transferred into the PBS solution and oocytes were put into the Eppendorf tube containing PBS, EDTA and Tween 100, and then vortexed and centrifuged at 6000 rpm for 30 minutes at 4 °C. Supernatants were collected as oocyte protein fraction and uterine fluids and then supplemented with 20 mM Tris-HCl 200 µl and stored in freezer at -10 °C (Aulanni'am 2004).

Preparation of TGF-β Protein with SDS PAGE. Protein sample was supplemented with buffer solution and then stored at -10 °C. The separating gel on SDS PAGE apparatus was prepared and added. About 1 ml butanol for 30 minutes. After the gel freezed, was added stacking gel and waited for approximately 30 minutes. Next was removed the comb.

About 10-20 µl proteins were put into a mold hole on stocking gel. Plates containing sample were entered into BioRad apparatus and anode was connected to lower reservoir and cathode connected to upper reservoir. Power supply was turned on with electrical current of 30 mA. Separation process (running) was stopped after the blue color of the marker reached a height of ± 0.5 cm then running buffer was poured and the gel was removed from the plate, then washed and stained with Coomassie Blue. Staining was performed by soaking the gel in staining

solution for 30-60 minutes. Electrophoretic results were then photographed.

Molecular weight was determined by comparing the electrophoretic results of protein fraction and the marker protein. Determination of molecular weight was performed by calculating retardation factor (Rf) values from each band where:

$$Rf = \frac{\text{migration distance of proteins from the initial place}}{\text{migration distance of color from initial place}}$$

Then we created a standard curve of markers protein with Rf value as the axis x and the logarithmic value of molecular weight as the axis y. Molecular weight of the protein fraction was determined by interpolation of the standard curve (Aulanni'am 2004).

Identification of TGF-β Proteins by Western Blotting. Gel protein was transferred to nitrocellulose membranes by cutting paper in line with the size of the gel. Nitrocellulose membrane was incubated in anode buffer 11 for 5 minutes and then we arranged 6 sheets of absorbent paper. Furthermore, they were furnished with electrical current of 125 V, 40 mA for 1 hour. After the proteins were transferred, nitrocellulose was washed with distilled water for 10 minutes and with TBS solution for 10 minutes and finally blotting was performed.

Nitrocellulose blot was blocked with 10% BSA for 30 minutes at room temperature and then washed with TBS solution 2 times. Furthermore, it was reacted with rabbit serum (polyclonal antibody-prolactin) and as control, reacted with PMSF. Afterward, it was incubated at room temperature for 1 hour. After being washed 3 times with TBS solution, it was reacted with alkaline phosphatase conjugates and P-NPP substrate and stained with fast-red. Finally was dried it at room temperature, and from this result, were determined specific protein, molecular weight of protein and isolation of specific protein from TGF-β.

RESULTS

TGF-β Expression in Cumulus Oocytes Complex (COCs). COCs as a source of TGF-β protein were collected from follicles with a superficial diameter of 3-8 mm. COCs used in this study are grade A oocytes (Figure 1). TGF-β expression in COCs observed using immunocytochemical method with monoclonal antibody TGF-β was identified by brown color (Figure 2).

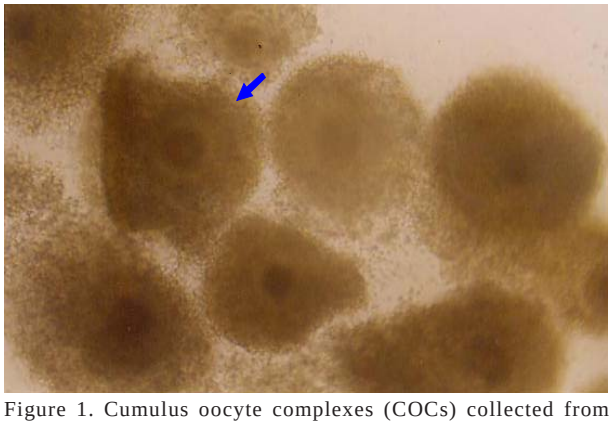

Figure 1. Cumulus oocyte complexes (COCs) collected from follicles.

Molecular Weight of the TGF-β. Molecular weight of the TGF-β determined using Western Blot showed protein band at 25 kDa recognized by Mab-bovine TGFβ (Figure 3). TGF-β performed using electro-elusion of the

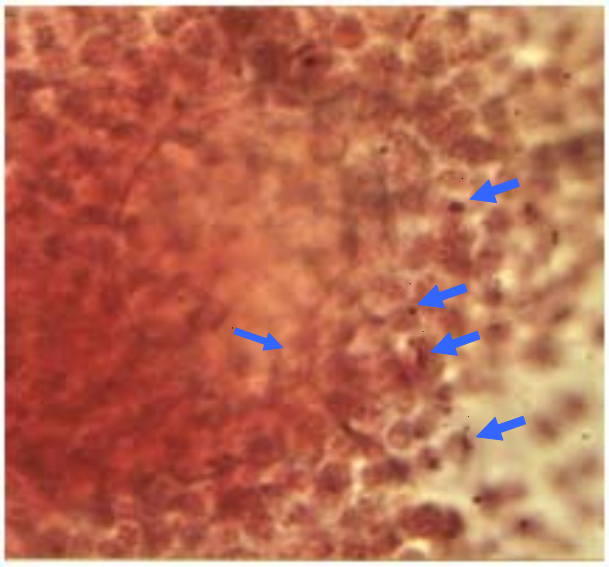

Figure 2. TGF-β expression in COCs. → TGF-β position recognized by anti-TGF-β.

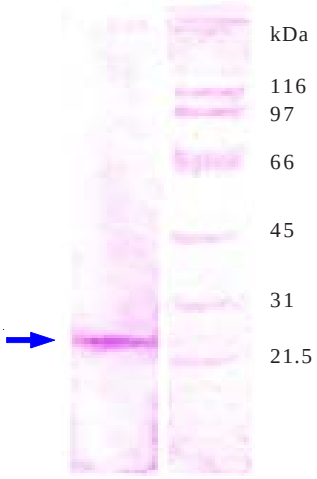

Figure 3. The result of Western Blotting for TGF-β antigen recognized by antibody anti-TGF-β Standard.

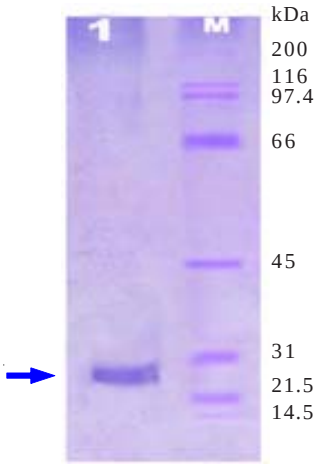

Figure 4. Electropherogram of the TGF-β. M: Marker, → molecular weight of TGF-β at 25 kDa.

SDS PAGE showed the band with molecular weight of 25 kDa (Figure 4).

DISCUSSION

The result of the antigen-antibody reaction can be identified in specimen because antibody is bound to a marker that can be visualized thereby it can indicate the presence of the active components. The active components should be accumulated in adequate number within cells or tissues so they can be bound to specific antibody and can be visualized (Nurhidayat 2002). Antibodies may be monoclonal or polyclonal. In this study, we used monoclonal antibody. Monoclonal antibody is a pure antibody containing one type of antibody for one antigenic side (epitopes) typical of the antigen. Monoclonal antibody is commonly used for immunohistochemical research with narrow specificity, so it does not allow cross reaction with other antigens (Nurhidayat 2002).

Staining in this method is the bond between the antigen-antibody attached either directly or indirectly to a marker, and the positive reaction will be visualized because of the cromogen bound to the marker (Nurhidayat 2002). The technique used in this research is a method of avidin-Biotin Complex (Nurhidayat 2002). Antigen, in this case TGF-β, is bound to antibody in two stages. Primary antibody binds directly to TGF-β, and then primary antibody binds to secondary biotynilated antibody. Each arm of secondary antibody has been conjugated with biotin which can bind to avidin molecules. Primary antibody binds to secondary antibody where arms of the secondary antibody are bound to biotin. This complex of avidin biotin-antibody bonds can identify a position of the TGF-β receptor.

TGF-β has three receptors consisting of TGF-β type I receptor (TβR I), TGF-β type II receptor (TβR II), and TGF-β type III receptor (TβR III). TGF-β transmits signals through receptor complex consisting of two types of serine-threonine kinase that bind to the membrane. The binding of ligand will activate kinases of TβR II, which then phosphorylates TβR I, resulting in kinase activation (Shi & Massague 2003; de Caestecker 2004). As a result, TβR I experiences phosphorylation and thus activates certain intracellular signaling molecule called as Smad (Miyazawa *et al.* 2002; Ten Dijke & Hill 2004).

Ligand of TGF-β includes homodimer and heterodimer bound to the receptor complex. Receptor complex consists of two TβRII subunits and two TβRI subunits. Aggregation of the receptor complex may occur; a binding can occur in TβR II first which then attracts TβR I or vice versa. Binding of ligand to the receptor complex can result in activation of serine threonine kinase of TβR II that then undergoes phosphorylation thereby activating TβR I. TβR I ultimately phosphorylates Smad receptor (rSmad). The phosphorylated rSmad comes into nucleus to regulate transcription through the interaction with certain DNA recognition site and other proteins regulating transcription (Newfeld *et al.* 1999; Shi & Massague 2003; Derynck & Zhang 2003). Protein already identified through SDS PAGE method doesn't necessarily contain a desired protein. Therefore, we need specific test to determine specifically the desired protein. One of the specific tests commonly used in the research is Western blotting test (Murray *et al.* 2002).

For such specific test, we used the TGF-β isolates as antigen identified through SDS PAGE method with anti-TGF-β monoclonal antibody and anti-mouse secondary antibody. The results showed that anti-TGF-β antibody demonstrated positive reaction against TGF-β from the SDS PAGE results with molecular weight of 25 kDa. Western blotting also indicated that the TGF-β molecules bind specifically to TGF-β antibody as primary antibody and anti-mouse as secondary antibody. Anti-TGF-β antibody is able to detect TGF-β as a band with molecular weight, thus we can infer that the band that emerged during electrophoresis by SDS PAGE method is a true band of TGF-β with molecular weight of 25 kDa.

Regarding the analytical results with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and specific test with Western Blotting, it is found that the protein profile of TGF-β can be seen clearly in the in vitro matured oocytes.

When maturation condition is not homogenous enough, the oocytes cannot reach optimal maturation which may produce a suboptimal outcome. TGF-β is secreted when an oocyte has experienced a maturation process and this is clearly shown from the results obtained (Roy *et al.* 1998).

Nuclear maturation process will take place when there is LH surge. In the course of the maturation process, there are various factors involved in protein synthesis, in addition to the growth factors such as Transforming Growth Factor- and other factors, which allow oocytes to have meotic competence. During the meiotic process, oocyte is regulated by maturation promoting factor (MPF), a protein kinase whose activity triggers a reaction that causes it to disappear so that the clear maturation process begins to take place (Hendriksen *et al.* 2000).

Meiotic competence is closely associated with the size of oocytes, in the sense, associated with size of follicles. Antral follicles with certain size have specific meiotic competence. Bovine oocytes of 2-3 mm are able to complete the GVBD and meotic stages. According to Abdon (2001), oocytes derived from large follicles have better capability to grow than those of medium follicles as large follicles can provide a macro-environment that could support their quality.

We concluded that TGF-β from cumulus oocyte complexes (COCs) can be identified and characterized well at 25 kDa. We can identify specifically that a protein with molecular weight of 25 kDa is true TGF-. Position of TGF-β receptor can be determined using immunocytochemical method.

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