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The Characterization of *Proprotein Convertase Subtilisin/Kexin Type*4 (PCSK4) on Human Sperm Membran for Developping Male Immunocontraception Candidates

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Abstract: Proprotein convertase subtilysin/Kexin Type4 (PCSK4) is a protein convertase enzymes in spermatozoa acrosome membrane which has molecular weight 54kDa, it has an important role in the acrosome reaction through endoproteolytic process that converts the precursor proteins into bioactive protein as a necessary substance to penetrate on the zona pellucida. This study aimed to characterize Proprotein Convertase Subtilisin/Kexin Type4 (PCSK4) of human spermatozoa to get an immunocontraception candidate. The research method; Characterization PCSK4 to identify the presence of convertase enzymes in the membrane proteins of human spermatozoa by immunohistochemistry technique, SDS PAGE and Western Blotting. The results of immunohistochemistry test showed that the molecule of PCSK4 54kDa expressed on the membrane of spermatozoa acrosome, the results of SDS PAGE showed that protein band which has molecular weight of 54kDa and confirmed as PCSK4 enzyme expressed and have possibility to develope as Male Contraception used as an antigen candidate of immunocontraception based on antibody againt to PCSK4.
Keywords: *Proprotein Convertase Subtilisin/Kexin Type*4 (PCSK4), immunocontraception, immunohistochemistry, SDS PAGE, Western Blotting.

Introduction

The world's population has used contraception more than 6.5 billion and this increased 75 million each year. The use of contraceptives is increasing in the worldwide, and more than 75% of couples use effective contraceptive methods in many countries. These days alot of women use contraception with wider range of choise but male contraception is limited only to condoms and vasectomy. The current methods of contraception are still not perfect, because they still leave side effects and discomfort. Multicultural surveys state that there is an awareness of men to participate using contraception, althought the choices are still limited. Therefore, a new contraception method is needed, which more effective, easier to use, and safer compared to existing contraception methods [1-3].

Immunocontraception is contraception which administered by injection using an antigen material and aims to prevent an attachement between both egg and sperm. The exist in membrane sperm actively interact in spem-egg interaction during fertilization process. One of molecule which has opportunity to be used as antisperma antibodies (ASA) in the development of male immunocontraception is convertase enzyme in sperm acrosome membrane, that enzyme is proprotein convertase subtilysin/ Kexin Type4 (PCSK4) which has a molecular weight of 54 kDa. PCSK4 molecule expressed on the cell membrane of sperm acrosome and has an

important role in the process on acrosome through endoproteolytic reaction that converts precursor protein into bioactive proteins as a substance which necessary to penetrate on the zona pellucida [4].

Proprotein convertase subtilysin / Kexin Type4 (PCSK4) will be active in the process of fertilization. PCSK4 is an enzyme that plays an important role in reproductive physiology. Impaired expression of PCSK4 cause premature acrosome reaction and deterioration of spermatozoa ability to attach to the zona pellucida. PCSK4 proteolytic activity disorder causing disruption of fertility. Inactivation of PCSK4 is a strategy in the development of non-hormonal contraception and immunocontraception [4].

The study aims to characterize Proprotein Convertase Subtilisin / Kexin Type4 (PCSK4) of human spermatozoa to get immunocontraception candidates.

Materials and Methods

Immunocytochemistry techniqur for the Confirmation of Proprotein Convertase Subtilisin/Kexin Type4 (PCSK4) on Human Sperm Membrane

The layered sample on glass was dipped in xylol twice, alcohol-rise (100, 90, 80, 70, 30%), and distilled water respectively, then washed in PBS pH 7.4 for 3 x 5 minutes, then it was soaked in 3% hydrogen peroxide (in DI water) for 5-10 minutes, washed in PBS pH 7.4 for 3 x 5 minutes, soaked in 1% BSA (or 1% NGS) in PBS for 10-30 minutes at room temperature, and washed in PBS pH 7.4 for 3 x 5 minutes. After that the primary antibody (anti PCSK4 antibody) was added on the preparate for 1 hour at room temperature, and washed in PBS pH 7.4 for 3 x 5 minutes. The secondary antibody Anti-Rabbit IgG biotin labeled was added for 1 hour at room temperature and folloowed by washing in PBS pH 7.4 for 3 x 5 minutes, and SA-HRP (Horseradish Peroxidase Strep-Avidin) was added for 30-60 minutes at room temperature, then it was washed in PBS pH 7.4 for 3 x 5 minutes. Chromogen DAB (3,3-diaminobenzidine tetrahydrochloride) were added for 10-20 minutes at room temperature, it was washed in distilled water for 3 x 5 minutes. Then counterstain with Hematoxylen was done for 5 minutes at room temperature and washed in distilled water for 3 x 5 minutes. The mounting with entellan was done and The observations of preparat was performed using a light microscope with 400x magnification. The used of human sperm were approved by Health Research Ethics Committee, Medical Faculty, Padjajaran University, Bandung, Indonesia (approval no. 458/UN6. C2.1.2/KEPK/PN/2013).

Isolation of Proprotein Convertase Subtilisin/Kexin Type4 (PCSK4)

The ejaculate of human sperm is transferred to the microtube, centrifuged at a speed of 6000 rpm for 10 minutes. 600 mL PBST-PMSF were added into pellet, it were mixed on the vorteks for 10 minutes, sonicated for 20 min and centrifuged at a speed of 10000 rpm. Pellet was crushed in PBST-PMSF and it were mixed on vorteks for 10 minutes, then sonicated for 10 min and centrifuged at a speed of 6000 rpm for 15 minutes. Ethanol 70% was added into supernatant in the ratio of 1: 1 and incubated overnight (overnight). Centrifugation for 15 minutes at 10000 rpm was done after incubation. Tris-Cl buffer was added into pellet in the ratio of 1 : 1 in order to obtain crude protein. The crude protein samples were stored at -20° C.

Determination Molecular Weight of PCSK4 by SDS PAGE.

SDS PAGE electrophoresis was using a discontinuous system with 12.5% separating gel and 3% stacking gel. The method was based on the method of Laemmli electrophoresis. RSB 20 µl were added into crude proteins from spermatozoa membrane isolation in the Tris-Cl buffer 20 mM with a ratio of 1: 1. Samples were heated for 5 minutes at a temperature 100°C. The samples were put into the wells (± 30 lµ). Running electrophoresis was conducted at constant current 28 mA until the tracking dye reached 0.5 cm above the bottom of the gel. The distribution of protein bands can be determined by staining the gel using Coomasie Brilliant Blue (CBB).

Confirmation of PCSK4 by Western Blotting Technique

Western blotting was performed using transfer equipment which is produced by BioRad. Proteins was transfered from the gel to the nitrocelulose membrane for 15 hours with a voltage of 25V. The result of transfer membrane was stained with Ponceau for 5-10 minutes, to determine whether the proteins in the gel had been transferred. Protein had been transferred if there is protein bands on the membrane after the membrane was

rinsed. Blotto nitrocelulose membrane was soaked in 5% (5% skim milk in PBS) for 60 minutes, then it was washed in PBST for 3 x 5 minutes.

The Nitrocellulose membrane was incubated with anti-human monoclonal antibody PCSK4 (1: 200 in 1% skim PBS) overnight at 4°C for confirmation of the band. Then it was washed in PBST 3 x 5 minutes after incubation. The secondary antibody (IgG Alkaline Phasphatase Conjugated 1: 2500 in TBS) was added on membrane for 1 hour at room temperature, then it was washed again with PBST 4 x 5 minutes. After that the membrane was soaked in Western Blue Substrate Solution (in the dark) overnight, then washed with distilled water to stop the reaction. The results seen by stained band on the membrane.

Data Analysis

The characterization of Proprotein Convertase Subtilisin / Kexin Type4 (PCSK4) on the membrane of human spermatozoa analysis was performed using Immunohistochemistry method, SDS PAGE and Western Blotting. The descriptive data were observated on the result from Immunohistochemistry, SDS PAGE and Western blotting.

Result

Immunocytochemistry of PCSK4 expression

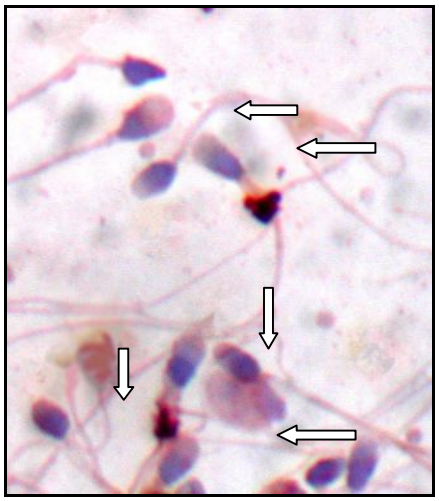

Fig 1. The arrows point to PCSK4 expression on the surface of sperm acrosome

Immunohistochemical technique in this study conducted to confirm the enzyme convertase (PCSK4**)** on the head of human sperm membrane in the ejaculate using Monoclonal antibody againt to human PCSK4 as a primary antibody. The results which had been obtained by immunohistochemistry test using DAB imunostaining showed that the sperm acrosome membrane looks dark brown (Fig.1).

SDS PAGE and Western Blot analysis fof PCSK4 isolated from human sperm

This study isolated crude protein convertase enzyme in the ejaculate which containing spermatozoa to get PCSK4 with molecular weight 54 kDa. The protein was tested by SDS PAGE to determine its characteristics.

Crude protein obtained from the isolation of ejaculate containing human spermatozoa process had been running by SDS PAGE. The results of SDS PAGE showed protein band profiles appear as eight type which have molecular weight of 20, 23, 37, 54, 190, 261 kDa. Protein with a molecular weight of 54 kDa been known have a high density (Fig 2).

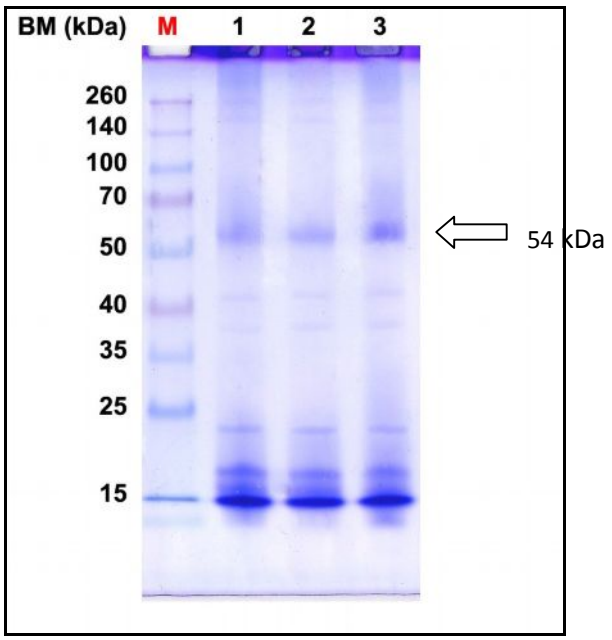

Fig 2. The protein band from SDS PAGE. The arrow point to protein band which has molecular weight of 54 kDa as PCSK4. M is a Marker, Lines 1,2 and 3 are the samples of protein from human sperm.

Western Blotting technique was performed by reacting the antigen (PCSK4**)** with Monoclonal antibody of PCSK4. The positive reaction antigen-antibody was showed by the appearance of purple-blue in Nitricellulose membrane (Fig 3). The protein band with molecular weight of 54 kDa in this study confirmed as PCSK4.

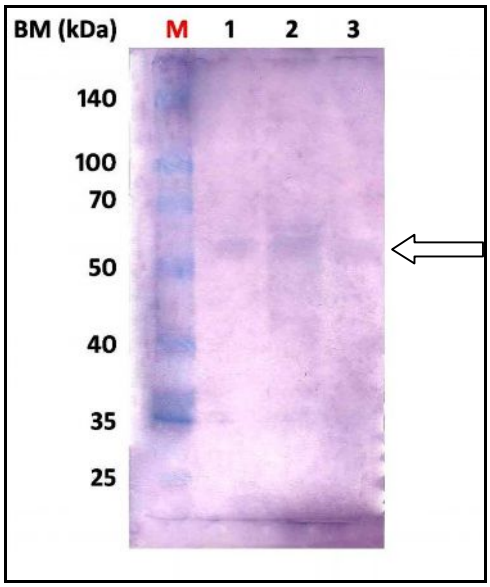

Fig 3. PSCK4 expression of isolast Human spermatozoa acrosome membrane with WB technique. M is a Marker, number 1,2 and 3 are the samples of crude protein from human spermatozoa membrane.

Discussion

In order to observed the existence of Proprotein convertase subtilysin / Kexin Type4 (PCSK4) in human sperm membrane, we develope staining procedure using immunocytochemistry technique, corfirmed by monoclonal human antibody PCSK4 (Fig 1)

Proprotein convertase subtilysin / Kexin Type4 (PCSK4) was a product of 9 kilobase (kb), gene exon 15 and intron 14, which in humans was located on chromosome 19. PCSK4 was synthesized in the endoplasmic reticulum (ER) as a preprotein multidominan consisting of signal peptide N -terminal followed by a pro-

domain, a catalytic domain, P domain and the C-terminal domain. The most abundant PCSK4 was located in spem membrane protein. Protein PCSK4 found significantly in testicular germ cells and spermatozoa [4-6].

The result of WB showed that monoclonal antibody of PCSK4 recognized with protein human sperm with molecular weight of 54 kDa. The previous studies mentioned that PCSK4 has a molecular weight 54 kDa. The band which showed a molecular weight of 54 kDa on the results of this study using Western blotting confirmed as PCSK4.

The results of this WB technique indicated that PCSK4 found with molecular weight of 54 kDa in ejaculated human sperm which will be used to develop a novel male immunocontraception candidate through the production of human antibodies againts to PCSK4. The protein with molecular weight of 54 kDa isolated from human sperm membrane recognized by monoclonal antibody to PCSK4 (Fig 1 & 3)

These antibody will cause a conformational change of protein in sperm membrane because that antibody will be bound to PCSK4 and fertilization receptors on the surface of sperm membrane will not recognized by its ligand in the oocyte cells. Sperm membrane protein has possibility to be used as candidate of male immunocontraception, because this protein only expressed in sperm membrane and not found in the other tissues.

PCSK4 was an enzyme that plays an important role in reproduction physiology, proteolytic activity PCSK4 disorders cause fertility disorders. Fertilization was a complex process, including capacitation, acrosome reaction linkage to the zona pellucida (ZP), penetration passes ZP, fusion with the plasma membrane of the oocyte [4,7]. The most important thing in the process of fertilization is the recognition and the linkage between the existing complement molecules on sperm and the ZP of oocyte. Molecules that play an important role in the recognition and attachement of spermatozoa and zona pellucida is an important molecule for the development of immunocontraception candidates. PCSK4 is an enzyme that essential in the process of reproduction. The expression and activity of PCSK4 disorder could be the cause of infertility in humans, therefore inactivation of this enzyme is a potential strategy for non-hormonal contraception or immunocontraception [8-9].

The protein which can be used as the antigen has a molecular weight at least 10 kDa. Appropriate immunogen will cause an immune response through activation of lymphocytes and induction repeatedly immunogen can enhance the proliferation of T cell and B cells specific to an antigen [10-11]. Protein PCSK4 of the isolated crude protein in ejaculated containing human spermatozoa had 54 kDa molecular weight, therefore PCSK4 Protein is a protein convertase enzyme that can be used as candidate antigens in immunocontraception.

Conclussion

The results of this study indicated that there was expression of Proprotein Convertase Subtilisin / Kexin Type4 (PCSK4) on acrosome sperm membrane which have molecular weight of 54 kDa. PCSK4 protein convertase enzyme is an immunogenic protein that can be used to develop a candidate male immunocontraception through the production of anti-PCSK4.

Conflict of Interest

There is no conflict of interest.

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