

CHARACTERIZATION OF FERTILIN BETA PROTEIN OF HUMAN SPERM MEMBRANE. A CANDIDATE FOR IMMUNOCONTRACEPTION

Reny I'tishom¹, Doddy M. Soebadi², Aucky Hinting¹, Umie Lestari³

¹Department of Medical Biology Department, Faculty of Medicine, Airlangga University, Surabaya

²Department of Urology, Faculty of Medicine, Airlangga University, Surabaya

³Department of Biology, State University of Malang, Malang

ABSTRAK

Di Indonesia peserta KB pria baru mencapai 1,3% dari total 58,3% dari seluruh peserta KB. Metode kontrasepsi pria selama ini hanya ada 2 macam, yaitu penggunaan kondom dan vasektomi. Namun kondom efektif mencegah kehamilan sebesar 75–80%, sedangkan vasektomi bersifat permanen. Untuk itu perlu pengembangan kontrasepsi baru bagi pria dengan khasiat jangka lama, tetapi reversibel dalam menyebabkan azoospermia. Vaksin kontrasepsi merupakan konsep baru untuk mengontrol kesuburan. Imunocontraceptive merupakan alternatif kontrasepsi berprinsip imunologis secara injeksi menggunakan bahan antigenik untuk mencegah pertemuan spermatozoa dan oosit. Salah satu kandidat vaksin imunocontraceptive adalah antigen spermatozoa. Salah satu antigen tersebut adalah protein fertilin beta membran spermatozoa manusia. Metode ini lebih aman, memiliki kespesifikan tinggi, efektif, praktis, efek samping lebih kecil dan kemungkinan memiliki peluang dapat diterima oleh pengguna dari pada teknik kontrasepsi lainnya. Penelitian eksploratif – laboratoris ini bertujuan mendapatkan isolat dan mengidentifikasi protein fertilin beta membran spermatozoa manusia. Koleksi spermatozoa dan analisis semen, imunositokina, pemisahan protein membran spermatozoa manusia menggunakan sonifikasi dan sentrifugasi, penentuan berat molekul protein fertilin beta membran spermatozoa pria menggunakan elektroforesis SDS-PAGE, pengukuran kadar protein dengan metode Biuret, penentuan konsentrasi protein fertilin beta, western blot untuk menentukan spesifitas fertilin beta, isolasi protein fertilin beta membran spermatozoa menggunakan teknik elektroelusi. Diketahui bahwa protein membran spermatozoa pria hasil SDS-PAGE memiliki profil pita protein sebanyak 4 pita dengan berat molekul 17, 35, 75 dan 140. Dari 4 profil pita tersebut dilakukan Western blotting dan diketahui bahwa protein fertilin beta mempunyai berat molekul (BM) 75 kDa. Protein fertilin beta 75 kDa memiliki kadar protein 2407 ug/ml. (FMI 2013;49:26-32)

Keywords: imunocontraceptive, fertilin beta, membran spermatozoa manusia

ABSTRACT

In Indonesia, male family planning participants reached 1.3% of total 58.3% of all participants. So far, there are only two kinds of male contraceptions, the use of condoms and vasectomy. Condoms are effective in preventing pregnancy by 75-80%, whereas vasectomy is permanent. We need new male contraceptive with long-term benefits, but reversible in causing azoospermia. Contraceptive vaccine is a new concept to control fertility. Injected imunocontraception is an alternative of immunological contraception using antigenic material to prevent the meeting between spermatozoa and oocytes. One potential candidate is spermatozoa antigen. One of these antigens is protein fertilin beta of human spermatozoa membrane, which is more secure, with high specificity, effective, practical, with less side effects and high probability of acceptance. The aims of this study were to isolate and identify protein fertilin beta of human sperm membrane. This exploratory laboratory study collected sperm and analyzed semen, undergoing immunocytochemistry, human spermatozoa membrane proteins separation with sonification and centrifugation, protein molecular weight determination of fertilin beta sperm membrane using SDS-PAGE electrophoresis, protein concentration measurements with the Biuret method, and determination of protein concentration fertilin beta, Western Blot was used to determine the fertilin beta specificity, and fertilin beta isolation with electroelution techniques. It was found that human sperm membrane proteins results of SDS-PAGE profiles of protein bands had four bands with molecular weights of 17, 35, 75 and 140. These bands were subjected to Western blotting and fertilin beta protein revealing a molecular weight (MW) of 75 kDa with higher levels of 2407 ug/ml. (FMI 2013;49:26-32)

Keywords: human sperm membrane, fertilin beta, imunocontraception

Correspondence: Reny I'tishom, Department of Medical Biology Department, Faculty of Medicine, Airlangga University, Surabaya. Email: ritishom@yahoo.com

INTRODUCTION

Global concern on population growth rate is related to family planning and reproductive health programs

which are safe and easy method to limit the number of family members. World population is estimated to reach 10 billion by 2050. Most of the population live in developing countries compared to developed countries.

In Indonesia, total population in 2004 has exceeded 200 million and population control implemented in Family Planning (KB). Male participants KB reached 1.3% of total 58.3% of all participants. Male contraceptive methods that have been done so far comprises only 2 kinds, namely the use of condoms and vasectomy. The failure rate of condoms is generally very high, which is about 12 percent, while vasectomy effectively prevents pregnancy permanently. Ideally there should be a male contraceptive with long term efficacy, but reversible in causing azoospermia.

Various studies have focused on male immuno contraceptive methods. Contraceptive vaccine is a new concept that is expected to be used to control fertility. Immunocontraceptive method is expected to be safe, effective, practical, fewer side effects and have a chance to be accepted by users than previous contraceptive techniques. Immunocontraceptive is an immunological contraception with the principles given by injection using an antigenic substance and intended to prevent a meeting between spermatozoa and oocytes. One of the materials that has potential as a contraceptive vaccine is a sperm membrane protein. (Naz 2006, Griffin 2012).

Membrane proteins of human spermatozoa have been found and has been tested as immunogenic proteins, and it has specific function in fertilization. These proteins include 17 kDa (Gritzzi et al 2003), 55 kDa and 95 kDa (Naz 1998), 57 kDa (Rajeev & Reddy 2004), 80 kDa (Bandivdekar et al 2005), PH -20 (Toshimori 2000), SPAG9 (Jagadish et al 2005). However, some researchers claimed that human sperm membrane protein that has been found so far have not met the requirements as immunocontraceptive material. Results of several studies indicate that the 17 kDa protein, in addition of being present in the testes and spermatozoa, are also found in epithelial cells of respiratory organs and nerve cells (Gritzzi et al 2003). SPAG9, in addition of being found in the anterior part of the head and the outer acrosomal membrane of spermatozoa is also found in nerve cells (Jagadish et al 2005). 95 kDa protein, in addition to be in the anterior part of the head was also found in human spermatozoa, and in some somatic tissues (Bull et al 2003).

Because proteins are still conserved in other cell types, we have to look for a membrane protein that is thought to exist only in spermatozoa so that the membrane proteins are immunogenic. The antibody induction outcome is not expected to inhibit the activity of other cellular tissues. One of sperm membrane proteins that have the potential of fertilin beta is present only in mature and ejaculated spermatozoa that serve as adhesion molecules. However, to date the identification

and characterization of the human sperm membrane fertilin beta is still not clearly disclosed.

Several studies have shown that sperm membrane protein has a function in the fertilization -related events, such as sperm motility, sperm membrane adhesion to the zona pellucida, the initiation of signal transduction, as well as binding and fusion with oocyte membrane. According to Naz et al (2000), sperm membrane protein used as an ingredient immunocontraceptive candidate must have specific criteria that is conserved only in spermatozoa, the spermatozoa lack of specificity (only acts on gametes and not in other organs), has a role in the process of antigen fertilization, and in immunogenicity it has the potential formation of antibody responses to block fertilization. Therefore, identification and characterization of proteins that are immunogenic sperm membrane is an important step for the development of such proteins as candidate foimmunocontraceptive materials.

MATERIALS AND METHODS

This was an explorative laboratory with the descriptive analysis of protein fertilin beta, exploring the human sperm membrane that is expected to be used for the manufacture of immunocontraceptive material candidates. Subjects were fertile men aged 21-40 years old, married, with the results of semen analysis normozoospermia and willing to participate in the study. This research was conducted in the Clinical Andrology Laboratory, Dr. Soetomo Hospital, Airlangga University Faculty of Medicine Department of Biology and Laboratory of Molecular Biology, Brawijaya University. The study subjects received an explanation of research objectives and agreed to participate in the study by signing a letter of willingness revelation that has been approved and tested by the Health Research Ethics Committee, Faculty of Medicine, Airlangga University. Semen sampling was conducted at Andrology Clinic Dr Soetomo Hospital, then semen analysis was performed.

Collection of human spermatozoa and semen analysis

Semen samples were obtained from fertile men (normozoospermia) which has been evaluated at Andrology Clinic, Dr Soetomo Hospital. Ejaculate was obtained from masturbation after 2-5 days of sexual abstinence. After liquefaction (37 °C, 30 min) semen analysis was performed (concentration, motility, viability and morphology) based on the guidelines of WHO (1999). Samples with a ratio of spermatozoa : leukocytes > 100 : 1, agglutination ejaculate was

examined immunobead test (IBT) (>10% agglutination of spermatozoa) were excluded (Rajeev & Reddy 2004).

Separation of human spermatozoa membrane proteins with sonification and centrifugation techniques

Spermatozoa samples were placed in a 15 mL Eppendorf tubes, added with PBS, and then centrifuged and vortexed at 3000 rpm (room temperature, 10 minutes). The process was done twice. The result was pellet, plus detergent NOG (1N-Octyl-beta-D-Glycopyranoside) (Rajeev & Reddy 2004) or 2% CHAPS (Cyclo Hexyl-Amino-1Propane Sulphonic acid) (Bohring et al. 2001). The vortex was done for 10 minutes, using an ultrasonic cleaner, and sonificated for 20 min and centrifuged at 6000 rpm for another 5 minutes. The supernatant obtained was added with ethanol at a ratio of 1:1 and put in refrigerator for 60 minutes. If no white blob appeared, the time was prolonged. If the white blob appeared, the sperm membrane proteins was subsequently centrifuged at 10,000 rpm (room temperature, 10 minutes) in order to settle a clot, then it was put in the refrigerator at -20 °C for 5 minutes. Ethanol in the extract was removed and aerated until the odor of ethanol is lost. The extract was diluted with Tris-Cl buffer at a ratio of 1:1 to the sediment volume of sperm membrane protein extracts. The extracts was stored in -20°C refrigerator.

Measurement of protein content by Biuret Method

Determination of Maximum Wavelength 5000 ppm BSA

A total of 200 µL solution of Bovine Serum Albumin Standard (BSA) concentration of 5000 ppm was put into eppendorf, added with 800 µL Biuret reagent, and then shaken and allowed to stand for 30 minutes. Furthermore, the absorbance was measured using a UV-VIS spectrophotometer in the wavelength range 500-600 nm. As the blank we pipetted 200 µL of distilled water and 800 µL Biuret reagent.

Preparation of standard curve of BSA

We prepared 10 eppendorf, each coupled with a 200 µL standard BSA (Bovine Serum Albumin) solution with variations in the concentration of 100-10000 ppm. Each was added with the 800 µL Biuret reagent, shaken and allowed to stand for 30 minutes at room temperature. Furthermore, the absorbance was measured with UV-VIS spectrophotometer at λ maximum measurement obtained from a standard solution of 5000 ppm BSA. Then, linear regression equation was made on the

relationship between concentration and absorbance in order to obtain a standard BSA curve.

Determination of male sperm membrane proteins concentration

A total of 200 µL sperm membrane fertilin β protein isolate solution was added with 800 µL Biuret reagent, then shaken and allowed to stand for 30 minutes at room temperature. Then we measured the absorbance at the maximum wavelength of 550 nm, namely BSA. The absorbance obtained was converted to the BSA standard curve, the blank was pipetted 200 µL of distilled water and added with 800 µL Biuret reagent, and then the absorbance was measured. The protein content was calculated by converting data absorbance to a concentration through BSA standard curve linear regression equation ($Y = 5 \times 10^{-5}X$). The minimum requirement of protein use as antigens was 300 µg/200 ml of solvent (Robert & Peter 1993).

Molecular weight determination of human sperm membrane proteins using SDS-PAGE electrophoresis

Identification of membrane proteins by SDS-PAGE aims to reveal protein profile in the form of area percentage (protein bands). Stages of works is as follows: the use of two layers of gels, as sample (stacking gel) and as medium for protein separation (separating gel). Separator gel was made by mixing ingredients of LGB, the T-Acryl, and dd H₂O, while the gel serving sample consisted of UGB, T acyl, dd H₂O. All materials were mixed each except ammonium persulfate (APS) and N, N, N', N'-tetramethylethylenediamina (TEMED). Bubbles were removed for 10 minutes, APS and TEMED were added shaken briefly and then put in a plate and allowed for 10-30 minutes until the gel hardened. Gel sample collection was made in the same way without removing the bubble. After the separator gel hardened, sample collecting gel solution was poured on top and fitted until the gel solidified and sinks were formed. Then, the comb was removed, mounted on the electrophoresis plate, and buffer was poured on the device.

Sample injection and running were done by mixing human sperm membrane protein isolate with reducing buffer, and then the sample was heated in a water heater at a temperature of 100 degree C for 2 min. Then it was cooled, and thereafter the samples were entered into the gel wells with a volume of 10 µL of each well. Standard protein as markers was treated the same as the sample. After that, the anode was connected to lower reservoir and the cathode was connected to upper reservoir. The

power supply was connected to main electric current of 600 mA at 30 volts for 2-3 hours. The separation process was stopped after the blue marker ± 0.5 cm from the lower limit of the gel plate.

Gel resulted from running was soaked in staining solution while being shaken for 30 minutes. Then it was soaked into color remover solution (destaining) for 30 minutes while being shaken until clear. The treatment above used automatic shaker. Molecular weight of the human sperm membrane protein was determined with the help of standard proteins as markers to calculate Rf (Retardation factor) of each ribbon.

Western blot test for specificity

This test method was based on Towbin's methods (Walker 1994) to determine whether the beta fertilin protein can react specifically to antibodies induced resulting from its induction. Gel from electrophoresis, Whatmann filter paper and nitrocellulose membrane (NC) were soaked in blotting buffer. These three components were arranged from bottom to top row as many as 7 sheets of filter paper, gel NC membrane, and 7 filters paper in a semi-dry transblot system (Biorad). The transfer was made on the condition of 0.16 V and 0.35 mA for 1.5 hours at room temperature.

Membrane transfer results were stained with Ponceau for 5 minutes to determine whether the proteins in the gel was transferred to a NC membrane, and then rinsed with distilled water. The protein was regarded as has been transferred if there was a pattern of protein bands on the membrane after being rinsed. NC membrane was soaked in 5% blotto (5% skim milk in PBST) for 60

minutes while being agitated, then washed in PBST 3 x for 5 minutes. NC membranes for antibody characterization were incubated in fertilin beta polyclonal antibodies as primary antibodies. Then it was incubated overnight in the refrigerator and thereafter washed in PBST 3 x 5 minutes. NC membranes was added with Anti-Rabbit IgG AP conjugated antibody characterization for treatment (1:2500) for 1 hour at room temperature, then washed again with PBST 4 x 5 minutes. The membrane was subsequently immersed in BCIP/NBT (KPL, Cat. No.. 50811) in dark room temperature overnight. The results obtained were compared based on the stained bands on the membrane.

Isolation of sperm membrane proteins fertilin beta with electroelution techniques

Uncolored SDS-PAGE gel was cut along the desired band. Each piece of gel was inserted into the bag cellophane. Furthermore, it was entered into glass block containing PBS, then it was stirred for 24 hours. PBS replacement was done every 6 hours. To know that the protein has been eluted, the acrilamid gel pieces were stained with silver staining, then the destainer was added. If there are no bands, protein elution has already been done. Furthermore, protein-containing fluid contained in a cellophane bag was removed and then precipitated and purified with absolute ethanol 1:1 for obtaining protein in question (Aulanni'am 2004)

RESULTS

Beta fertilin protein immunocytochemistry of human sperm membrane

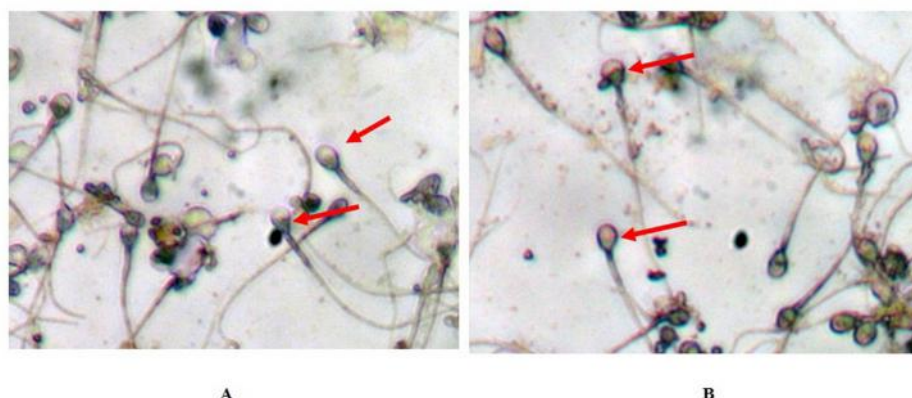


Figure 1. The results of immunocytochemistry on human sperm membrane A (control) : not found fertilin beta protein and B (given fertilin beta polyclonal antibodies) are proteins fertilin beta.

Figure 1 shows that the fertilin beta protein is found in the membrane of human spermatozoa. In control, the sperm head is transparent, indicating the absence of the protein fertilin beta. Those with fertilin beta antibodies on the sperm head appear to be in brown color. This proves that the fertilin beta protein is found in the membrane of human spermatozoa.

SDS PAGE of Human Sperm Membrane Fertilin Beta Protein

Human sperm membrane was isolated using detergent Tween-20 from fertile men. We had obtained Ethical Eligibility of Health Research Ethics Committee of the Faculty of Medicine, Airlangga University. The profile of the isolated sperm membrane protein was confirmed by SDS PAGE as in Figure 2.

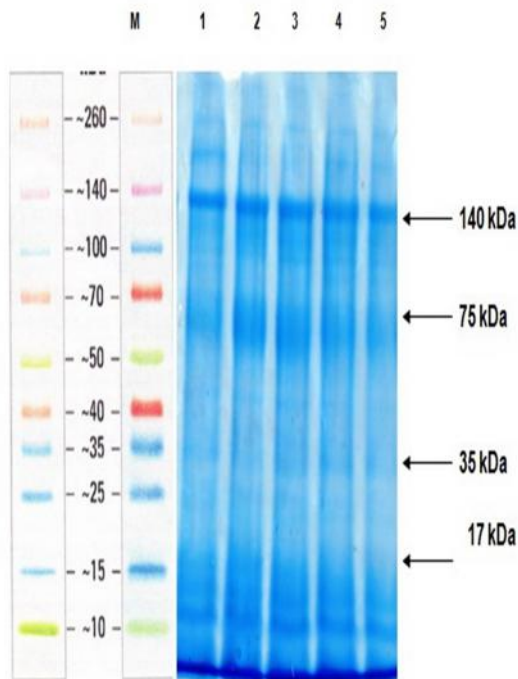


Figure 2. The profile of human sperm membrane fertilin beta protein bands resulting from SDS PAGE

Figure 2 shows that the male sperm membrane proteins results of SDS PAGE has protein bands profile as many as 4 bands with molecular weight of 17, 35, 75 and 140. 4 profiles of the most powerful bands are 75 kDa. The results of research by Rajeev and Reddy (2004), there are a few human sperm membrane protein with molecular weight between 5 kDa to 120 kDa, including the protein with a molecular weight of 75 kDa. From the results of SDS PAGE, western blotting was then performed to determine fertilin beta protein by using

polyclonal antibodies as primary antibodies. The results of western blotting could detect only one band, the 75 kDa. Therefore, it can be ascertained that fertilin beta protein has a molecular weight 75 kDa (Figure 3).

DISCUSSION

One of requirements for protein can be used as a contraceptive vaccine is that it always conserves the target organ, which, in this study, was the human sperm membrane. Immunocytochemistry results proved that protein fertilin beta is at the head of human spermatozoa. It is highly possible that this protein can then be used as candidate for immunocontraceptive material.

Fertilin beta protein with a molecular weight of 75 kDa is always present in human sperm membrane band profiles. The existence of sperm membrane proteins fertilin beta male indicates that this protein has a specific function and role in fertilization process. In general, proteins that are immunogenic has several requirements, i.e. molecular weight (MW) of more than 10,000 dalton (Subowo 1993) and protein levels of 300 μ G/200 ml or 1.5 μ G/ml (Robert & Peter 1993). In addition to the foreignness, the complexity of chemical structure, genetic constitution, and antigen route of administration also greatly affect the immunogenicity of a protein.

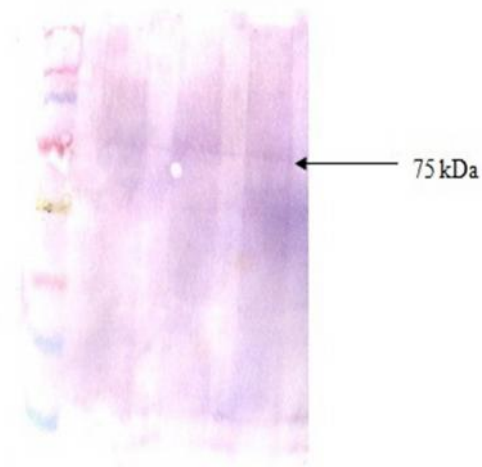


Figure 3. Fertilin beta protein band profiles of human sperm membrane from Western blotting results. Description: arrow (\uparrow) shows fertilin beta with 75 kDa MW.

Fertilin beta protein 75 kDa protein had higher levels of 2407 µg/ml. It is expected to induce the formation of antibodies. Through immunization with fertilin beta protein, fertilin beta protein polyclonal antibodies is supposedly be produced through humoral immune response. According to Subowo (1993), antibodies generated from humoral immune response are generally in the form of IgG, which are largely dissolved in blood. The development of fertilin beta as a candidate material for the production of monoclonal antibodies immunocontraceptive, according to Naz et al (2000) requires other criteria that must be met in addition to the specific immunogenic proteins that are only expressed by spermatozoa and is not present in other tissues. Therefore, if fertilin beta proteins are immunogenic, the monoclonal antibody induction results can be used to determine the location of the fertilin beta in spermatozoa, reproductive and somatic tissues. It also means the target is specific, meaning that these antibodies have properties that can only recognize a specific target and specific, the protein which is distributed in the sperm membrane that has function in fertilization (Naz et al 2006, Vernekar et al 2004), and the antibody should be safe and be able to prevent fertilization. In addition, it is also necessary to know the presence of fertilin beta protein in some other species, so these species can be used as experimental animals for research.

The location of the protein on spermatozoa determines the function of these proteins in process fertilization. These functions are performed by morphological structures located in certain areas, such as the membrane on the head that serves to penetrate the egg. Membrane proteins on the back of the acrosome has a function of early contact and fuse together with oolema egg. Whereas, the membrane on the tail has the function to obtain substrate spermatozoa energy for sperm motility and function (Susilowati 2007). When the sperm membrane protein binds to the antibody, the protein function will be disrupted. The function of the 75 kDa beta fertilin distributed in the acrosome will experience interference when bound to anti fertilin beta. Disruption of protein fertilin beta function in acrosome region will be possible to inhibit fertilization process, especially in the initiation process of fertilization. Spermatozoa fertilin beta has function for binding and fusion with oocytes.

In this study, there are limitations that may affect the results of study. Semen samples used for protein isolation should be at least 50 ml in one isolation of. As for obtaining semen of 50 ml we must collect a minimum 25-30 volunteers. Less protein isolates collection will affect the results of SDS-PAGE and Western blotting. The band would appear thin on

Western blotting and polyclonal antibodies fertilin beta proteins cannot recognize the desired protein. So it should be repeated several times to obtain satisfactory result. From this study, further research needs to be conducted to determine the immunogenicity of the fertilin beta protein of human sperm membrane in experimental animals for the production of its polyclonal antibodies.

CONCLUSION

Fertilin beta protein was isolated from human sperm membrane. Fertilin beta protein is present in human spermatozoa membrane with molecular weight (MW) 75 kDa and protein content 2407 µg/ml.

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