HUMAN SPERM PROTEIN 116 KDA: A CANDIDATE ANTIGEN FOR IMMUNOCONTRACEPTION TECHNOLOGY

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ABSTRACT

Spermatozoa membrane antigenic protein utilized as the substance candidate of immunocontraception has important criteria, that are only expressed in spermatozoa, cannot be found in the other tissue, and also should not have kinase activities. The observations done finally conclude 116kD, a sperm head's protein is within the above criteria. It's antigenic characters are shown with ability of it's polyclonal antibody to bind the human spermatozoa, and interestingly this polyclonal antibody enable to block fertilization of other species in an in-vitro test experiments. This reseach also acquired that 116 kDa protein is specific and exists only in spermatozoa, and not in the other tissue such as spleen, kidney, pancreas, and epididymic. This 116 kDa protein distribute along the whole area of the sperm, but mostly accumulated around the head up to the the neck. The imunohistological staining of the testis also indicate that this protein exists in the spermatid of the testes, but not be found in other somatic tissues, and it's antibody is evidently was recognised by the goat and cow spermatozoa membrane protein resulted in blocking the fertilization of both species respectively. And the conclussion, 116 kD is Non-kinase protein specific only exists in the area of the head of human spermatozoa. It is therefore believed to be adequate candidate for antigen substance of immunocontraception technology.

Keywords : *human spermatozoa membrane, immunocontraception*

INTRODUCTION

Development of immunocontraception, an immunological concept of blocking interaction between sperm membrane protein and zona pellucida (ZP3) are widely proposed (Cheng and Palacios, 1994; Grudzinskas and Yovich, 1995; Thaler et al., 1998; Florman et al., 1998; Naz and Arjun, 2000; Talbot et al., 2003). The principle is immune response induction to bound the receptor exist in the ovum with antigenic ligand blocking the spermatozoa to bind on fertilization (Griffin, 2003; Toshimori, 2000, Bandivdekar et al., 2005; Cesari et al., 2005, Hinsch et al., 1999).

The protein exist on sperm head membrane, actively interacts with the membrane zona pellucida protein during fertilization (Gahmberg et al.,1996; Yanagimachi, 1998; Patrat et al.,2000; Evans, 2002; Talbot et al., 2003). These permatozoa membrane proteins considerably have important criteria when considerated for candidate antigenic substance in immunocontraception. The criteria are as the following: 1).The protein should only exist on the sperm membrane, and not be expressed and presented in the other tissue, 2). Having immunogenic characters and involved in fertilization process (Naz et al. 2005) allowing raising antibody against these proteins to target the sperm membrane proteins to impair the sperm function in binding the ovum.

This research aimed to obtain the antigenic proteins for the antigen of male immunocontraception which are isolated from human sperm that is evidently only expressed in the sperm head. The protein has non-kinase characters, and should has significant role of the interaction between ovum and sperm.

MATERIALS AND METHODS

Ethical clearance and sperm quality evaluation

The study had got an ethical clearance from the Institutional Ethics Commite of Brawijaya University. Semen samples were obtained by masturbation from healthy donors 3-4 days of sexual abstinence. On liquefaction (37oC, 30-45min) sperm count, motility, and morphology were evaluated as per Word Health Organization guidelines (WHO, 2003). Only specimen with count > 60 x 106 /mL, motility rate > 50% and normal morphology ≥ 50% were used.

Protein isolation and characterizations

The sperms membrane proteins are isolated from lytic sperms in buffer containing Tween-20 (Rajeev and Reddy, 2004). The proteins are isolated from spots of Two Dimensional Gel Electrophoresis. The proteins ware then evaluated their kinase potential as repoted on Lestari, et al, (2008). The 116 kDa protein used has antigenic potential to produce polyclonal antibody isolated from blood serum of 8 female rabbits. The isolated polyclonal antibody of 116 kDa protein, then quantified using of indirect ELISA method at optical wave length 405 nm. Their existence in somatic tissues observed by means of Immunological staining procedure. Western blot procedure confirmed that this antibody against human protein 116kDa enable to recognize mice, rats, goats and bulls spermatozoa membrane proteins. We then conclude to use goat ovum and sperm for in vitro fertilization experiments. These experiments are aimed to evaluate the inhibition potential of antibody anti 116 KDa protein on ovum sperm interaction. Further evaluation of The 116 kDa proteins distribution was done using mice tissues especially on the testicular, epididymic, pancreas, lymph, and kidney.

Cross reaction test of antibodies against human 116kD protein using goat spermatozoa

A total of 100 µL goat sperm suspension in concentration of 1.43 Milion/1mL, taken from the swim-up layer of the sperm suspension, was diluted up to 500 µL, and placed in the rosette drop filled with polyclonal antibody anti protein 116 kDa protein of 1/160, 1/80 and 1/40 dilution consecutively. This cross reaction was incubated in 5% CO_2 controlled incubator, which is set at humidity 95% and temperature of 38.50C, for 15 minutes.

Goat Oocyte Maturation

Oocyte maturation techniques are taken from Hozumi (2001). Fresh goat ovaries filled into 0.9% NaCl + 0.006 g + 0.01 g penicillin-Streptomycin. Oocytes derived from follicles by aspiration using a syringe of 18g, and inserted into the as-

piration medium (TCM 199 without FBS), soaked for 10 minutes. From the aspiration medium, the oocyte was inserted into (TCM 199 + 10% FBS), then inserted into the medium drop (10% + media surrounding paraffin). Incubation performed in 5% CO_2 incubator, humidity 37°C and 95%, for 24 hours.

In vitro Fertilization (IVF)

The goat ova and sperms were used for IVF after 15 minutes of incubation with polyclonal antibody against 116 kDa proteins isolated from human sperm. IVF was conducted by mixing 100 µL spermatozoa suspension in flacon containing maturated oocyte drop. Each flacon contains 4 drops, each drop contained 5 maturated oocyte. Observation of goat spermatozoa binding to oocyte was done using inverted microscope.

Immuno-staining of sperms and somatic tissue

In order to observe the existence of protein 116 kDa in the tissues, we develop a staining procedure of the polyclonal antibody of protein 116 kDa labeled with Hueing DAB (3,3 diaminobenzidine tetrahidrocloride). The stained human tissues of lymph, pancreas, and kidney are then observed under light microscope, and the distributions of the protein on the surface of human sperm are shown by means of confocal laser microscopy.

The result in Western Blot has showed that the 116 kDa protein polyclonal antibody is cross-reacting with the goat and bull spermatozoa membrane proteins (see figure 4). The observation of the number of goat oocytes zona pellucida binding sperma-tozoa showed that incubation with 116 kDa protein polyclonal antibody in 1/40 dilution resulted in the least number of spermatozoa binding to the zona pellucida while the number of zona pellucida binding spermatozoa increases in the incubation of 116 kDa protein polyclonal antibody in 1/80, and 1/160 dilution consecutively.

Table 1 Numbers of goat spermatozoa after given antibody of poli\ yclonal protein 116 kDa

Concentration of Antibody Protein 116 kDa	Number of Sperm in category
Pre-imun	++++++++
Concentration of (1/160) Ab 116 kDa	++ +++
Concentration of (1/80)Ab 116 kDa	++
Concentration of (1/40)Ab 116 kDa	+

The in-vitro culture of binding test indicated that head to head agglutination between sperms occurred along with the

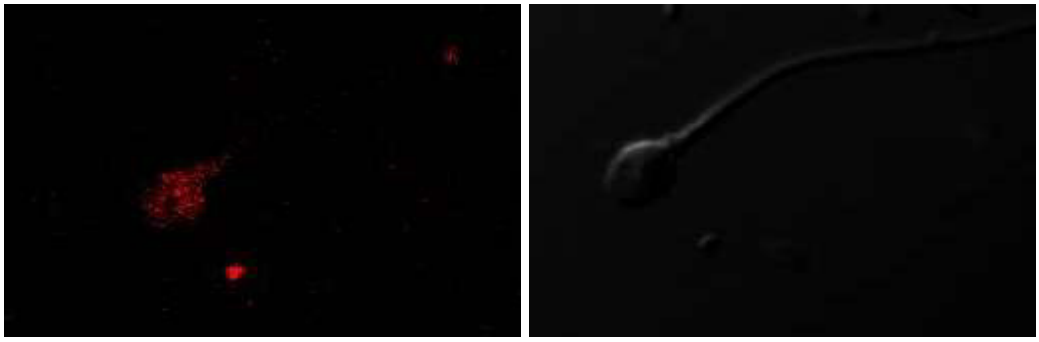

Figure 1: Immunolocalization of protein 116 kDa in head area of human spermatozoa by using polyclonal antibody of protein 116 kDa by dillution 1/2560. The expression of protein 116 kDa in spermatozoa (A), (B). It shown that the location of protein 116 kDa in head area, neck area (midpiece) tied by Rhodamin which resulted in red appearance. The observation by using olympus laser convocal microscope by 100 times magnification and zoom 1.5 times (B)

RESULTS AND DISCUSSION

The protein 116 kDa isolated from human spermatozoa membrane is recognized by polyclonal antibody of protein 116 kDa under the Western blot procedures. This result is consistent with the immunocytochemical staining applied to human sperms using antibody of 116 kDa protein conjugated with Rhodamin Hueing. The sperms showed expression of protein 116 kDa distributed in head area, neck area (midpiece) of spermatozoa as illustrated by figure 1 (A-B). Conversely, mice tissues do not showed any indication of protein 116 kDa of neither their cytoplasm nor the cell surface (figures 2). If we compare the supplies of tissues which only administered with pre-immune, there is no difference. This indicates that there is no such protein recognized by the polyclonal antibody of human protein 116 kDa in the mice somatic tissues.

The results of immunohystochemical on testicular are shown in figures 3. By the same hue, there are brow colored in lumen tubulus-seminipherus of the testis. Hystologically, this cell is small and circular, point to spermatid cell. This shows that the existence of protein in spermatid known by polyclonal antibody of protein 116 kDa.

inhibition effect of antibody preventing spermatozoa to bind to the oocyte zona (see Figure 6). However, the Laser Convocal Microscope observation of spermatozoa by using stained with polyclonal antibody of protein 116 kDa labeled with fluorosenced rhodamin Hueing, showed that the anti body anti bZP3 were distributed in head and neck of sperm membranes. This result indicates that protein 116 kDa are distributed only on spermatozoa membrane especially on head to necks area, and profoundly around acrosome matrices. According to Wassarman (1999), the distributed protein in head membrane and matrices have been predicted involved in acrosome reaction or fusion process of spermatozoa membrane with oocyte membrane. Based on this result, then we might predict that protein 116 kDa of human spermatozoa membrane has the chance to function in acrosome reaction and fusion with oocyte membrane.

Protein 116 kDa distributed in head area, in a case tied with glycoprotein ZP3, suspected to role in activation of G-coupled path (Patrat et all 200). And, if protein 116 kDa bonded, then this bond with glycoprotein ZP3 will be inhibited, which annihilate acrosome reaction.

The 116 kDa protein polyclonal antibody recognizes no protein exists in spleen, kidney, and pancreas. However, it recognizes the protein distributed in the area of the human spermatozoa head, mid-piece and in acrosome as well as recognizes the protein existing in the spermatid around the testicle lumen. Colorazation by using immunohystochemical method with polyclonal antibody of protein 116 kDa, resulted in protein 116 kDa location in testis, brown colored of chromogen DAB (3,30diaminobenizidine tetrahydro chloride) in spermatozoid certain characteristics such as the smallest dimension of cell among other germ cells, and it is localized in lumen area (Junqueira et all 1971).

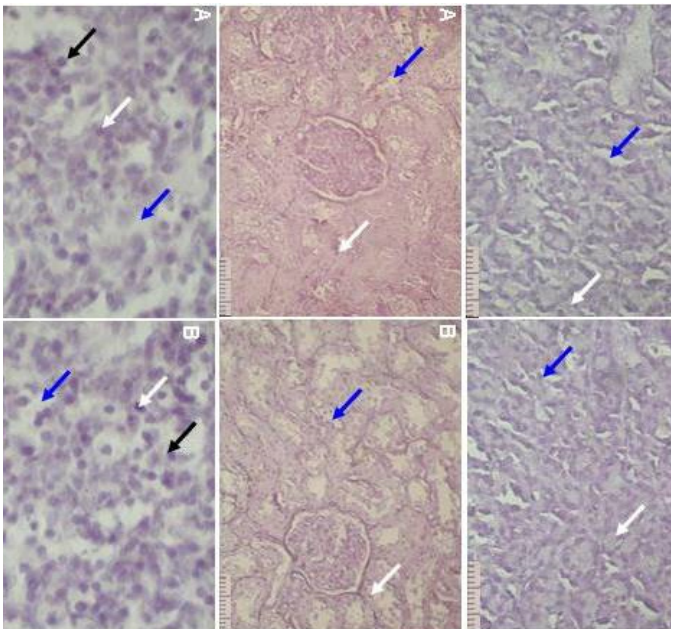

Figure 2 : Immunolocalization of Protein 116 kDa on pancreas, kidney, lymph tissues. The control given pre-immune (A); administered polyclonal antibody of protein 116 kDa (B), white arrow indicates that the cell nucleus doesnot show brown colored; blue arrow indicate that there is no brown colored intercellular gap, black arrow indicate that cytoplasma does not show brown colored. The examination is using ligh microscope by 400 times magnification

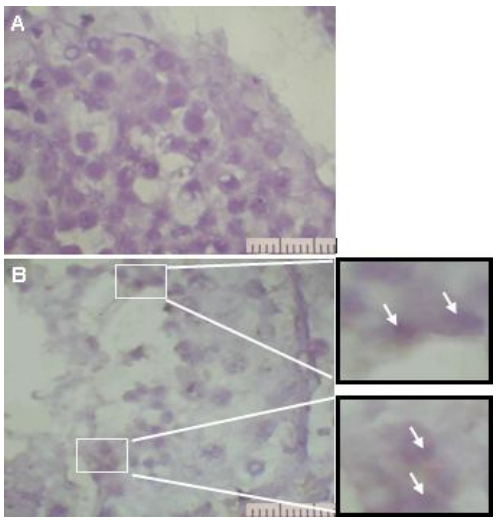

Figure 3: Immunolocalization of protein 116 kDa on testical tissue. (A.)The control given pre-immune; (B) administered polyclonal antibody of protein 116 kDa; white arrow indicates brown colored on spermatoid; The examination is using ligh microscope by 400 times

Protein 116 kDa in spermatoid, it might be synthesized during spermatogenesis, that is the formation of spermatoid, known as transformation process of spermatoid to form spermatozoa, or spermiogenesis. Spermiogenesis is a transformation process which engage in meiosis, and resulting specifical protein synthesis.

As has been done by Toshimori et al., (1997) shows that in spermatosite on leptotene step, they found mRNA, which functions to encode protein in order the protein synthesis proceed, and it is predicted this protein to develop spermatoid. Some authors such as Kleene (1993); Eddy (2002); Dadoune et al (2004) believe that during spermatosite development on

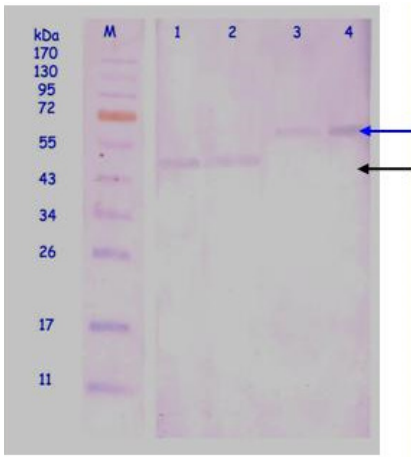

Figure 4 : The results of western blot showing the introduction between 116 kDa protein antibodies with goat and bull spermatozoa membrane proteins. A single band of goat molecular weight 50 kDa (blue arrow head) and bull molecular weight 60 kDa (black arrow head) . M = Marker; Column 1,2= sample isolat for protein membrane goat spermatozoa 3,4 = sample isolat for protein membrane bull sper-

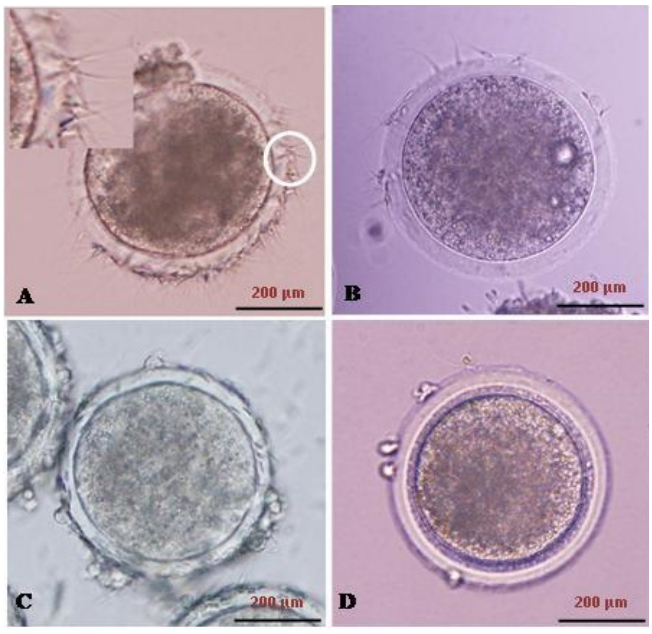

Figure 5: Results of in vitro culture showed the binding of goat spermatozoa to the oocyte zona pellucida, after it is incubated with 116 kDa protein antibodies in various dilutions. Control, spermatozoa were treated with preimmune (A), spermatozoa were treated using 116 kDa protein Polyclonal antibodies, 1/160 (B), 1/80 (C), 1/40 (D) dilutions consecutively. Observations using inverted microscope in 200x magnification.

leptoten step, 14-15 spermatoids, there are organels engaged in protein synthesis such as golgi apparatus, ribosome, mitochondria and endoplasma reticulume. These organels encapsulated in transparent cytoplasma which surrounds the nucleus during transformation process (spermiogenesis). Therefore, protein 116 kDa detected in spermatoid, and does not exist in other germ cells, assumed that biosynthesis during spermiogenesis of protein. 116 kDa protein polyclonal antibody resulted from the induction of 116 kDa membrane protein of human spermatozoa is proven to bind to the goat sperm membrane protein. This is supported by the results of western blot (Figure 4) showing the introduction between 116 kDa protein polyclonal antibodies with goat spermatozoa membrane proteins and it is observed from the in vitro culture showing the reduction of spermatozoa number that binds into the goat oocytes zona pellucida.

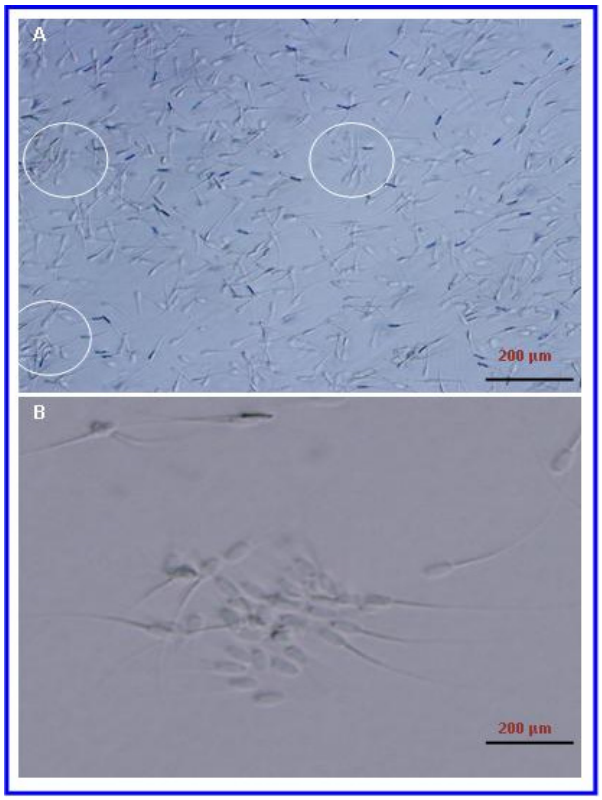

Figure 6: In-vitro Head to head agglutination of goat spermatozoa. It shows the bond between spermatozoa with 116 kDa protein polyclonal antibody. Goat spermatozoa were incubated with 116 kDa protein antibodies Polyclonal with a dilution of 1/80 (A, B). White circle indicates the clustering spermatozoa (A), revealing a head to head Agglutination of goat spermatozoa (B). Observation was conducted using inverted microscope (100x magnification for A, magnification

The observation of the number of goat oocytes zona pellucida binding spermatozoa showed that incubation with 116 kDa protein polyclonal antibody in 1/40 dilution resulted in the least number of spermatozoa binding to the zona pellucida while the number of zona pellucida binding spermatozoa increases in the incubation of 116 kDa protein polyclonal antibody in 1/80, and 1/160 dilution consecutively.

The researchers conducted binding test through in-vitro culture believed that head to head agglutination mechanism in spermatozoa as a spermatozoa binding inhibiting factor to the oocyte zona pellucida (Cesari et al., 2005; Chiu and Chamley, 2004). From a female reproduction infertility test

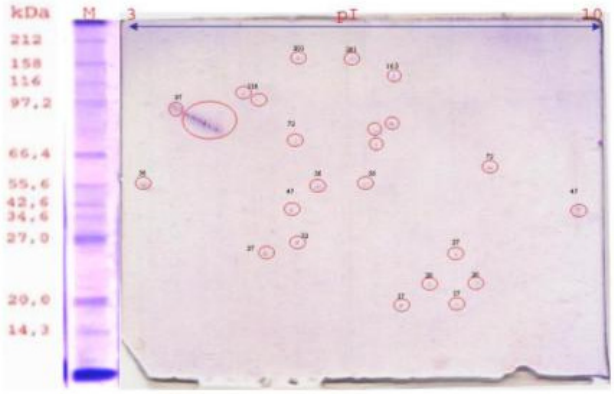

Figure 7: Two dimentional electrophoresis of crude proteins extracted from human sperms. The 116 KDa spot which is known as nonkinase characters then were use for antigen

ing clinic, a test on antibody obtained from cervical secretions section scrap which then incubated with spermatozoa resulted in a head to head agglutination indicating the presence of bond between antigen and antibody (Chiu and Chamley, 2004). Thus, other factors preventing the interaction of spermatozoa with the oocyte is the head to head Agglutination that occurs between spermatozoa after it is treated with 116 kDa protein polyclonal antibodies, by which it decreased motility and inhibited its chance to move toward the oocyte.These phases of research also showed the mechanism of interaction between spermatozoa with goat oocytes, namely in the medium without 116 kDa protein polyclonal antibody/control. In control medium, there was the absence of spermatozoa binding to the oocyte zona pellucida. This mechanism shows the bond between the receptor in spermatozoa with the oocyte ligand. The 116 kDa protein polyclonal antibodies medium demonstrated the existence of barriers toward the oocyte - spermatozoa binding.

This observation may explain a few things, first, that there had been a bond between goat spermatozoa membrane proteins with 116 kDa protein antibody polyclonal. The second is that the goat sperm membrane proteins having the ability of 116 kDa protein polyclonal antibody binding is a receptor from goat spermatozoa.

This in vitro study suggests that IVF outcome observation needs to be developed and followed-up further up to the embryo stage so that the data on the effectiveness of 116 kDa protein polyclonal antibodies resulted from the induced 116 kDa membrane protein of human spermatozoa can be further revealed.

And the conclussion,116 kDa protein antibodies resulted from the 116 kDa human spermatozoa membrane induction specifically targets the 116 kDa protein existing in the human spermatozoa head, mid-piece and acrosome. 116 kDa polyclonal antibodies played a role in resisting the binding of spermatozoa toward the goat oocyte zona pellucida therefore it is to be believed that it can be developed as the immunocontraception substance candidate.

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