Asian Pacific Journal of Reproduction

Journal homepage: www.apjr.net

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Anti−VDAC3 recombinant antibody decreased human sperm motility and membrane integrity: A potential spermicide for contraception

Asmarinah[1]✉, Tri Panjiasih Susmiarsih[2,3], Amalia Shari[2], Putri Ratri[2], Dwi Ari Pujianto[1], Endang Winiati Bachtiar[4]

[1]*Department of Medical Biology, Faculty of Medicine University of Indonesia*
[2]*Postgraduate Program in Biomedical Sciences, Faculty of Medicine, University of Indonesia*
[3]*Faculty of Medicine, YARSI University, Jakarta, Indonesia*
[4]*Department of Oral Biology, Faculty of Dentistry University of Indonesia*

ARTICLE INFO

Article history:
Received 10 August 2017
Revision 30 August
Accepted 7 September 2017
Available online 1 November 2017

Keywords:
Recombinant VDAC3
Antibody
Sperm motility
Membrane integrity
Spermicide

ABSTRACT

Objective: To express recombinant protein that comprises an important fragment of human sperm specific voltage dependent anion channel 3 (VDAC3) protein as a potential molecule for generation of antibody, which can affect sperm function, aiming at spermicide development. **Methods:** The produce of VDAC3 recombinant protein encoded by cDNA sequence of human VDAC3 exon 5-8, based on experimental design of VDAC3 knock-out mice study. And after the purification of various human sperm VDAC3 recombinant proteins, epitope has been predicted in our recombinant protein determined by ElliPro program. Polyclonal antibody was produced for 14 wk. Then anti-VDAC3-exon 5-8 recombinant antiserum was inoculated to human sperm. After the process, antibody VDAC3 protein in human sperm was incubation with anti-VDAC3 recombinant antibody. Finally evaluation the effect of VDAC3 antiserum to human sperm motility and plasma membrane integrity was proceeded. **Results:** Human VDAC3 recombinant protein was successfully over-expressed in Escherichia coli and purified by affinity chromatography method. Purified human sperm VDAC3 recombinant protein could stimulate immune response in rabbit producing an antibody against VDAC3. Anti-VDAC3 recombinant antibody recognized VDAC3 antigen in human sperm could decrease human sperm motility and membrane integrity significantly. **Conclusions:** Anti-VDAC3 recombinant polyclonal antibody that we produced in rabbit by ourselves could decrease sperm motility and sperm membrane integrity. The authors suggest this polyclonal antibody could be used as a candidate agent for male contraception in the future. Furthermore, the authors intend to explore the effect of this antibody into sperm function aiming at male contraceptive vaccine development.

1. Introduction

Development of male contraception methods is required to overcome population growth, especially in countries with high population growth. One of the strategies to develop male contraception is to create an agent that could block human sperm motility. Transmembrane proteins and ion channels are also thought to play an important role in sperm motility[1,2]. To maintain their motility, sperm undergo a process of regulatory volume decrease wherein ions and organic osmolytes are fluxed from or into the sperm[3]. Many ion channels in sperm have been proposed as regulating sperm activity and motility[4]. Voltage-dependent anion channels (VDACs) which are found in plasma membrane and abundant in outer mitochondrial membrane are a channel protein for Na^+, K^+, Ca^{2+}, Cl^- ions as well as for small molecules such as

✉First and corresponding author: Asmarinah, Department of Medical Biology, Faculty of Medicine, University of Indonesia, Jln. Salemba Raya No. 6, Jakarta Pusat 10430, Indonesia.
Tel/fax: +622131907281
E−mail: asmarinah.si@gmail.com

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How to cite this article: Asmarinah, Tri Panjiasih Susmiarsih, Amalia Shari, Putri Ratri, Dwi Ari Pujianto, Endang Winiati Bachtiar. Anti-VDAC3 recombinant antibody decreased human sperm motility and membrane integrity: A potential spermicide for contraception. Asian Pac J Reprod 2017; 6(6): 257-263.

ATP, succinate and malate[5]. Localization of VDACs in sperm has been reported[6,7]. Study for the examination of regulation of VDACs to human sperm function in capacitation suggested that VDACs are a key factor of male fertility[8]. It has been known that male mice containing deletion of the last four exons of *VDAC3* gene were infertile and showed decrease in sperm motility in comparison with wild-type male mice[9]. Various mutations in human *VDAC3* gene, especially in exon 5-8 occur in infertile asthenozoospermic patients[10,11].

Based on previous studies it has been suggested that VDAC3 protein could be used for development of male immunocontraception. Obtaining high amount of VDAC3 protein by genetic engineering would be an important step in applying a strategy for development of male contraception. Here, we present methods to express and purify recombinant protein that comprise an important fragment of human sperm specific VDAC3 protein as a potential molecule for generation of antibody, which can affect sperm function aimed to spermicide development.

2. Materials and methods

2.1. Sperm collection and production of insert fragment of human sperm VDAC3 (hVDAC3) gene

Human normozoospermic sperm was collected using a 45% and 90% discontinuous percoll (Pharmacia, Uppsala, Sweden) gradient centrifugation[12] in Cremer medium. Following centrifugation at 500 g for 30 min, purified spermatozoa were recovered from the base of the 90% percoll fraction, resuspended in the medium and washed twice in phosphate buffered saline (PBS). Total sperm mRNA was extracted using TRIzol reagent (Invitrogen, Carlsbad USA) and DNA contamination was digested by DNase. Synthesis of cDNA (reverse transcription) was catalyzed by superscript III RT (Invitrogen, Carlsbad USA). Amplification of VDAC3 cDNA target sequence was performed by polymerase chain reaction (PCR) technique (Kapa Biosystem, Boston, USA). The PCR reaction was performed for 35 cycles at 94 ℃ for 2 min, 94 ℃ for 30 s, 60 ℃ for 30 s, 72 ℃ for 30 s and 72 ℃ for 5 min. The primers for the amplification of cDNA were designed from GenBank Ref NM 005662.6 (homo sapiens *VDAC3*, transcript variant 2, mRNA) that cover exon 5-8 of the *hVDAC3* (hVDAC3-ex5-8). The primer sequence was *VDAC3*F 5'-CACCTTGGCTGAAGGGTTGAAACT-3' and *VDAC3*R 5'-TCAAGTTCTACAATCCA GCATGTA-3' which produced an amplicon size of 435 bp. To obtain a blunt end PCR product (5 764 bp) to be cloned into an expression vector of pET 100/D-TOPO (Invitrogen, Carlsbad USA), CACC overhang linker was added to the forward primer, whereas TGA stop codon was added at 5'-end of the reverse primer as described in user manual of Champion pET Directional TOPO Expression Kits (Invitrogen, Carlsbad USA).

2.2. Cloning of human sperm VDAC3 gene to pET 100/D-TOPO vector

The fragment of human sperm *hVDAC3 ex5-8* gene was inserted to pET 100/D-TOPO vector (Figure 1) and was transformed into *Escherichia coli* (*E. coli*) TOP 10 competent cells using TOPO directional cloning system. Transformation reactions were performed according to the Invitrogen protocol (Invitrogen, Carlsbad USA). Positive transformed *E. coli* was identified and selected on Luria Bertani medium that contains ampicillin with the final concentration of 100 μg/mL and was subsequently purified by the alkaline lyses method using Genejet Plasmid Purification Kit (Fermentas, Vilnius Lithuania). The insertion of the *VDAC3* target sequence was confirmed by restriction enzyme using *Hin*d III as well as by PCR colony method.

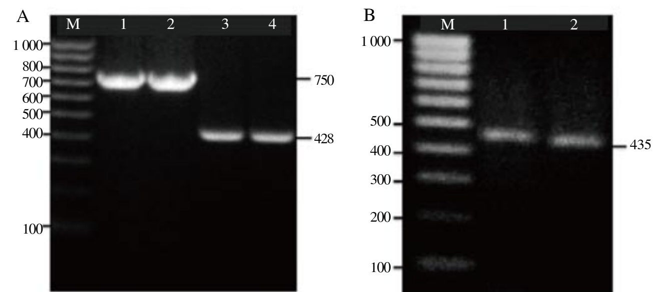

Figure 1. Production of insert fragment of *hVDAC3-ex5-8* transcript.
A: Reverse transcription-PCR (RT-PCR) product of control positive with specific primer result in a single band of 750 bp (line 1 and 2), and with *VDAC3-ex5-8* primer pairs result in a single band of 428 bp (line 3 and 4). B: Insert fragment of *hVDAC3-ex5-8* with additional nucleotide of CACC overhang and stop codon result in a single band of 435 bp (line 1 and 2). M=Marker.

2.3. Expression and purification of human sperm VDAC3 recombinant protein

Recombinant vector pET 100/D-TOPO – hVDAC3-ex5-8 was transformed into *E. coli* strain BL21 Star^{TM} (Invitrogen). Transformant were grown to optical density (OD) 600 = 0.4-0.6 at 37 ℃ and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 37 ℃. Cells were harvested at 8 000 *g* for 10 min and ultrasonicated. Supernatant containing recombinant VDAC3 protein was applied to a Ni-NTA Agarose column (Qiagen) pre-equilibrated with binding buffer (50 mM NaH_2PO_4•$2H_2O$; 300 mM NaCl, 10 mM imidazole) and was extensively washed with washing buffer (50 mM NaH_2PO_4•$2H_2O$; 300 mM NaCl, 20 mM imidazole). Recombinant hVDAC3-ex5-8 protein was eluted with elution buffer (50 mM NaH_2PO_4•$2H_2O$; 300 mM NaCl, 200 mM imidazole). The eluent fraction containing recombinant VDAC3 protein was collected and analyzed. Eluted

proteins were analyzed by 100 g/L Tris/Tricine SDS–PAGE. The protein concentration was determined by Bradford technique. For western blot analysis, nitrocellulose membranes were probed with anti-His (C-term) antibody (Invitrogen) and peroxidase-conjugated goat anti-mouse IgG second antibody. Bound antibody was detected by chloronaftol. The concentration of purified hVDAC3-ex5-8 protein was determined by enzyme-linked immunosorbent assay (ELISA) method using anti-His (C-term) antibody (Invitrogen) and mouse anti IgG HRP conjugate (Invitrogen). Positive immune reaction was detected by tetramethylbenzidine substrate and the optical density of reaction was measured quantify at wavelength 450 nm of ELISA reader. The second-dimensional structure of a VDAC3 recombinant protein sequence was predicted by the Phyre2 servers and the tertiary structure of hVDAC3-ex5-8 proteins were predicted by the PyMOL servers. The characteristic of its epitope prediction was determined by ElliPro program.

2.4. Production of VDAC3 polyclonal antibody

The time period for producing polyclonal antibody was 14 wk. Immunizations of rabbit with hVDAC3-ex5-8 recombinant proteins were carried out in two series interspersed with 4 wk wash out time. The first series of immunization was conducted for 5 wk with wash out for 4 wk. The second series was performed for 4 wk with intervals of 7 d. In the first immunization, 300 μg hVDAC3 recombinant protein was emulsified with 700 μL of Freund's complete adjuvant, whereas next immunizations was performed by injections of 300 μg recombinant protein that was combined with 700 μL of Freud's incomplete adjuvant. The antigen and adjuvant were mixed thoroughly to form a stable emulsion that was injected beneath the skin of New Zealand white rabbit in the area around the shoulders and intra-muscularly into muscle of the rear legs. Blood was collected at 6th, 9th and 12th wk from the central ear artery with a 19-gauge needle and allowed to clot and retract at 37 ℃ for 1 h. The clotted blood of before and after immunization was centrifuged at 4 500 r/min for 20 min, and the supernatant was collected as antiserum and pre-immunserum.

2.5. Determination of binding of anti–VDAC3–ex5–8 recombinant antiserum to human sperm

The semen was gently stratified on top of the 90% percoll column (Amersham). Spermatozoa from the collection samples were prepared an air-dried onto poly-lysine-coated slides. Then samples were fixed with 4% paraformaldehyde in PBS for 1 h and permeabilized with 0.2% Triton X-100 in PBS for 20 min at 37 ℃. After three 5-minute washes with PBS, slides were blocked in PBS containing 2% bovine serum albumin for 2 h and then incubated with a 1:25 dilution of 12th week-collected anti-VDAC3-ex5-8 recombinant antiserum over night at 4 ℃, as a control without rabbit IgG antibodies as well as pre-immunserum. After incubation with goat anti-rabbit IgG labeled with fluorescein isothiocyanate (FITC) (KPL) at 1:500 for 1 h, the slides were washed in PBS and cover slipped. They were viewed with a confocal fluorescent microscope (Olympus).

2.6. Evaluation of VDAC3 antiserum effect on human sperm motility and plasma membrane integrity

Sperm samples were obtained from 30 fertile normozoospermic donors. Sperm with high quality in motility were isolated by swim-up procedure in Cramer medium. Two observers blindly assessed sperm motility in this study. Evaluation of the percentage of immotile (unmotile) sperm (million per milliliter) and velocity of motile sperm (second for 0.1 mm distance in slide chamber) were observed under light microscope at 0, 30 and 60 min after addition of 12th week-collected anti-VDAC3 recombinant antiserum and pre-immunserum as a control with dilution ratio 1:1 between antiserum and sperm suspension.

Sperm plasma membrane integrity was measured by applying of hypo-osmotic swelling test. Sperm samples were incubated with pre-immunserum and anti-VDAC3 recombinant antiserum in the ratio 1:1 (100 μL:100 μL) for 30 min. After incubation, these mixtures were added with 1 mL hypo-osmotic solution and incubated for 30 min at 37 ℃. Hypo-osmotic solution contained 0.90 g fructose and 0.49 g sodium citrate, and dissolved with double-distilled water up 100 mL. Sperm samples were then observed by light microscope (Olympus) with a magnification of 400 ×. Sperm with coiled tail were determined sperm with good sperm membrane integrity[13]. Uncoiled sperm defined as poor sperm membrane integrity were calculated in 200 sperm. The study was approved by the local ethics committee and informs consent was obtained from donors.

2.7. Statistical analysis

The effect of anti-VDAC3 recombinant antiserum on human sperm motility and membrane integrity compared to preimmunserum was analyzed statistically using SPSS program version 22.0 for Windows. Data of non-motile sperm with poor membrane integrity were analyzed by *t*-test paired, and significance was considered at $P<0.05$.

3. Results

3.1. Production of human sperm VDAC3 insert fragment

Total RNA concentration of human sperm was 3.1 ng/μL.

Amplification of cDNA using primers covered *hVDAC3–ex5–8* (VDAC3F and VDAC3R) transcript gene produced a single band with the size of 428 bp (Figure 1A). The sequence of this PCR product was completely matched with *hVDAC3* sequence. Using primer VDAC3F+CACC overhang and primer VDAC3R+TGA stop codon, a 435 bp insert fragment of *hVDAC3–ex5–8* was obtained (Figure 1B).

3.2. Cloning of human sperm VDAC3 gene fragment

A 435 bp fragment of human *VDAC3* was obtained from pET 100/D-TOPO - hVDAC3-ex5-8 recombinant plasmid as well as from 4 colonies using PCR colony method (Figure 2A). The pET 100/D-TOPO-VDAC3 recombinant plasmid from four colonies were confirmed by digesting of recombinant plasmid with *Hin*d III restriction enzyme producing 6 199 bp-fragment visible in the gel agarose (Figure 2B).

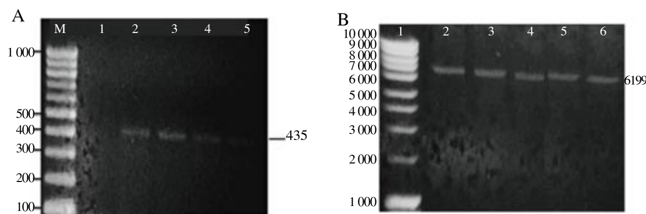

Figure 2. Construction of pET 100/D-TOPO - hVDAC3-ex5-8 recombinant plasmid.
A: The insert fragments that were obtained by using PCR colony method from 4 *E. coli* colonies containing pET 100/D-TOPO-VDAC3 recombinant plasmid (colony 2-5 in line 2-5 respectively; M=1 000 bp DNA marker). B: The pET 100/D-TOPO-VDAC3 recombinant plasmid from colony 2, 3, 4 and 5 cut by *Hin*d III showing a 6 199 bp fragment (M=10 000 bp DNA marker).

3.3. Expression, purification of VDAC3 recombinant protein and generation of anti–VDAC3 antiserum

Total protein concentration in *E. coli* strain BL21 Star™ (DE3) that was measured after induction of IPTG in various concentrations (0 to 1 mM). The IPTG optimal concentration for the expression of protein in *E. coli* was 0.5 mM (P<0.05) (Table 1).

Table 1
OD and concentration of protein expression in *E. coli* strain BL21 Star™ (DE3) after IPTG induction at various concentrations.

IPTG (mM)	OD in λ 655 in various time (h)			Protein Concentration (mg/mL)
	0	2	4	
0.00	0.458	0.520	0.531	1.596
0.01	0.443	0.480	0.500	1.168
0.05	0.532	0.541	0.561	1.224
0.10	0.475	0.504	0.551	1.636
0.25	0.411	0.423	0.435	0.909
0.50*	0.492	0.691	0.785	1.879*
1.00	0.491	0.496	0.499	1.417

ANOVA test (P<0.05), *P=0.012.

The recombinant hVDAC3-ex5-8 fusion protein with a His-tag at N-terminus was expressed in the *E. coli* BL21 Star as indicated by a clear band with a molecular weight of about 22 kDa (Figure 3A). Using Ni-NTA agarose column hVDAC3-ex5-8 recombinant protein was purified and diluted in the buffer of purification system. Expression of hVDAC3-ex5-8 was quantified by IMAGE J tool in the amount of 7.06% of total proteins before purification (Figure 3B). The presence of protein was detected at first and second elution and continuously decreased at the next elution. The protein was finally not detected at the fifth elution (Figure 3B). The expression level of purified VDAC3 recombinant proteins in different of elution was presented in Figure 3C.

The prediction of tertiary dimension of hVDAC3-ex5-8 recombinant protein structure and its continuity and discontinuity is depicted in Figure 4. A hVDAC3-ex5-8 protein structure composed of coil and 12 β-strand/sheet shape. The protein contains five continuous epitopes and four discontinuous epitopes for B-lymphocytes.

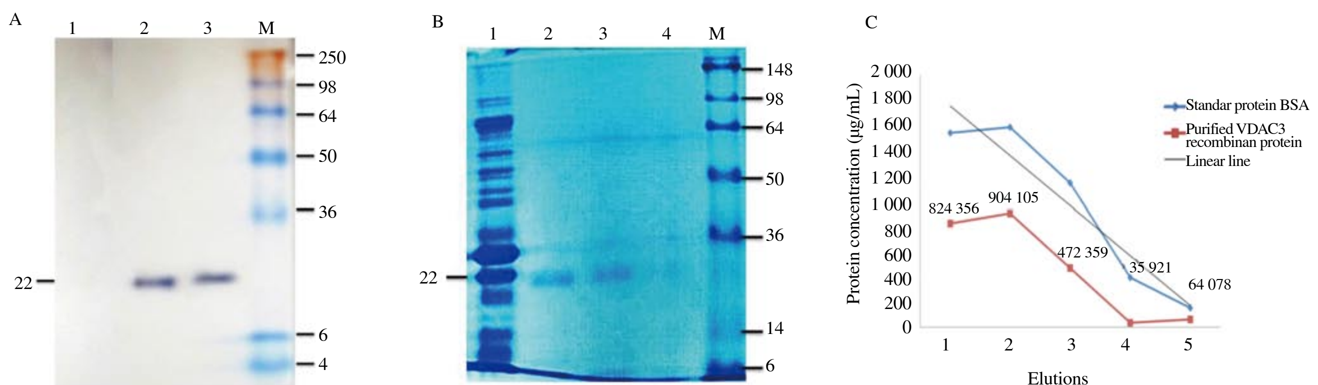

Figure 3. Expression of hVDAC3-ex5-8 recombinant protein in *E. coli* strain BL21 Star™ induced by 0.5 mM IPTG.
A: Using anti-His tag antibody, the expression of protein in the size of 22 kDa was detected in line 2 and 3 respectively; while line 1 constitutes a negative control. B: Purification of hVDAC3-ex5-8 proteins: line 1 total protein before purification; line 2, 3 and 4 are protein expression after purification respectively from 1st, 2nd and 5th elution. C: Concentration of purified VDAC3 recombinant protein (μg/mL) in 1st to 5th elutions was determined.

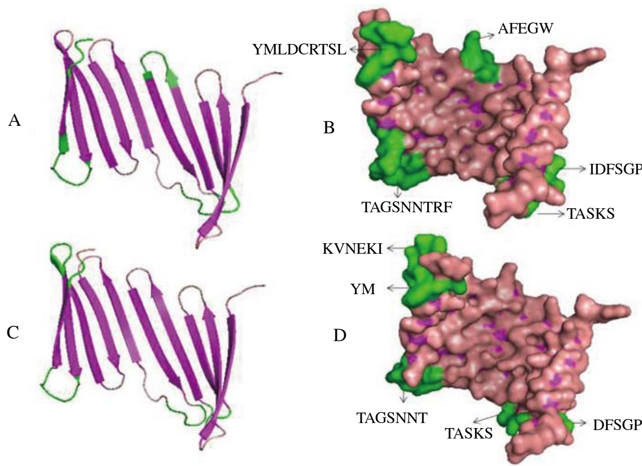

Figure 4. Prediction of the structure of hVDAC3-ex5-8 recombinant protein. Figures showed coil and β -sheet structure and the prediction of its continued epitope (A and B) and discontinues epitope (C and D).

Spectrophotometric ELISA showed that hVDAC3-ex5-8 proteins can induce an immune response in immunized rabbit, which is characterized by an increase in the absorbance value of the polyclonal antibody titers at 6th, 9th and 12th week. The profile of absorbent value of VDAC3 antiserum as well as pre-immunserum is depicted in Figure 5.

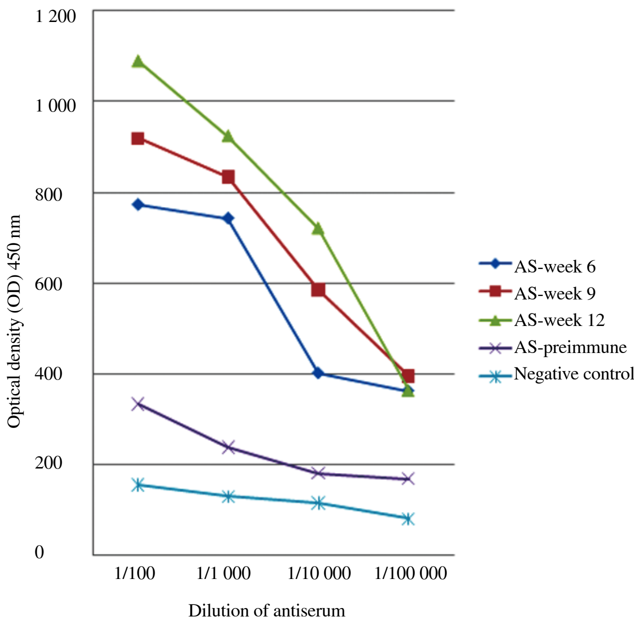

Figure 5. Titer of anti-VDAC3 antiserum.

3.4. Evaluation of anti-VDAC3 recombinant antibody effect on human sperm

Anti-VDAC3 recombinant antibody recognized VDAC3 protein in human sperm, especially in the midpiece region of the sperm tail. Incubation human sperm with pre-immune serum anti-VDAC3 recombinant antiserum without FITC-labeled rabbit IgG antibody, and FITC-labeled rabbit IgG antibody without anti-VDAC3 recombinant antiserum as a negative control showed there were no positive signal of its binding to the sperm antigen (Figure 6).

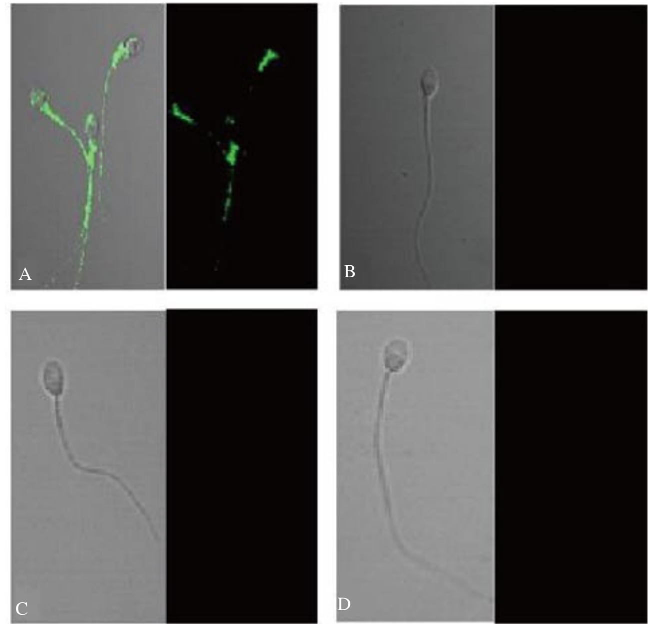

Figure 6. Binding anti-VDAC3 recombinant antiserum into human sperm observed by confocal microscope.

A: Positive binding of antiserum to VDAC3 protein was detected in midpiece and tail region of sperm. B, C and D: Negative reaction were observed in sperm that were treated with pre-immunserum, anti-VDAC3 recombinant antiserum without FITC-labeled rabbit IgG antibody, and FITC-labeled rabbit IgG antibody without anti-VDAC3 recombinant antiserum, respectively, as a negative control.

Evaluation of anti-VDAC3 recombinant antibody on human sperm motility and membrane integrity showed that incubation of this antibody could increase the percentage of non-motile (unmoved) sperm significantly (P=0.041) after 60 min incubation with anti VDAC3 recombinant antibody compared to pre-immunserum. Incubation sperm in time of 30 min could not yet increase unmoved sperm significant statistically. The number of moved sperm after 30 and 60 min addition of VDAC3-antiserum is presented in Figure 7. Anti-VDAC3 recombinant antibody could increase the percentage of sperm with poor membrane integrity significantly (P=0.000) compared to the pre-immune serum (Figure 8).

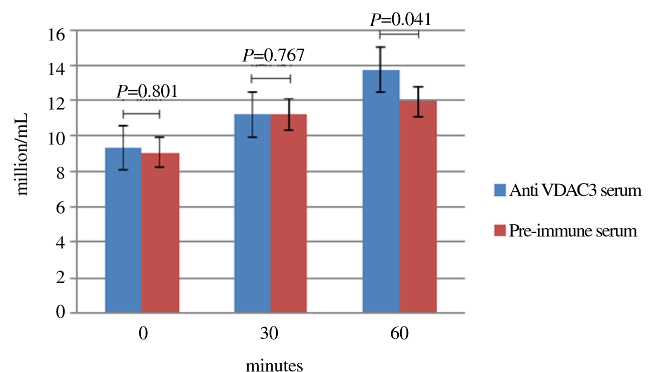

Figure 7. Evaluation of effect of VDAC3 antiserum into sperm motility.

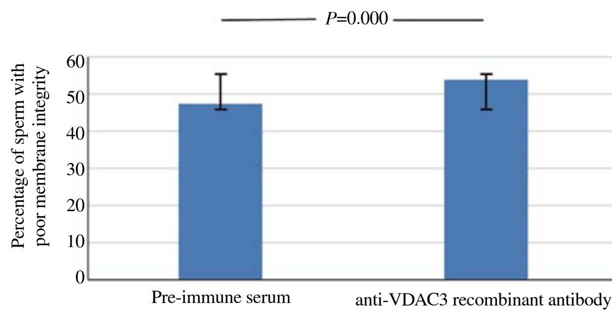

Figure 8. Evaluation of effect of VDAC3 antiserum on sperm plasma membrane integrity.

4. Discussion

In the present study, we evaluated the effect of polyclonal antibody against VDAC3-ex5-8 recombinant proteins that were produced by ourselves through genetic engineering method to sperm motility and sperm membrane integrity. We have produced VDAC3 recombinant protein that encoded by cDNA sequence of *hVDAC3–ex5–8*, based on experimental design of VDAC3 knock-out mice study conducted by Sampson *et al*[9]. They deleted the 4 last exons of mouse VDAC3 gene, *i.e.* exon 5-8, and demonstrated there was significantly reducing of sperm motility in homozygote mutant male mice[9]. Furthermore, mutation analysis of human *VDAC3* gene in exon 5-8 showed there were various mutations in analyzed exons[10,11]. These findings suggested exon 5-8 of *VDAC3* gene are important region for the activity of protein, because a part of this region in the channel structure located in extracellular[14], and might have proper antigenic determinants (epitope). Various epitopes have been predicted in our recombinant proteins. The ability of our recombinant protein to induce immune response generating antibody in rabbit was proven by high concentration of VDAC3 antibody. This polyclonal antibody could recognize VDAC3 protein mainly in principal piece of human sperm tail had confirmed the result study before that reported the localization of VDACs in human sperm[7]. We showed that this antibody recognize VDAC3 protein in human sperm and after 1-hour incubation with anti-VDAC3 recombinant antibody, human sperm motility decreased significantly and this antibody could increase sperm with poor membrane integrity, compared to the pre-immunserum treatment. Liu *et al*[15] demonstrated that commercial anti-VDAC antibody that can react to three isoforms of VDAC and could decrease significantly three motility parameter of sperm, *i.e.* straight line velocity, curvilinear velocity as well as average path velocity; but not significantly decrease total sperm motility[15]. Our data suggested that self-generated antibody was more specific to bind VDAC3 isoform protein in sperm, which could make defect sperm plasma membrane integrity, block channel activity and finally might give impact to ion flux as well as ATP intake for sperm motility. It has been known that the open state of VDAC channel mediates ATP flux and VDAC closure stop this flux despite closing state having a pore diameter of 0.9 nm approximately. The conformational changes of VDAC are influenced by transmembrane voltage[16,17]. VDACs are abundant proteins found in outer mitochondrial membrane and their channels allow diffusion of small hydrophilic solutes through the membrane[18,19]. Several studies reported that VDACs are found also in plasma membrane[20,21]. Phosphorylated VDAC1 isoform in plasmalemmal work together with channel modulator can regulate cell volume and extracellular apoptotic pathway[22]. Our study using self-generated anti-VDAC3 recombinant antibody indicated that the presence of VDAC3 in human sperm flagellum suggested the location of this channel was also in sperm tail membrane. Sperm mitochondria located only in midpiece region of sperm tail producing energy source to drive the tail. VDAC3 in sperm membrane might facilitate flux of ions required to initiate and support sperm motility. Sperm with rapid and progressive movement is required to penetrate an egg at fertilization. Incubation of sperm with anti-sperm protein antibody that can lower sperm movement is a promising method for development of contraceptive agent such as spermicide.

Anti-VDAC3 recombinant polyclonal antibody that we produced in rabbit by self could decrease sperm motility and sperm membrane integrity. We suggest this polyclonal antibody could be used as a candidate agent for male contraception in the future. Furthermore, we intend to explore the effect of this antibody into sperm function aiming at male contraceptive vaccine development.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This study was supported by Competitive Research Fund of University of Indonesia. Asmarinah conceived and designed the research, as well as coordinated the research. Tri Panjiasih Susmiarsih, Amalia Shari and Putri Ratri performed the research. Asmarinah and Dwi Ari Pujianto analyzed the data and wrote the paper. Endang Winiati Bachtiar participated in study design.

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