

## Polyclonal VDAC3 antibody decreases human sperm motility: a novel approach to male contraception

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### Abstrak

**Latar belakang:** Voltage dependent anion channel (VDAC) merupakan protein spesifik yang memperlancar transport anion, kation dan ATP dan berperan penting pada motilitas sperma. Penelitian ini bertujuan mengevaluasi pengaruh antibody VDAC3 poliklonal terhadap motilitas sperma manusia.

**Metode:** Antibodi VDAC3 poliklonal diproduksi dengan mengimunisasi kelinci dengan peptid sintetik spesifik VDAC3. Serum kelinci sebelum diimunisasi dikoleksi menjadi preimunserum untuk kontrol percobaan. Pengenalan antiserum VDAC3 yang diproduksi terhadap antigen VDAC3 pada sperma dilakukan dengan menggunakan metode western blot. Sperma dengan motilitas baik dari 30 pria fertile dicuci dan diisolasi dengan menggunakan metode Percoll gradient. Evaluasi pengaruh antibody VDAC3 terhadap motilitas sperma dilakukan dengan mengukur kecepatan gerak sperma (detik/0,1 mm) dan menghitung jumlah sperma tidak bergerak (juta/ml) pada 0 menit, 30 menit, 60 menit setelah penambahan antiserum dan preimunserum. Data kecepatan sperma dan jumlah sperma tidak bergerak dianalisis dengan menggunakan program statistic SPSS 13.0.

**Hasil:** Antiserum VDAC3 dapat mengenali protein VDAC3 pada sperma dan dapat meningkatkan jumlah sperma tidak bergerak setelah 60 menit secara bermakna dibandingkan preimunserum. Kecepatan gerak sperma menurun secara bermakna setelah penambahan antiserum VDAC3 pada menit ke 0, 30 dan 60 dibandingkan dengan preimunserum.

**Kesimpulan:** Antiserum VDAC3 poliklonal dapat menurunkan motilitas sperma manusia, sehingga diharapkan dapat dikembangkan untuk vaksin kontrasepsi pria di masa dating. (*Med J Indones 2011; 20:5-10*)

### Abstract

**Background:** Voltage dependent anion channels (VDAC) mediate transport of anions, cations and ATP which play an important role in sperm motility. This study was aimed to examine the effect of polyclonal VDAC3 antiserum to human sperm motility.

**Methods:** Polyclonal VDAC3 antiserum used in this study was produced in rabbits by immunization of VDAC3-specific synthetic peptides. Preimmunserum was collected before immunization and used for control experiment. Recognition of VDAC3 antiserum to antigen in human sperm was performed by western blot. Thirty sperm samples obtained from fertile men which had high quality of sperm motility were washed and collected by Percoll gradient. Sperm motility was assessed by means of evaluation of sperm velocity (seconds per 0.1 mm distance) and the number of unmoved sperm (million per ml) which were observed 0 minute, 30 minutes and 60 minutes after addition of VDAC3 antiserum and preimmunserum as a control. Both data were analyzed by SPSS 13.0 software.

**Results:** VDAC3 antiserum recognized VDAC3 protein in human sperm. Statistical analysis demonstrated that there were increasing numbers of unmoved spermatozoa after addition of anti-VDAC3 antiserum in vitro for 60 minutes observation compared with preimmunserum (control). We found also that sperm velocity decreased significantly after giving anti-VDAC3 antiserum in vitro for 0 minute, 30 minutes, and 60 minutes compared with pre-immunee serum (control).

**Conclusion:** VDAC3 antiserum can decrease motility of human sperm. and may provide a novel principle of male contraception in the future. (*Med J Indones 2011; 20:5-10*)

**Key words:** VDAC3 antiserum, sperm, motility, contraception

The rate of world population growth especially in developing countries is presently still high. Family planning through use of contraceptive methods for couples is proposed in many developing countries to suppress growth of the population. Traditionally, most of contraceptive methods have been targeted to women. The numbers of contraceptive methods for men are limited to condom and vasectomy, both of which have their limitations.<sup>1</sup>

Development of new satisfactory male contraceptive methods is needed to provide alternatives that might aim to encourage men to actively participate in successful family planning. There are several target approaches to develop male contraceptive methods, such as hormonal approach, control of epididymal function as well as nonhormonal and post-testicular approaches. One of post-testicular approaches for the

development of male contraception methods is anti-spermatozoal immunocontraception: antibodies against sperm-specific protein may be potential candidates for immunocontraception.<sup>2</sup>

Voltage Dependent Anion Channels (VDACs) also known as porins are pore-forming 30-35 kDa proteins abundant in the outer mitochondrial membrane and also found in plasma membrane of eukaryotes. There are three different VDAC genes encoding distinct isoforms in mammals, i.e. VDAC1, VDAC2 and VDAC3. Each of these proteins is highly conserved in human, rat and mouse.<sup>3</sup> They mediate transportation of anions, cations ( $\text{Ca}^{2+}$ ), ATP, and metabolites between mitochondria and other intra- and extra-cellular compartments.<sup>4-6</sup>

A knock-out mouse study with deletion of the last four exons (i.e. exons 5, 6, 7 and 8) of mouse VDAC3 demonstrated that mutant male mice were healthy, but infertile. The mutant mice had normal sperm counts, but low sperm motility compared to that of wild type mice. In sperm flagella of the knock-out mice, structural defects were observed.<sup>7</sup> Our genetic studies on human VDAC3 gene of asthenozoospermic patients brought evidence to raise the hypotheses that about 50% of these patients exert various mutations in the last 4 exons of hVDAC3 gene and that these mutations can cause the observed asthenozoospermia.<sup>8,9</sup>

It has been reported that VDACs are found in bovine testis and sperm flagellum as well as in the acrosomal region of bovine sperm head. VDAC2 was found in the acrosomal region of the bovine sperm head.<sup>10,11</sup> Anti-VDAC antibodies against VDAC isoforms structurally and functionally lead to surface alterations of the sperm head with a loss of the acrosomal cap, to coiled sperm tails and sperm cell volume disturbances.<sup>12</sup>

The aim of this study was to examine the effect of anti-VDAC3 polyclonal antibody produced in our own lab on human sperm motility.

## METHODS

### Production of anti-VDAC3 polyclonal antibody

Polyclonal anti-VDAC3 antiserum used in this study was collected from rabbits after five times every ten days (AS1R2) *sub-cutaneous* injection of a VDAC3 synthetic peptide of ten amino acids (SVFNKGYGFM). This procedure was followed by 30 days wash out of the immunization (AS2R2) and four times every ten

days injection after the end of the wash out period (AS3R2). We also took the serum before the injection (preimmunserum) as control. Immunization with the synthetic peptide was carried out after conjugation with *keyhole limpet hemocyanin* (KLH) and glutaraldehyde using modified *Single-Step coupling* method.<sup>13</sup> The specificity of anti-VDAC3 antiserum to recognize its antigen was analyzed by ELISA method using ABTS peroxide substrate system (KPL, Netherland).

### Evaluation of the presence of VDAC3 protein in human sperm

Human normozoospermic sperm was isolated by Percoll gradient (90% and 45%) centrifugation method in Cramer medium. Sperm pellet residing in 90 % Percoll solution was collected and subsequently the extraction of total protein in the sperm was accomplished using 2% triton-100 solution containing protease inhibitor cocktail.<sup>10</sup> Protein extract from sperm was electrophorised in SDS polyacrylamide gel electrophoresis (SDS-PAGE) consisting of 6% stacking and 12% separation gel. Electrophoresis was carried out at 200 mV for 45 minutes. Subsequently, the separated proteins were transferred from the electrophoresis gel to nitrocellulose membrane at 70 mV in 90 min time.

The presence of VDAC3 protein in human sperm was determined by immune blot method using the produced VDAC3 antiserum (AS3) and protein A – HRP (Sigma, USA) as a second antibody. VDAC3 protein was then detected with ECL chemiluminescence (Amersham, USA) and exposed to X-ray film (Fuji, Japan).

### Evaluation of the effect of VDAC3 antiserum on the motility of human sperm

Sperm samples were obtained from 30 fertile men with high quality of sperm motility (normozoospermia). Sperm analysis profile is depicted in Table 1. Spermatozoa of high quality sperm motility were isolated by “swim-up” procedure in Cramer medium. The concentration of sperm samples was adjusted to 50 million spermatozoa per ml.

Sperm motility in this study was assessed blindly by means of evaluation of sperm velocity (seconds per 0.1 mm distance in *improved Neubauer* chamber) and the number of unmoved sperm (million per ml) which were observed under light microscope at 0, 30 and 60 minutes after addition of anti-VDAC3 antiserum (AS3R2) and preimmunserum (S0R2) as a control, respectively,

with 1:1 of dilution volume between antibody and sperm solution. Data from sperm velocity and number of unmoved sperm evaluation both from VDAC3 antiserum-treated samples and preimmunserum-treated

samples was analyzed statistically with Saphiro-Wilk, Levene, Mann-Whitney, or T-Test using SPSS 13.0 software ( $P < 0.05$ ).

Table 1. Assessment of motility parameters of spermatozoa

No. Samples	Total amount of sperm (million per ejaculate)	Motility of sperm			
		Rapid progressive motility (%)	Slow or sluggish progressive motility (%)	No progressive motility (%)	Immotility (%)
1	168	5	43	17	35
2	161	5	40	10	45
3	210	17	43	10	30
4	177	11	43	21	25
5	60	8	43	17	32
6	75	5	73	7	15
7	227	5	64	16	15
8	120	10	65	15	10
9	70	10	70	13	7
10	276	5	60	15	20
11	76	10	55	15	20
12	134	8	42	17	33
13	224	8	43	18	31
14	207	7	44	17	32
15	381	9	42	18	31
16	232	9	43	17	31
17	429	6	43	16	35
18	224	7	44	26	23
19	390	12	42	20	26
20	306	10	42	18	30
21	130	8	43	16	33
22	126	9	43	17	31
23	156	14	50	16	20
24	66	10	37	23	30
25	432	10	41	17	32
26	63	7	42	14	37
27	119	8	42	20	30
28	60	8	43	21	28
29	204	6	44	23	27
30	108	10	40	21	29

## RESULTS

Anti-VDAC3 antiserum produced against the synthetic 10 amino acid peptide- analogue of VDAC3 protein revealed its antigenic activity by ELISA method at different dilution levels. The profile of the antibody ELISA titer is displayed in Figure 1. The absorbance value of the preimmunserum in the ELISA reader system was 0.1495 at 1:100 dilution.

By using western and immune blot methods, the presence of VDAC3 protein in human sperm could be detected as a band in the molecular weight range of about 30 kDa. No positive reaction could be detected in the sperm protein extract with ELISA method using preimmunserum (Fig. 2).

The measurement of the velocity of sperm motility immediately after addition (0 min) of the VDAC3

antiserum (AS3R2) or at incubation times of 30 and 60 minutes demonstrated that the required time (seconds) of sperm to reach 0.1 mm distance increased significantly as compared to preimmunserum treatment (Fig. 3). The differences in seconds required to reach 0.1 mm distance increased with duration of incubation: 0.28 sec at 0 min < 0.32 sec at 30 min < 0.44 sec at 60 min (values of antiserum-incubated samples minus values of preimmunserum-incubated samples from the table in Fig. 3).

The number of unmoved sperm 60 min after addition of VDAC3 antiserum increased also significantly as compared to the incubation with preimmunserum, while the number of unmoved sperm at 0 and 30 minutes was not significantly different (Fig. 4).

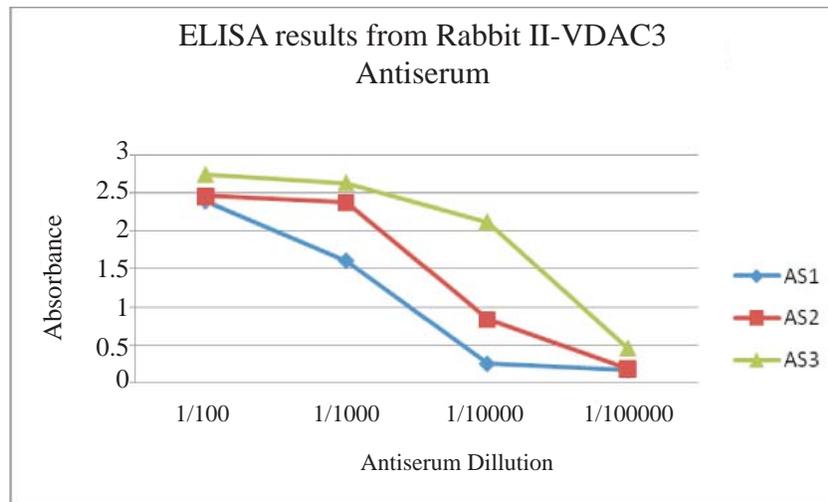


Figure 1. Diagram of ELISA-titer of VDAC3 antiserum

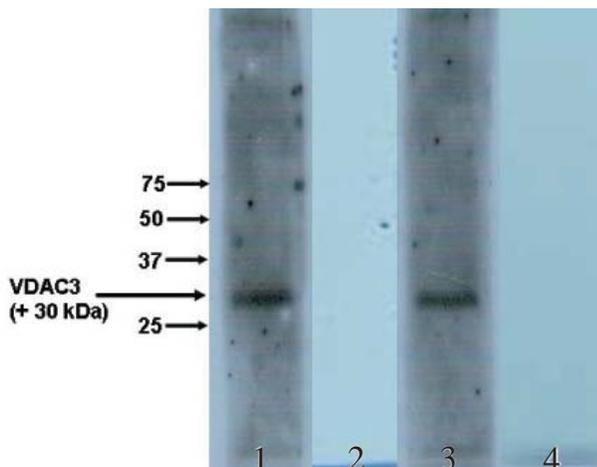


Figure 2. Evidence of the presence of VDAC3 protein in human sperm. Line 1 and 3, protein sperm extract treated with VDAC3 antiserum; line 2 and 4 treated with preimmunserum

## DISCUSSION

Antiserum produced in rabbits against a synthetic peptide of ten amino acids analogous to the VDAC3 sequence could specifically recognize VDAC3 polypeptide in human sperm. High titers of ELISA-optical antibody (AS3R2) density was reached in the 3<sup>rd</sup> collection (2.7409) compared with preimmunserum (S0R2) titer (1.495) at dilution of 1:100. Subsequently, polyclonal VDAC3 antiserum (AS3R2) and its control preimmunserum (S0R2) were used to identify and characterize VDAC3 protein extracted from human sperm. Positive reaction in the molecular weight range of approximately 30 kDa was detected in AS3R2-treated immunoblot while reaction with preimmunserum was negative.

Our study demonstrates that VDAC3 antiserum can decrease human sperm movement measured by two parameters: firstly, by reducing sperm velocity and secondly, by increasing the number of unmoved spermatozoa. Both effects were time-dependent in our experiments with maximum effect after 60 min of incubation of the sperm with VDAC3 antiserum.

This finding suggests that VDAC3 protein plays a role in sperm motility. Movement of the sperm flagellum can be induced by the alteration of the composition and concentration of ions in the sperm microenvironment.<sup>14</sup> Voltage dependent anion channels (VDACs) play a role in transportation of ATP, ions, and metabolites in the outer mitochondrial membrane and in the plasma membrane of cells regulating various activities and cell volume.<sup>6,15</sup>

It has been reported that VDAC1 and VDAC2 isoforms are localized in bovine testis and high amounts of VDAC2 and VDAC3 protein were found in bovine sperm flagella, especially in a subcellular component named outer dense fibres (ODF).<sup>11</sup> Anti-VDAC antibodies against VDAC isoforms functionally lead to a loss of the acrosomal cap, surface alterations of the sperm head, coiled sperm tails and to disturbance of the volume of spermatozoa.<sup>12</sup> Our preliminary study reported that anti-VDAC2 antibody can decrease bovine sperm motility but these results were not statistically significant.<sup>16</sup>

A knock-out mouse study of VDAC3 gene demonstrated that mutant males had normal sperm counts, but their sperm flagella showed structural defects and the spermatozoa low motility.<sup>7</sup> Our evaluation of VDAC3 protein function for sperm motility showed that VDAC3 antiserum can significantly decrease human sperm motility.

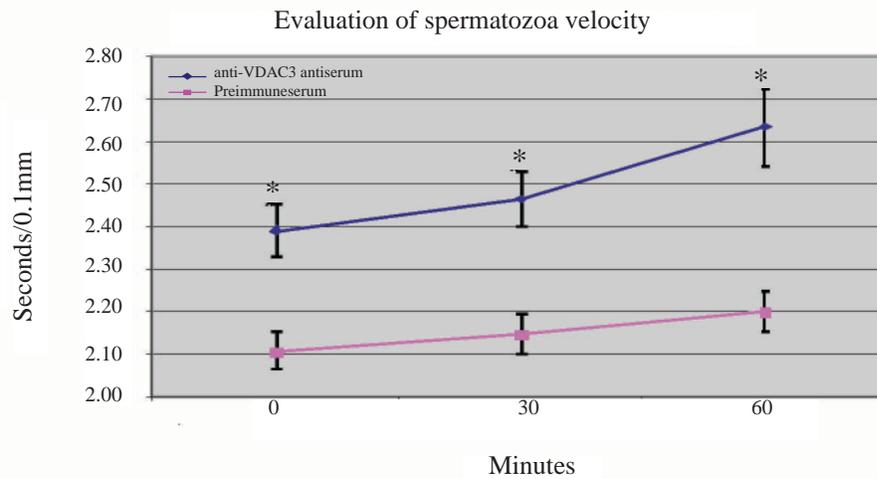


Figure 3. Diagram of sperm velocity (second/0,1mm) after 0, 30, and 60 minutes treatment with VDAC3 antiserum and preimmuneserum (\*, P<0,05)

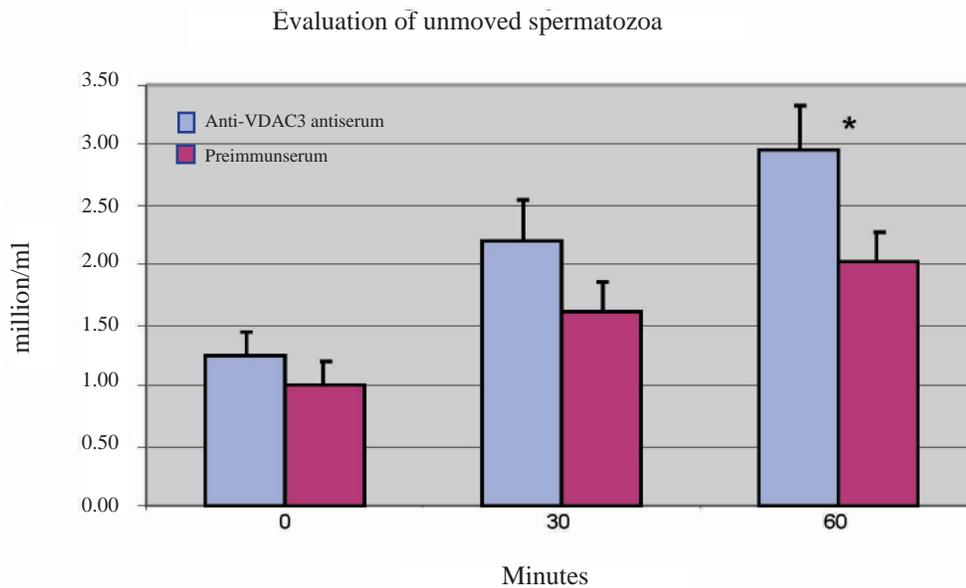


Figure 4. Diagram of the number of unmoved sperm (million/ml) after 0, 30, and 60 minutes treatment with VDAC3 antiserum and preimmuneserum (\*, P<0,05)

After we now demonstrated that the principle of anti-VDAC3 antiserum functions, the following questions have to be addressed in future studies: How can the efficacy of the anti-VDAC3 principle be optimized in humans, e.g., i) by using monoclonal instead of polyclonal antibodies, ii) how can the incubation time of 60 min in our experiments be improved, iii) how can these *in vitro* experiments be transferred to *in vivo*, especially

iv) how can the anti-VDAC antibody be administered to humans and v) how to overcome reactions of the human immune system towards the antibody.

In conclusion, our result suggests that VDAC3 as a sperm-specific protein may be a target and anti-VDAC antibodies may be potential candidates for the development of male contraception concepts in the future.

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