J Assist Reprod Genet (2010) 27:719–724
DOI 10.1007/s10815-010-9466-8

GAMETE BIOLOGY

Analysis and difference of voltage-dependent anion channel mRNA in ejaculated spermatozoa from normozoospermic fertile donors and infertile patients with idiopathic asthenozoospermia

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Received: 27 May 2010 / Accepted: 12 August 2010 / Published online: 1 September 2010

Abstract
Purpose To analyze the abundance and difference of voltage-dependent anion channel (VDAC) mRNA in ejaculated spermatozoa from normozoospermic fertile donors and infertile patients with idiopathic asthenozoospermia.
Methods High motile and low motile spermatozoa were separated respectively from ejaculates of 36 donors and 40 patients using a discontinuous Percoll gradient centrifugation. Real-Time PCR was performed to detect mRNA abundance and difference of three VDAC subtypes between two groups with different sperm motility.
Results Real-Time PCR demonstrated that three VDAC mRNAs were present in mature spermatozoa. The VDAC2 mRNA level in ejaculated spermatozoa of patients was significantly higher than that of donors. No significant differences of VDAC1 and VDAC3 mRNA levels were found between two groups.
Conclusion The high abundance of VDAC2 mRNA seemed to have a positive correlation with low sperm motility. The abnormal expression of VDAC might be related to male infertility with idiopathic asthenozoospermia.

Capsule The high abundance of VDAC2 mRNA seemed to have a positive correlation with low sperm motility in male infertility with idiopathic asthenozoospermia.

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Keywords Voltage-dependent anion channel · mRNA · Spermatozoa · Asthenozoospermia

Introduction

Voltage-dependent anion channel (VDAC) is firstly identified in the mitochondrial outer membrane of *Paramecium aurelia* as a channel protein [1]. Now it has been found in the mitochondrial outer membrane of all eukaryotes [2]. In higher eukaryotes, three homologous genes can encode three different proteins, each of them shares approximately 70% identity to the other subtypes [3, 4]. VDAC displays a very sensitive voltage dependence and regulates mitochondrial outer membrane permeabilization to ions and metabolic molecules [5–8]. It is now recognized that VDAC is involved in many physiological and pathophysiological processes, including energy metabolism and cell apoptosis [6, 9–11]. Furthermore, VDAC is found in the plasma membrane or other non-mitochondrial cellular components, which implies that VDAC has novel functions [12–14].

Although VDAC has been extensively studied in somatic cells, the presence and function of VDAC in mammalian germ cells are still unclear. Few recent studies have reported that VDAC is present in mammalian spermatozoa and plays putative roles in spermatogenesis, sperm maturation and fertilization [15–19]. However the respective expression, localization and function of three VDAC subtypes remain uncertain. In addition, the relationship between VDAC and sperm motility has been investigated in a recent study. Male mice lacking VDAC3 exhibit reduced sperm motility and infertile, but their testicular structure and numbers of sperm are normal [20]. However the mechanism of VDAC affecting sperm motility has not yet been established.

Asthenozoospermia, or low sperm motility, is a common cause of human male infertility. Asthenozoospermia is reportedly found in approximately 19% of infertile patients [21]. Numerous causes can lead to asthenozoospermia such as abnormal semen liquefaction, anti-sperm antibodies, varicocele, endocrine abnormality, physical and chemical factors, inflammation, drug injury and some basic diseases. However, no clear causes can be found in some cases using routine clinical examinations, which are named as idiopathic asthenozoospermia. We have demonstrated for the first time that three VDAC genes are transcribed during spermatogenesis and VDAC proteins are localized in sperm flagella [22]. Since VDAC functional defect can lead to reduced sperm motility without changes in testicular structure and sperm concentration, we presume that there is a relationship between VDAC and idiopathic asthenozoospermia. The purpose of this study is to analyze the abundance and difference of VDAC mRNA in ejaculated spermatozoa from normozoospermic fertile donors and infertile patients with idiopathic asthenozoospermia.

Materials and methods

Semen collection

Approval for this study was granted by the ethics committee of Nanjing Medical University (China) prior to sample collection. Freshly ejaculated human semen samples were collected by masturbation after 3–7 days of sexual abstinence. Routine semen assessments were carried out according to the World Health Organization guidelines [23]. Normal semen samples were obtained from normozoospermic fertile donors at Human Sperm Bank, The First Affiliated Hospital of Nanjing Medical University. The mean±SD age of 36 donors was 24.5± 1.9 years. The semen had the following characteristics: liquefaction time (min) <60; volume (ml) ≥2; sperm concentration ($\times 10^6$/ml) ≥ 20; motility (%) ≥70; progressive motility (%) ≥50; Leukocytes ($\times 10^6$/ml) <1; pH 7.2~7.8. Asthenozoospermic semen samples were obtained from the infertile patients at the Center of Clinical Reproductive Medicine, The First Affiliated Hospital of Nanjing Medical University. The semen showed normal parameters except progressive motility (%) <50 or rapid motility (%) <25. These patients were failed to make their wife pregnant from 2 to 5 years. The mean±SD age of 40 patients was 26.7±3.0 years. Clinical examinations revealed that they were well-developed men. No acute or chronic inflammation, varicocele or anti-sperm antibodies were found. Serum testosterone, LH, and FSH levels were within the normal range.

Sample preparation

For removing the round cells (immature germ cells and leukocytes) and debris, and collecting high motile and low motile spermatozoa respectively from normal and asthenozoospermic semen, the liquefied semen samples were washed in a discontinuous Percoll (GE Healthcare, USA) gradient consisting of four successive layers (90%, 76%, 57% and 45%), which was a modification of the protocol described previously [24–26]. After centrifugation at 300 g for 20 min at room temperature, high motile spermatozoa in donors' semen and low motile spermatozoa in patients' semen were separated respectively from the 90% layer and from the interface 76%-57%. The two kinds of sperm fractions were then washed three times with Earle's balanced salts (Sigma-Aldrich, USA). Microscopy inspections were performed to ensure the quality of the sperm fractions before last centrifugation.

RNA extraction

Total RNA was extracted from sperm fractions prepared as above using TRIzol reagent (Invitrogen, USA). Briefly, after centrifugation the spermatozoa were dissolved and shaked in TRIzol and chloroform. After standing and centrifugation at 12,000 g for 15 min at 4°C, the aqueous phase was collected and an equal volume of isopropanol was added. The RNA was extracted and precipitated with isopropanol, washed with 75% ethanol in Diethyl pyrocarbonate (DEPC)-treated water and then stored at -80°C until use. The total RNA samples were quantified using the DU800 spectrophotometer (Beckman, USA).

Reverse transcription

Aliquots of total RNA (1 μg) from each sample were reverse-transcribed to cDNA using ReverTra Ace® qPCR RT Kit (TOYOBO, Japan) as follows: denaturation at 65°C for 5 min, reverse-transcription at 37°C for 15 min in the 10 μl reaction solution containing 2 μl 5× RT Buffer, 0.5 μl RT Enzyme Mix, 0.5 μl Primer Mix and the appropriate volume of Nuclease-free Water, and termination at 98°C for 5 min. The cDNA samples were stored at -20°C until use.

Polymerase chain reaction (PCR)

In order to confirm the separated sperm fractions free from round cells, the c-kit (as the marker of immature germ cells) and CD45 (as the marker of leukocytes) were amplified from cDNA samples as previously reported [25, 26]. GAPDH was amplified as an internal control. Primer sequences were shown in Table 1. The total reaction volume was 20 μl, containing 14 μl ddH_2O, 2.0 μl 10×PCR Buffer, 1.6 μl

Table 1 Primer sequences and cycle profiles for RT-PCR analysis

Gene		Sequence (5'-3')	Product (bp)	Cycle profile (°C/s)
c-kit	forward	AGTACATGGACA TGAAACCTGG	780	95/60;52/60;72/ 60
	reverse	GATTCTGCTCAG ACATCGTCG		
CD45	forward	TGCAGATGCCTAC CTTAATGC	844	95/45;60/45;72/ 60
	reverse	CACATTGCAGC ACTTCCATT		
GAPDH	forward	TGCACCACCAAC TGCTTAGC	106	94/45;55/30;68/ 60
	reverse	TCTTCTGGGTGG CAGTGATG		

$MgCl_2$ (25 mM), 0.4 μl dNTP (10 mM), 0.4 μl of forward and reverse primers (10 μM), and 0.2 μl DNA polymerase (2.5 U/μl), in which 1 μl cDNA served as the template. The cDNA samples extracted and reverse- transcribed from untreated sperm samples were used as positive controls. Negative controls (ddH_2O as the template) were included. PCR cycle profiles were performed according to the previous protocols [25, 26] (see Table 1). The PCR products were analyzed on a 2.5% agarose gel stained with ethidium bromide and visualized under UV transillumination.

Real-time PCR

The mRNA expression of VDAC1, VDAC2 and VDAC3 in ejaculated spermatozoa was analyzed using ABI 7300 Real-Time PCR System (Applied Biosystems, USA). β-actin was analyzed as an internal control. Primer sequences were shown in Table 2. The cDNA samples from aliquots of total RNA were diluted five times before analyses. The reaction solution contained 6.4 μl of ddH_2O, 10 μl of SYBR® Green Real-Time PCR Master Mix (TOYOBO, Japan), 8 pmol of forward and reverse primers, and 2 μl of cDNA. Amplification was performed at 95°C for 60 s, followed by 40 cycles at 95°C for 15 s, 60°C for 15 s and 72°C for 45 s. The cycle numbers where the amplification curve crossed the threshold were noted as C_T values. Three replicates of each reaction were performed and the C_T values were averaged. The $2^{-\Delta C_T}$ was calculated to represent the amounts of VDAC normalized by β-actin, where $\Delta C_T = (C_{T,\ VDAC}\text{-}C_{T,\ \beta\text{-actin}})$. The average relative difference of the VDAC mRNA abundance between donors and patients was assessed using $2^{-\Delta\Delta C_T}$ method.

Table 2 Primer sequences for real-time PCR analysis

Gene		Sequence (5'-3')	Product (bp)
VADC1	forward	ACAAGAAGTTGGAGACCGCT	136
	reverse	CCTATCAGGCTGGAGTTGTTC	
VDAC2	forward	CTGGGAACAGAAATCGCAA	183
	reverse	TGAACCATGGATTGCAGGT	
VDAC3	forward	GCTGAAGGGTTGAAACTGACT	189
	reverse	CTGATAGCCAGCAAGCCAC	
β-actin	forward	TCACCCACACTGTGCCCATCTACGA	295
	reverse	CAGCGGAACCGCTCATTGCCAATGG	

Statistical analysis

Data were expressed as mean±SD. All data were initially tested to screen for normality and homogeneity of variance, and *T*-test or Nonparametric Test were performed for the comparison of different groups. Statistically significant differences were determined at $P<0.05$.

Results

Semen parameters of 76 subjects were shown in Table 3. We collected high motile and low motile spermatozoa respectively from normozoospermic fertile donors and infertile patients with idiopathic asthenozoospermia using a discontinuous Percoll gradient centrifugation. By microscopy inspections, we checked the quality of the two kinds of separated sperm fractions and did not observe round cells (Fig. 1).

Total RNA was then extracted from sperm preparations and reverse-transcribed to cDNA. We could not amplify c-kit and CD45 in prepared samples, which confirmed the purity of sperm fractions (Fig. 2). Real-Time PCR was performed for analyzing the abundance and possible difference of VDAC mRNA between two groups. As shown in Table 4, three VDAC mRNAs were all detected in ejaculated spermatozoa. Data showed a significant difference of VDAC 2 mRNA level between high motile spermatozoa from donors and low motile spermatozoa from patients. The relative mRNA abundance in ejaculated spermatozoa of patients was more than 4-fold higher than that of donors. There was an almost same VDAC1 mRNA abundance between two groups. Although showing rela-

Table 3 Semen parameters of subjects

	Donors (n=36)	Patients (n=40)
Volume (ml)	4.3±1.3	3.6±1.5
Concentration ($\times10^6$/ml)	73.28±29.94	53.28±22.04*
Motility (%)	76.95±4.76	63.62±8.68*
Progressive motility (%)	59.69±5.29	36.01±10.74*
Rapid motility (%)	23.33±8.32	12.72±5.42*

Data were shown as Mean±SD

*$P<0.05$ versus Donors

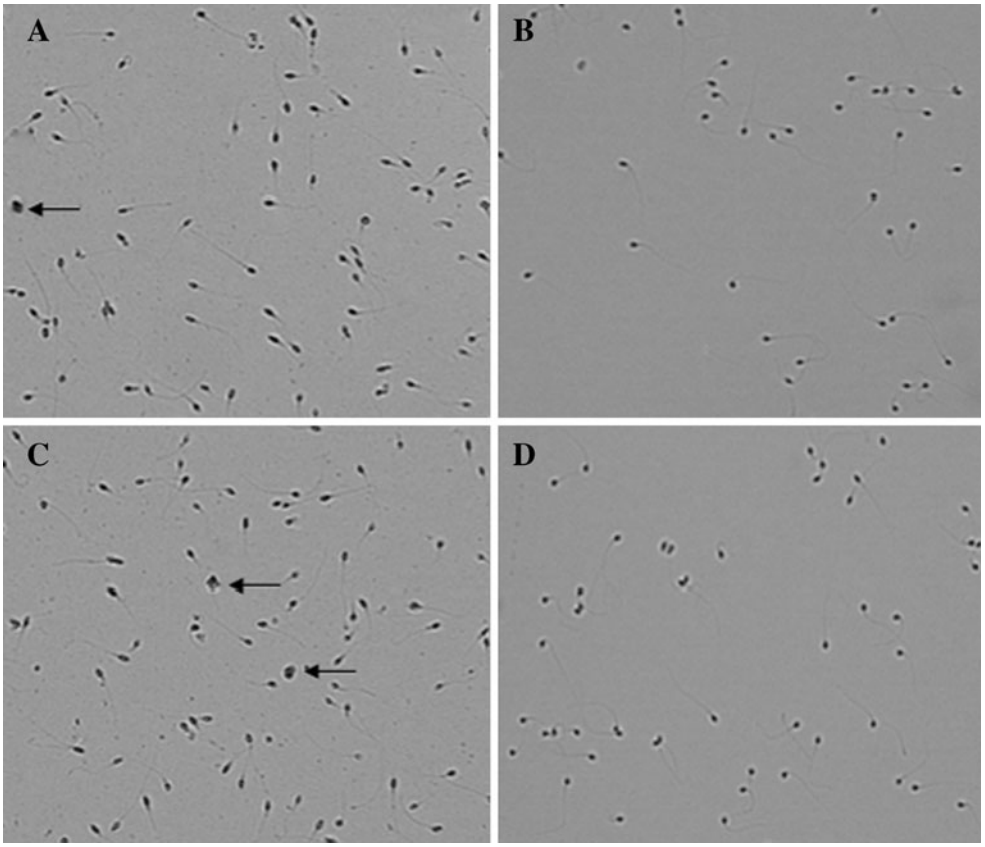

Fig. 1 Microscopy inspections of semen samples and spermatozoa preparations. **a** human semen samples from donors. **b** spermatozoa separated from the 90% Percoll gradient. **c** human semen samples from patients. **d** spermatozoa separated from the interface 76-57% Percoll gradient. Round cells were indicated by arrows. Original magnification ×200

tively low level of VDAC3 mRNA in ejaculated spermatozoa of patients compared with donors, no significant difference was found.

Discussion

In the conventional view, human mature spermatozoa are transcriptionally inactive. There is almost no mRNA in mature spermatozoa. However, some recent studies have shown that a diversity of mRNAs exist in human mature spermatozoa, which might be responsible for motility, capacitation, acrosomal reaction and fertilization [27–30]. The origin and function of mRNAs are controversial. It is now generally agreed that these mRNAs represent the remnant of stored transcriptions from post-meiotically active genes. Thus, the mRNA profiles in mature spermatozoa should be roughly similar to mRNAs in the testis and can reflect the transcription and translation of genes during spermatogenesis [25].

Human three VDAC genes, being located on autosomes, are not previously considered a kind of male infertility genes [31, 32]. Some recent studies had reported that VDAC protein was expressed at testicular level, incorpo-

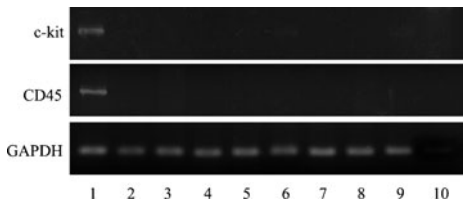

Fig. 2 RT-PCR products of c-kit, CD45 and GAPDH amplified from human sperm total RNA extracts and showed by agarose gel electrophoresis. Lane 1: untreated sperm samples as positive controls; Lane 2–5: sperm samples from donors; Lane 6–9: sperm samples from patients; Lane 10: negative controls with RNA extracts omitted

Table 4 The mRNA abundance of three VDAC subtypes in ejaculated spermatozoa from normozoospermic fertile donors and infertile patients with idiopathic asthenozoospermia analyzed by Real-Time PCR

		ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C}{}_T$
VDAC1	Donors	5.45±0.75	0.00±0.75	1 (0.59–1.68)
	Patients	5.31±0.90	-0.14±0.90	1.10 (0.59–2.06)
VDAC2	Donors	6.08±1.24	0.00±1.36	1 (0.39–2.57)
	Patients	4.07±1.07*	-2.01±1.07	4.03 (1.92–8.46)
VDAC3	Donors	3.98±0.84	0.00±0.84	1 (0.56–1.79)
	Patients	4.72±1.41	0.74±1.41	0.60 (0.23–1.59)

Data were shown as Mean±SD

*$P<0.05$ versus Donors

rated into mature spermatozoa, and was implicated in sperm motility. In our present study, we analyzed the mRNA abundance of three VDAC subtypes in ejaculated spermatozoa from normozoospermic fertile donors and infertile patients with idiopathic asthenozoospermia. In order to obtain high motile and low motile spermatozoa respectively from donors and patients for RNA extraction, we prepared semen samples using a discontinuous Percoll gradient centrifugation. This sperm separation technique could effectively separated sperm populations according to different motility. Furthermore, round cells (immature germ cells and leukocytes) in human semen were successfully excluded via sample preparations. VDAC existed in the mitochondrial outer membrane of leukocytes. Although the presence and localization of VDAC in human reproductive system remained unclear, VDAC2 was reportedly expressed in immature germ cells [33]. Removing the non-mature cells would avoid the contamination of redundant VDAC mRNA.

Our current study documented that three VDAC mRNAs existed in human mature spermatozoa, which would help to study VDAC in human reproductive system at the genetic level. The mRNA level of VDAC2 in ejaculated spermatozoa from normozoospermic fertile donors was significantly higher than that from infertile patients with idiopathic asthenozoospermia. It suggested that VDAC2 was correlated with some male infertility with idiopathic asthenozoospermia. The exact role and mechanism of VDAC affecting human sperm motility are unknown. It is possible that VDAC2 and VDAC3 in sperm flagellum control sperm motility by regulating energy metabolism. Evidence exists that VDAC in somatic cells contains an ATP binding site and mediates ATP transport through the mitochondrial outer membrane [6, 34]. VDAC2 in the mitochondrial sheath of sperm flagellum can provide required energy for sperm motility via participating in ATP transport and sperm energy metabolism. VDAC2 is also identified in the plasma membrane of sperm head and might be implicated in acrosomal reaction [35]. VDAC, as a channel protein, has been validated to mediate ions transport such as Na^+, Ca^{2+}, Cl^- and HCO_3^-. These ions are indispensable not only for acrosomal reaction, but also for sperm motility [36–38]. VDAC2 in the plasma membrane of sperm head could affect sperm motility through mediating the transport of these ions. In addition, the outer dense fiber of sperm flagellum is the important source of ATP for sperm motility through glycolysis [39]. VDAC3 in the outer dense fiber might be also involved in regulating sperm motility. However, the signaling pathways and exact subtypes of VDAC regulating sperm motility remain a mystery. Although VDAC3-deficient mice exhibited reduced sperm motility, it was interesting that VDAC3 mRNA level in spermatozoa of asthenozoospermic patients did not reduce significantly, and meanwhile VDAC2 mRNA level in spermatozoa of patients increased markedly. Why the high abundance of VDAC2 mRNA exhibited a negative correlation with sperm motility? The high abundance mRNA roughly reflected its transcription level during spermatogenesis, being not the necessarily representative of the protein translation and activity in the mature sperm. Another possibility was that the high abundance mRNA could not be the cause of reduced sperm motility but instead was the consequence of compensating motility defect. Overall, VDAC, especially VDAC2, was related to male infertility with idiopathic asthenozoospermia. Our findings would be beneficial to further explore the mechanism of asthenozoospermia and research new diagnostic and therapeutic methods.

Acknowledgments We thank Key Laboratory of Reproductive Medicine, Institute of Toxicology, Nanjing Medical University for the technical assistance. This work was supported by the grant from National Natural Science Foundation of China (30872575).

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