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Research Article

Alteration Expression of Bax, Bcl-2 and VDAC1 Genes in Oligozoospermic and Fertile Subjects

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Abstract

One of factors causing oligozoospermic circumstances is excessive apoptosis during spermatogenesis. Spermatogenesis known involves Bcl-2 family proteins in cytoplasm and Voltage Dependent Anion Channel 1 (VDAC1) in outer mitochondrial membrane to facilitate releasing of apoptosis factor such as cytochrome-c from inter-membrane space into cytoplasm. The study was aimed to analyze the mRNA expression of pro-apoptotic Bax, anti-apoptotic Bcl-2 and VDAC1 genes derived from 45 oligozoospermic subjects and 20 fertile subjects as control. Analysis of transcript expression was performed by two-steps real-time (PCR) and calculating by standard curve method. Stages of works were followed: Analysis of sperm basal characterization, isolation of spermatozoa to separate it from cement and resulted pellets. Pellets were saturated with PBS to obtain mRNA and reversed into cDNA. The cDNA were sequenced to investigate SNP of Bax, Bcl-2 and VDAC1 genes. Results showed that comparison of log mRNA copy number of Bax, Bcl-2 and VDAC1 genes for oligospemic and fertile subjects varied. The Bax, Bcl-2 and VDAC1 were significantly different between oligozoospermic and normozoospermic subjects ($p = 0.000$, $p = 0.041$, $p = 0.000$, respectively). It was suggested that oligozoospermia may be occurred by inducing the increase of Bax pro-apoptotic and VDAC1 genes expression and decreasing of Bcl-2 expression to lead the excessive of apoptosis.

Key words: Oligozoospermia, apoptosis, Bax, Bcl-2, VDAC1

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

High infertility in Indonesia still becomes health problem. Approximately 15% married couples or about 626.5 millions couples get difficulties to have a child and require a medical assistance. Despite the more pressing matter in Indonesia is birth control problem, infertility case should also be attended as a concern by reproduction experts (Suryandari, 2005).

One of male infertility is marked by a sperm count of less than normal. Patients with low number spermatozoa are categorized as oligozoospermia, with cutoff number is 15 millions per milliliter or 39 millions per ejaculate. Low cement concentration may be decreased concentration can be together with significant abnormalities in the morphology and motility of spermatozoa. Oligozoospermia may occur due to the disruption of mitotic and meiotic division process at all stages of spermatogenesis and or the occurrence of excessive apoptosis during spermatogenesis (Blanco-Rodriguez, 2006). Programmed cell death (apoptosis) plays an important role in regulating spermatogenesis, although the molecular mechanisms in regulating apoptosis have not been characterized. Spermatogenic cells initially undergo mitotic, meiotic and differentiation processes to be a mature spermatozoa in Sertoli cells with a closed process. In early phase of mammalian development (before puberty), Sertoli cells supply the nutrients and protection of spermatogenic cells are limited, thus over-propagation of the cells is prevented with the mechanism of apoptosis. These processes are persisted toward at the beginning of puberty or during the maturation of the reproductive function of male animals (Bartke, 2000).

Apoptosis is a normal cell response to the developmental differentiation and environmental pressure. Spontaneously, spermatogenic cell apoptosis can be increased by various stimuli including testicular injury, low hormone level, radiation, toxic compounds and heat exposure. In eukaryotic cells, apoptosis may be induced by endogenous endocrine changes, Tumor Necrosis Factor (TNF), or by an exposure to toxic chemicals with detrimental effects to cell cycle. Therefore, the viability of spermatogenic cells is determined by complex signals, including the paracrine signals such as Stem Cell Factor (SCF), Leukemia Inhibitor Factor (LIF), desert hedgehog (DHH) and endocrine signals such as gonadotrophine pituitary and testosterone. The signals tend to lead expression of the gene that induces the rapid cell death (Cheng *et al.*, 2003).

Transcription factors bound to basal factor and DNA at promoter regions act together with RNA polymerase to regulate initial transcription. Transcription and other proteins are bound to basal promoter at TATA books. Gene transcription is accelerated when proper interaction between proteins, basal promoter complex and RNA polymerase occurred (Clark, 2006). Based on reasons the above, this study elucidated the biological molecular aspects which contributing to oligozoospermia by investigating gene expression of a protein family of Bcl-2 in apoptosis and ion channel protein VDAC1 in the mitochondrial, known to regulate outer membrane permeability and interact with Bcl-2.

MATERIALS AND METHODS

This study was an observational study with cases control design. Forty five subjects were with oligozoospermia and 20 fertile donors as control of study. Subjects were recruited from patients who come for cement analysis to Laboratory of Biology Department, Faculty of Medicine, University of Andalas Padang. Participants were volunteers, proper with inclusion criteria and each approval inform consent was obtained. Analysis of basal characterization was performed by investigating volume, pH, viscosity, number of spermatozoa ($10^6\times$) and sperm concentration ($10^6\times$ cell per milliliter) and compared between oligozoospermic and normozoospremic subjects. Volume, pH and viscosity of cement were conducted by macroscopic analysis. Number of spermatozoa and cement concentration was evaluated by microscopic analysis. Data were analyzed with T-independent test with 5% of confident interval ($p<0.05$).

Spermatozoa were separated from seminal components. In a 15 mL tube, each 1 mL percoll 90% and Percoll 45% was added; cement was pippeted at very top layer and centrifuged by density gradient at 15.000 rpm at 20°C for 30 min. Pellets and supernatant were transferred into a new tube, added 5 mL Cramer and centrifuged at 17.000 rpm at 20°C for 7 min. Supernatant was added with 1 mL Cramer and sperms were counted. Suspension was centrifuged at same condition, washed twice with 1 mL PBS (pH 7.4). Pellets were resuspended with 1 mL PBS, transferred into microtube and stored at -80°C before using. The RNA of sperm was extracted by using Roche RNA Isolation Kit protocol (11,828,665,001). The RNA was used as template for cDNA.

The RNA was transferred to cDNA by using the Roche Kit (lot: 040 379 012 001). Reverse reaction consisted of two preparation. First contained 2 µL anchored oligoprimer, 22 µL water PCR, 1 mL RNA and 12 µL of mix. Mixture was denaturated at 65° for 10 min. Second preparation consisted of 4 µL transcriptor reverse reaction buffer, 1 µL protector RNAse inhibitor, 2 µL deoxynucleotide mix and 1 µL

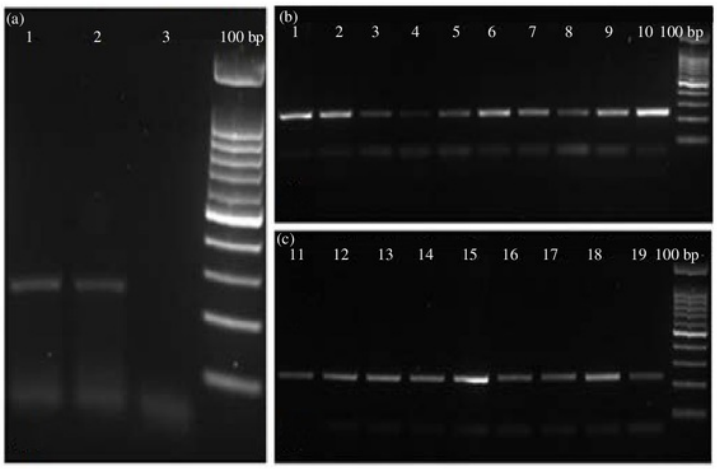

Fig. 1(a-c): Electrophoresis result of Reverse Transcription (RT)-PCR VDAC1 result with optimization temperature of annneling. The best result was (a) at 59°C with 226 bp in size, (b) Electrophoresis of Bax with 59°C annealing temperature, 253 bp in size and (c) Electrophoresis of Bcl2 with 59°C annealing temperature, 253 bp in size, 100 bp: ladder, 1, 2, 3 ..... : Samples

transcriptor reverse transcriptase. Preparation-1 and preparation-2 were mixed and amplified at 50°C for 60 min for step one and at 85°C for 5 min for step two. The PCR profile was described in Roche Kit Protocol.

Primer pairs and probes for Bax, Bcl-2 and VDAC1 were designed with Primer 3 Plus. Specificity of primers was assessed with BLAST. The Bcl-2 probe was labeled with 6-carboxyfluorescein (FAM) at 5′ and Black Hole Quencher (BHQ) at 3′. Bax probe was labeled with Cy5 at 5′ and BHQ at 3′. The VDAC1 probe was labeled with FAM at 5′ and BHQ at 3′. The β-actin was as a housekeeping gene used for internal control.

The Bax, Bcl-2, VACD1 and β-actin were amplified by using LightCycler® FastStart DNA Master SYBR® Green kit (Roche). Reaction was running with Real Time PCR machine (LightCycler® 2.0 Real-Time PCR System, Roche). The PCR profile program was Roche protocol. Real Time PCR stages consisted of four steps, denaturation of template DNA, amplification of target DNA, melting curve and cooling. Annealing temperature was established based on optimization temperature results (Fig. 1). The sequencing was performed in Macrogen Seoul, Korea. Results were checked in BLAST NCBI to determine the homologues of obtained sequences and compared with GeneBank data result to confirm the position of genes.

Gene expression was investigated with two measurements; absolute and relative. Data of absolute measurement was demonstrated with log copy number, beside relative measurement was showed as gene ratio between oligozoospermic and fertile subjects. Ration was obtained by calculating the subtraction of Ct value of Bax, Bcl-2 and VDAC1 with Ct value of β-actin as housekeeping gene. Absolute expression was obtained by calculating previously curve standard of housekeeping gene expression and Bax, Bcl-2 and VDAC1 expression was extrapolated to curve standard to get number of log copy number. Both data were not homogenous and did not normally distribute and analyzed with Mann Whitney-U test.

RESULTS

Data of spermatozoa basal characterization were showed in Table 1. Volume and viscosity of sperm result between oligozoospermic and fertile subjects were not significantly different ($p = 0.103$, $p = 0.472$, respectively), whereas, data was significantly different for pH cement, number of spermatozoa and sperm concentration between oligozoospermic and fertile subjects ($p = 0.001$, $p = 0.000$ and $p = 0.000$, respectively). Macroscopic variables (volume, pH, viscosity of cement) of oligozoospermic subjects were assessed within normal limit condition, meanwhile microscopic variables (number and concentration of spermatozoa) were not in normal condition.

Based on ensemble genome browser Bax located on chromosome 19, at position 49.457.472-49.465.655 nucleotides. The size of genes was 8.184 bp, consisted of 4 exons and 13 introns. The Bcl-2 located on chromosome 18,

Table 1: Seminal characteristics of fertile and oligozoospermic subjects

Variables	Fertile (n = 20)	Oligozoospermia (n = 45)	p-value (student's t-test)
Volume (mL)	2.88±0.93	2.43±0.5	0.103
pH	7.61±0.1	7.73±0.1	0.001
Viscosity	2.80±1.2	2.54±1.4	0.472
Sperm count (10^6×)	115.15±52.5	26.25±8.2	0.001
Concentration (10^6×cell per milliliter)	42.10±14.4	11.06±3.9	0.001

Table 2: mRNA log copy number of Bax, Bcl-2 and VDAC1 in oligozoospermic and control subjects

Genes	Control (n=20)	Oligozoospermia (n = 45)	p-value (Mann-Whitney U-test)
Bax	8.76±0.44	9.22±0.29	0.001
Bcl-2	8.84±0.59	8.49±0.64	0.041
VDAC1	8.13±0.45	8.57±0.50	0.001
β-actin	7.61±0.46	7.68±0.65	0.065

Table 3: Transcript expression ratios of Bax, Bcl-2 and VDAC1 in oligozoospermia and normozoospermia

Genes	Minimum	Maximum	Mean	SD
Bax ratio	0.070	56.890	6.63311	11.025702
Bcl-2 ratio	0.000	13.550	1.04067	2.169172
VDAC1 ratio	0.090	59.300	7.60311	13.749485

SD: Standard deviation

nucleotide positions was at 60.790.579-60.987.361, 196.782 bp in size, consisted of two exons and five introns. The VDAC1 located on chromosome 5, nucleotide position at 133.307.606-133.340.824, the size of 33.219 bp, consisted of nine exons and seven introns.

Analysis of Bax, Bcl-2 and VDAC1 consisted of absolute and relative measurement. Absolute measurement result between oligozoospermic and fertile subjects was showed in Table 2. Expression of Bax and VDAC1 were statistically significant for oligozoospermic subjects, higher than control ($p = 0.001$, $p = 0.001$, respectively). The Bcl-2 expression was lower in oligozoospermic subjects ($p = 0.041$) than in fertile subjects. Relative measurement (ratio of transcript expression) was showed in Table 3. Result demonstrated that mean of Bax and VDAC1 expression level increased in oligozoospermic subjects ($\bar{x} = 6.63311$ $\bar{x} = 7.60311$, respectively) while Bcl-2 expression was quite similar with fertile subjects. The recent study investigated the relationship between Bax, Bcl-2 and VDAC1 expression, correlation analysis was carried out using Spearman's correlation coefficient and results were showed in (Fig. 2a and b). Level expression between VDAC1 and Bax demonstrated no correlation of expression ($p = 0.844$, $r = 0.025$) and no correlation expression between VDAC1 and Bcl-2 ($p = 0.055$, $r = 0.239$).

DISCUSSION

In the concept of molecular cell, gene expression control is a necessity. It plays an important role in existence of cell processes. Epigenetic mechanism, in the form of methylation, histone modification, regulation at transcription level by enhancer and silencer system is a developmental model by every cell in regulating gene activity. Disruption in this system may results the changes of gene expression patterns. Continuous disorder in this system may affects rate of reducing number of cell (oligozoospermia), or cellular proliferation or regeneration like malignancy (Phillips, 2008). Gene expressions in eukaryotes include pre-regulation at the level of transcription, transcription, post-transcription and translational (Yuwono, 2008). Regulation of eukaryotic genes work is occurred at the level of transcription, pre-mRNA processing, mRNA transport, mRNA stabilization, translation, post-translational processing of proteins, protein stabilization and function of enzymes. Current studies indicate that gene expression is basically regulated at the level of transcription and pre-mRNA processing because both processes impact to organism phenotype (Gardner *et al*., 1991).

Each cell contains mechanisms to death or survival, apoptosis resulting from an imbalance between the two signals. Excessive or lack apoptosis may lead to abnormality. Multicellular organisms control the process and pathway of apoptosis performing by genes and proteins (Hussein, 2005).

One of apoptotic process, initiation phase was a phase consisting of several "Caspases" as activated catalyst and an executor which trigger the degradation of other cellular components. Initiation of apoptosis occurs by signals from two different pathways, the intrinsic pathway or mitochondrial and extrinsic pathways involving death receptors. This pathway induced by different stimuli and involves different sets of proteins, although there are some cross paths in between. Both lines meet to activate caspases, which are the actual mediators of cell death (Rautureau *et al*., 2010; Kumar *et al*., 2010).

Chronology of intrinsic apoptosis results the elevating of permeability of mitochondria and releasing of pro-apoptotic

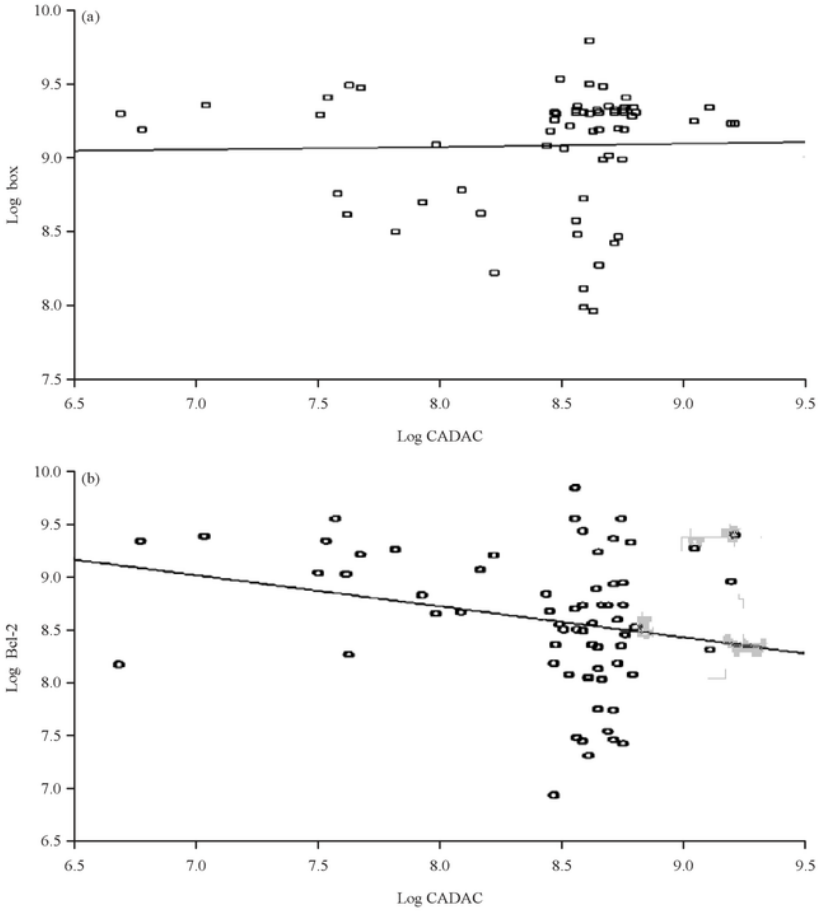

Fig. 2(a-b): Correlation analysis between (a) VDAC1 and Bax and between (b) VDAC1 and Bcl2 by comparing with standard curve

molecules into the cytoplasm. Mitochondria contain proteins such as cytochrome-c which is essential for life, if some similar cytochrome-c are released into the cytoplasm and initiate the "Suicide" program of apoptosis. The releasing of cytochrome-c was regulated through Bcl2 which is pro- and anti-apoptosis (Cory and Adams, 2002; Kumar *et al*., 2010).

One of factor led to apoptosis in mitochondrial pathway is the release of cytochrome-c from mitochondria through the pore formed by mitochondrial Permeability Transition Pore (PTP) and pro-apoptotic protein, Bax. Association of PTP and Bax forms a specific channel for cytochrome-c and other factors inducing apoptosis.

The findings of this study were gene expression of Bax and VDAC1 which was higher in oligozoospermia compared with control. High ratio of Bax and VDAC1 expressions played a role in germinal cells which led low number of spermatozoa. Bcl-2 gene (the anti-apoptotic) expression in oligozoospermia was not significantly different with fertile subjects, further investigation will support the hypothesis of excessive apoptosis in oligospermia.

Previous studies showed that VDAC1 was an important protein for the opening of PTP and apoptosis induction. The N-terminal α-helix chain of VDAC1 played role in facilitating cytochrome-c interaction with pro-apoptotic proteins, the process led to the development of apoptosis. This feature was found in almost eukaryotes (Abu-Hamad *et al*., 2009).

The increased expression of Bax and VDAC1 along with the absence of increase of Bcl-2 led to the formation of pores involved in apoptosis and may triggered apoptotic reaction cascades. The cascade was initialized by mitochondrial cytochrome-c release into cytosol and interacted with Apaf-1 to form apoptosom which recruited

and activated procaspase-9. Activated caspase-9 cleaved its effectors; caspase-3, caspase-6 and caspase-7. Caspase effectors are then cleaved many substrates in cell and leading to characteristics of morphological changes in apoptosis.

The VDAC1 expression serves as an important factor in the process of apoptosis mediated by mitochondria. The VDAC1 over-expression in yeast, rice, fish, mice and humans induced apoptosis. Over-expression of VDAC1 in murine and rats in U-397 cells lead to cell death. Over-expression of VDAC1 caused inner mitochondrial membrane depolarization and activation of mitochondrial membrane permeability. The mechanisms underlying cell death induced by over-expression of the gene in VDAC1 has not been understood yet. However, recent studies prove that over-expression of VDAC1 accompanied VDAC1 oligomerization, by forming monomers, dimers, trimers and tetramers, hexamers and higher order oligomers.

A study in Japanese male showed that seven of 117 subjects (6.0%) with azoospermia and four of 34 (11.8%) subjects with severe oligozoospermia with Y chromosome microdeletion. Frequency of Y chromosome microdeletion was lower in azoospermia than with oligozoospermic subjects. Frequency of Y chromosome microdeletion was between 6.2-25.9% (Yamada *et al*., 2010).

Nagata *et al.* (2005) suggested that genetic abnormality may underlie azoospermia in Japan. Eight of 11 patients with chromosome Y microdeletion have complete deletion of AZFc. The phenotype of patients with AZFc deletion is highly variable from azoospermia to severe oligozoospermia. Progressive regression of germinal epithelia may explain the variable phenotype. On the other hand, Oates *et al.* (2002) reported that four patients with AZFc deletion have stable sperm production.

CONCLUSION

The result of study suggested that oligozoospermia may be in part induced by an increase of the pro-apoptotic Bax and VDAC1 genes expression and by a decrease in the expression of anti-apoptosis Bcl-2, which together supported the excessive apoptosis hypothesis.

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