Survivin Downregulation Is Associated with Vasectomy-Induced Spermatogenic Damage and Apoptosis

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Key Words

Survivin • Vasectomy • Apoptosis • Spermatogenesis

Abstract

Objective: To evaluate the expression of the apoptotic genes survivin, Bax and Bcl-2 in vasectomized rabbits and to determine their relation with vasectomy-induced spermatogenic impairment and germ cell apoptosis. **Materials and Methods:** Twelve adult rabbits (6–12 months old) were divided into three groups: sham control, unilateral vasectomy or bilateral vasectomy. Six months after vasectomy, testicular tissue was analyzed for germ cell apoptosis and DNA fragmentation by the TUNEL assay and gel electrophoresis, respectively. Spermatogenesis was assessed using the Johnsen score. The relative gene expression of survivin, Bax and Bcl-2 was measured using reverse transcription followed by real-time PCR. **Results:** Compared to sham animals, a significant decrease in testicular survivin mRNA levels was measured in the two vasectomy animal groups ($p < 0.05$). This was accompanied by a significant increase in the Bax:Bcl-2 ratio in the vasectomized animals ($p < 0.05$). In addition, these data showed positive correlation with enhanced apoptotic index, damage to spermatogenesis and DNA fragmentation after vasectomy. **Conclusion:** These findings demonstrate that vasectomy-induced damage to spermatogenesis due to testicular apoptosis may be associated with survivin downregulation and Bax overexpression.

Introduction

Worldwide, vasectomy is the most relied upon contraceptive method among an estimated 33 million male partners aged 15–49 [1]. In the United States approximately half a million vasectomies are performed each year. Postvasectomy complications such as hematoma, infections or orchalgia are infrequent and minor and do not require hospitalization or serious medical management. Vasectomy is therefore a popular, safe and cost-effective method of contraception.

Apoptosis plays a prominent role in human male infertility, which could be caused by either increased or decreased rate of apoptosis since both will lead to disrupted testicular homeostasis [2]. In states of impaired sperm production, the formation of intratesticular apoptotic bodies has been observed with implication of several factors of the apoptotic machinery in the process [3]. Thus, an interest was instigated to explore the molecular determinants that control germ cell apoptosis and their signaling pathways. One of the well-known survival-promoting genes is survivin, an inhibitor of apoptosis. Its role in regulating cellular proliferation, cell cycle and programmed cell death made it a good candidate gene for regulating germ cell apoptosis [4]. One of the early indications of its possible role in human spermatogenesis came from a study by Weikert et al. [5]. The group demonstrated that survivin is downregulated in spermatogenesis.

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genic disorders, which implicated it as a regulatory factor in germ cell proliferation and apoptosis with a possible role in human spermatogenesis. Other reports also suggested a role of survivin in infertility-related processes like its involvement in ensuring optimal development of bovine blastocysts and suppression of apoptosis of preimplantation embryos [6] and increasing the chance of patients to get pregnant during in vitro fertilization [7].

Against this background we opted to compare the gene expression of survivin and evaluate its biological significance in vasectomy-induced apoptotic germ cell death in a rabbit model. We also aimed to establish whether there is a correlation between the gene expression of survivin, Bax and Bcl-2 and impaired spermatogenesis 6 months after vasectomy.

Materials and Methods

Experimental Animal Model

Six- to twelve-month-old New Zealand White rabbits were used. They were cared for according to the guidelines of the Kuwait University Animal Research Center. All experiments were performed under anesthesia using intravenous ketamine and xylazine mixture (Sigma-Aldrich Co. Ltd., Pool, Dorset, UK). Three animal groups (4 animals/group) were studied: group A animals (sham, control group) were subjected to left scrotal incisions with exposure of the testis and the wounds were closed using 3/0 chromic catgut (CCG) suture (Ethicon Ltd., Edinburgh, UK). Group B animals underwent unilateral vasectomy whereby 4 cm of the left vas deferens was excised and the ends of the vas were tied with 3/0 silk sutures. The wound was closed with 3/0 CCG suture. Group C animals had a bilateral vasectomy during which 4 cm of the vas deferens (left and right) was excised and the ends were tied with 3/0 silk sutures. The wounds were closed with 3/0 CCG suture. The right testis served as a positive internal control in animals in groups A and B. Six months after vasectomy, the two testes were excised under anesthesia. The harvested testis was divided into four equal parts and each quarter used as follows: assessment of testicular histology using Johnsen's score, TUNEL assay, gene expression measurement and evaluation of DNA fragmentation.

Histological Examination

The mean testicular biopsy score (Johnsen score) was used to assess morphological damage of testicular tissue caused by vasectomy using hematoxylin and eosin staining [8]. Testicular histology was assessed blindly for both right and left testes of the three experimental groups of animals. Any other important histological features were noted. The Johnsen score is based on the premise that with testicular damage, there is successive disappearance of the most mature cell type, with progressive degeneration of germinal epithelium: first, spermatozoa and spermatids, then spermatocytes and finally Sertoli's cells disappear in that order. For experimentally induced damage to the testis, this premise has been found to be largely correct [9].

TUNEL Assay

The TUNEL assay was performed using the in situ Cell Death Detection Kit according to the manufacturer's protocol (Roche Applied Sciences, Mannheim, Germany). Briefly, harvested testicular tissues were fixed and digested with 100 µg/ml Proteinase K (Roche Applied Sciences, Foster City, Calif., USA). Permeabilized samples were incubated with the TUNEL reaction mixture that contains TdT and fluorescein-dUTP. During incubation, TdT catalyzes the addition of fluorescein-dUTP at free 3'-OH groups in single- and double-stranded DNA. After washing, the label incorporated at the damaged sites of the DNA was marked by an anti fluorescein antibody conjugated with the reporter enzyme peroxidase. Immune complex visualization and stained cell analysis was done by light microscopy. The number of apoptotic cells was evaluated within the seminiferous tubules of the testes using the following simplified system: a score of <1 indicates few tubules involved with up to 1 apoptotic cell/tubule; a score of 1+ indicates scattered tubules involved with <4 apoptotic cells present per tubule; a score of 2+ indicates several tubules involved with 4–6 apoptotic cells present per tubule, and a score of 3+ indicates many tubules involved with >6 apoptotic cells present per tubule.

RNA Extraction, Reverse Transcription and Real-Time PCR

Total RNA was purified from freshly isolated testes from control and experimental animals using TRIzol (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's instructions. Purified RNA samples were incubated with DNase I to remove contaminating DNA molecules. Reverse transcription was performed using 1 µg of total RNA using random hexamers. Specific Taqman primers and probes were used to detect the gene expression of survivin (forward primer: 5'-GGCCCAGTGTTCCTTCTGCTT-3', reverse primer: 5'-CAACCGGACGAATGCTTTTT-3' and Taqman probe: 5'-AGCCAGATGACGACCC-CATAGAGGAACA-3'), Bcl-2 (forward primer: 5'-GGACGC-GAAGTGCTATTGGT-3', reverse primer: 5'-TCACGATCTCCGGTTATCATA-3' and Taqman probe: 5'-CCGGAAGGATGGCGCAAGCC-3') and Bax (forward primer: 5'-TGACATGTTTTCTGATGGCAACT-3', reverse primer: 5'-GGTGCACAGGGCCTTGAG-3' and Taqman probe: 5'-TGTTGCCCTCTTCTACTTTGCCAGCAA-3') by real-time PCR using the 7000 Sequence Detection System from Applied Biosystems (Carlsbad, Calif., USA).

The expression of the housekeeping gene β -actin was measured in all samples to normalize for sample-to-sample differences in RNA input, quality, and reverse transcription efficiency. The relative expression of mRNA species was calculated by the comparative ΔC_T method.

Evaluation of DNA Fragmentation

Portions of excised testicular tissue were snap-frozen in liquid nitrogen, powdered and stored at -80°C . Subsequently, these tissues were subjected to extraction of genomic DNA. Briefly, cells were lysed in a hypotonic lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 0.2% Triton X-100, pH 7.5) followed by centrifugation. Isolated DNA was incubated in a proteinase K solution at 37°C for 12 h and precipitated with isopropanol at -20°C . Equal DNA concentrations (10 µg/lane) were loaded and run on a 1.0% agarose gel containing 0.001% ethidium bromide and then visualized under ultraviolet light.

Table 1. Histological analysis and TUNEL assay

Animal group	Histological analysis			
	Johnsen score		apoptotic score	
	right testis	left testis	right testis	left testis
Control sham	9.67 ± 0.57	9 ± 0	1+	1+
Unilateral left vasectomy	10 ± 0	6.7 ± 1.35 ¹	<1	2+
Bilateral vasectomy	4.33 ± 0.57 ¹	4 ± 1.57 ¹	3+	2+

Values are expressed as means ± standard deviation from 4 animals per group.

¹ p < 0.05 is considered significant data as compared to sham levels.

Measurement of Serum Level of Sex Steroid Hormones

Five milliliters of blood was obtained from the ear veins in plain tubes at 0 h and at 24 weeks. Serum was separated using standard laboratory methods and stored at -80°C. From each serum sample, the levels of the following hormones were determined using ELISA technique: luteinizing hormone (LH), follicle-stimulating hormone (FSH) and dihydrotestosterone (DHT). The ELISA kits were purchased from Diagnostic System Laboratories, Inc. (Brea, Calif., USA). The absorbance of the immunoreactions was measured using a Sunrise microplate reader (Tecan, Männedorf, Switzerland) set to dual wavelength measurement at 450 nm with background wavelength correction set at 620 nm.

Statistical Analysis

The data was analyzed using the Statistical Package for the Social Sciences (SPSS) version 13. Comparison between groups was done by Student's t test; p < 0.05 was considered statistically significant. Morphological assessment was expressed as a mean (± standard error of the mean) of the Johnsen testicular biopsy score.

Results

Assessment of Germ Cell Apoptosis

The mean testicular biopsy scores (Johnsen score) for testes subjected to either unilateral (group B) or bilateral (group C) vasectomies are shown in table 1 and illustrated in figure 1A–E. The histological features showed significant damage in group C (p < 0.05) and less severe damage in group B (p < 0.05) compared to sham, indicating the damaging long-term effect of the surgical procedure on the viability of testicular germ cells. Furthermore, cellular apoptosis was detected using the TUNEL assay. Positive germ cell apoptosis was scored using the described scoring system in 'Materials and Methods' and histologically represented in figure 1F–J. Increased scores of apoptotic cell death corresponded more with the damaged testicular tissues from groups B and C in comparison to the sham control group A (table 1).

Effect of Vasectomy on the Gene Expression of Apoptosis Genes

mRNA levels of all three apoptosis genes and the housekeeping gene β-actin were detected in all testicular tissue samples. The calculated relative gene expression values are shown in table 2. Survivin expression was significantly downregulated (p < 0.05) in animals with unilateral (left testis only) and bilateral vasectomies. Bax expression was significantly upregulated in the two vasectomy groups compared with the sham control (p < 0.05), while Bcl-2 expression levels were not significantly changed after vasectomy (p > 0.05). Accordingly, the calculated ratio of Bax to Bcl-2 relative gene expression showed a significant increase (p < 0.05) in both vasectomy animal groups.

Hormonal Assessment

There were no significant changes in the hormonal levels before and after the vasectomy procedures (table 3).

Evaluation of DNA Fragmentation after Vasectomy

Significant DNA fragmentation, shown as a distinctive DNA ladder, was observed in animals subjected to bilateral vasectomy and to a lesser degree in those with unilateral vasectomy while it was absent in control samples as shown in figure 2.

Discussion

In the present study, we have looked at the gene expression of survivin, an inhibitor of apoptosis, to determine its involvement in the long-term vasectomy-induced spermatogenic damage due to germ cell apoptosis. The use of the 6-month period after vasectomy was based on preliminary findings from a study done by our group [9],

Fig. 1. Histological evaluation of testicular biopsy. **A–E** Johnsen score. Sham control: score 9 (**A**), score 9.67 (**B**); unilateral vasectomy: score 10 (**C**), score 7 (**D**); bilateral vasectomy: score 4 (**E**). **F–J** Apoptosis score. Sham control: score 1+ (**F**); unilateral vasectomy: score <1 (**G**), score 2+ (**H**); bilateral vasectomy: score 3+ (**I**), score 2+ (**J**).

Table 2. Relative expression of apoptosis genes

	Control sham		Unilateral vasectomy				Bilateral vasectomy			
	RT	LT	RT	p value	LT	p value	RT	p value	LT	p value
Survivin	1	1	1.1 ± 0.06	0.41	0.3 ± 0.01	0.03*	0.2 ± 0.03	0.03*	0.15 ± 0.02	0.03*
Bax	1	1	1.3 ± 0.05	0.28	2.5 ± 0.07	0.02*	3.5 ± 0.04	0.02*	3.8 ± 0.07	0.03*
Bcl-2	1	1	1.1 ± 0.06	0.41	1.4 ± 0.03	0.31	1.2 ± 0.04	0.52	1.5 ± 0.04	0.05
Bax:Bcl-2 ratio	1	1	1.3 ± 0.08	0.31	1.8 ± 0.05	0.02*	2.9 ± 0.1	0.02*	2.6 ± 0.08	0.03*

Values are expressed as means ± standard error of the mean from 4 animals per group. RT = Right testis; LT = left testis.

* p < 0.05 is considered significant data as compared to sham levels.

Table 3. Effect of vasectomy on serum levels of LH, FSH and DHT

	Control sham			Unilateral vasectomy			Bilateral vasectomy		
	0 h	24 weeks	p value	0 h	24 weeks	p value	0 h	24 weeks	p value
LH, mIU/ml	0.69 ± 0.01	0.62 ± 0.02	0.08	0.59 ± 0.02	0.56 ± 0.02	0.34	0.70 ± 0.02	0.69 ± 0.03	0.74
FSH, mIU/ml	1.9 ± 0.04	1.9 ± 0.01	0.41	1.9 ± 0.04	1.8 ± 0.05	0.34	1.8 ± 0.03	1.81 ± 0.02	0.9
DHT, pg/ml	171 ± 1.5	175 ± 1.95	0.14	171 ± 1.8	176 ± 1.7	0.14	167 ± 1.1	166 ± 3.1	1.0

Values are expressed as means ± standard error of the mean from 4 animals per group.

which showed that following experimental torsion of testis or vasectomy, changes at the molecular and cellular levels continue to take place in the testis for about 3–6 months.

A positive correlation was established in this study between the downregulation of survivin gene expression and the increase in apoptosis and the degeneration of the germinal epithelium. Survivin expression was decreased in testicular tissues from animals with unilateral and bilateral vasectomy ($p < 0.05$). Decreased survivin mRNA levels also coincided with an increased apoptotic score ($p < 0.05$) as demonstrated by the TUNEL assay, which further supports the role of survivin as an inhibitor of apoptosis and in this case germ cell apoptosis. Although there have been no reports about the role of survivin in vasectomy-induced germ cell apoptosis and impaired spermatogenesis, several studies have looked at its role in germ cell production. Survivin gene expression was found to be altered in certain spermatogenic disorders. The studies of Weikert et al. [5] have shown that testicular survivin levels in infertile patients were related inversely to the severity of spermatogenic failure ($p < 0.001$), with a lack of expression in most specimens with premeiotic spermatogenic arrest and in all those with germ cell aplasia [10, 11]. Another study by Roshdy and Mostafa [12] showed that seminal survivin was significantly high in fertile control males in comparison with oligo-asthenozoospermic and nonobstructive azoospermia patients. Survivin expression was undetectable in individuals with obstructive azoospermia. This further supports a potential role of survivin in male gametogenesis and its possible involvement in determining male fertility.

Another apoptosis marker is the Bax (an apoptosis promoter) to Bcl-2 (an apoptosis inhibitor) ratio. The modulation of cellular levels of these proteins in response to the onset or regression of apoptosis has established them as a reliable apoptosis 'rheostat' for the cell. In our study, Bax levels were significantly higher ($p < 0.05$) after both surgical procedures in contrast to the Bcl-2 gene expression, which is in agreement with other studies using testicular tissue from animal models and vasectomized men. Testicular biopsies from vasectomized men (>5 years) showed reduced sperm yields after vasectomy which correlated with increased apoptosis through the Fas-FasL and Bax pathways and increased DNA fragmentation [13]. In vasectomized rats, damage to spermatogenesis was associated with increased p53, increased Bax expression and germ cell apoptosis more than 8 weeks after vasectomy [14]. The role of Bax in regulating apop-

Fig. 2. Detection of DNA fragmentation in testicular tissue. 10 μ g of purified genomic DNA was separated on 1.0% agarose gel electrophoresis. Lanes 1–3 represent the right testis, while lanes 4–6 represent the left testis. From left to right: sham control (lanes 1 and 4), unilateral left vasectomy (lanes 2 and 5) and bilateral vasectomy (lanes 3 and 6). The right testis served as the internal positive control for the sham control and unilateral left vasectomy.

toxis in the testis is hypothesized to occur via association with other Bcl-2 family members, Bak and Bcl-w, followed by their translocation into the mitochondria to trigger the release of cytochrome C and induce the mitochondrial apoptosis pathway [15].

Since spermatogenesis is mainly controlled by the endocrine effect of FSH and the paracrine effect of testosterone, the observed vasectomy-induced testicular dysfunction could also have been a result of an endocrine disruption. Although some studies reported increased levels of FSH, LH and testosterone in men 1 year after vasectomy [16, 17], other studies found that there were no significant changes in the levels of these hormones after vasectomy [18, 19]. In our study, serum levels of FSH, LH and DHT did not show any serum level changes 6 months after vasectomy (table 3).

Conclusions

Our data indicate that germ cell apoptosis after vasectomy is associated with survivin downregulation. Whether survivin expression is regulated through the mitochondrial pathway or another parallel death-signaling pathway leading to germ cell apoptosis needs to be

established by further studies. The involvement of survivin in the spermatogenic damage induced by vasectomy identifies another player in germ cell apoptosis and adds to our understanding of the mechanism(s) causing male infertility.

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