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Both protamine-1 to protamine-2 mRNA ratio and Bcl2 mRNA content in testicular spermatids and ejaculated spermatozoa discriminate between fertile and infertile men

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BACKGROUND: Human sperm contain similar amounts of protamine-1 (P1) and protamine-2 (P2). Although aberrant protamine ratios have been observed in infertile men, functional evidence is provided by protamine knockout mice exhibiting male infertility. As sperm DNA integrity is known to be linked with DNA fragmentation and apoptosis, we investigated whether the protamine ratio or Bcl2 content represent a reliable biomarker to discriminate fertile and infertile men. METHODS: Real-time quantitative RT-PCR was used for P1, P2 and the apoptotic marker Bcl2 in testicular biopsies (TB; 74 infertile men versus 17 controls) and ejaculates (E; 95 infertile men versus 10 controls). RESULTS: The P1–P2 mRNA ratio differed significantly between groups, namely 1:4 versus 1:3.2 in TB ($P = 0.0038$) and 1:1.7 versus 1:1 in E ($P = 0.0002$), for infertile men and controls, respectively. Bcl2 mRNA content was correlated with protamine mRNA ratio ($P = 0.0250$ for TB; $P = 0.0003$ for E). Infertile men exhibit a more than 10-fold ($P = 0.0155$ for TB; $P = 7.0 \times 10^{-6}$ for E) higher Bcl2 mRNA content versus controls. No correlation was found between absolute sperm density and the protamine mRNA ratio or Bcl2 mRNA content. No significant correlation was demonstrated with fertilization rate after ICSI and either protamine ratio or Bcl2 content. CONCLUSIONS: We found significantly aberrant protamine ratios and a higher Bcl2 content in TB and E of infertile men compared to controls, suggesting that these molecules may be useful biomarkers for predicting male infertility.

Keywords: Bcl2; ejaculate; protamine; testicular sperm extraction; testis

Introduction

Genes for protamines 1 and 2 are transcribed in Step 1–4 spermatids (Steger *et al*., 2000), while synthesis of the corresponding proteins starts, with temporal delay, in Step 4 spermatids (Le Lannic *et al*., 1993). Protamine–DNA interaction, subsequently, results in chromatin condensation and termination of gene expression (reviewed in Steger, 1999, 2001). Consequently, comparative microarray analysis revealed no differences in the gene expression pattern between testicular spermatids and ejaculated spermatozoa (Ostermeier *et al*., 2002).

In human sperm, the histone to protamine exchange is only ~80% complete (Tanphaichitr *et al*., 1978) and the protamine-1 (P1) to protamine-2 (P2) ratio is 0.8–1.2 (Balhorn *et al*., 1988). Numerous studies, however, demonstrated that male infertility is associated with an abnormal histone to protamine ratio (Zhang *et al*., 2006) and an aberrant P1–P2 ratio at both the protein level in ejaculated spermatozoa (Balhorn *et al*., 1988; Belokopytova *et al*., 1993) and the mRNA level in testicular spermatids (Steger *et al*., 2001, 2003). Recently, abnormal protein synthesis has been reported to be associated with aberrant mRNA retention suggesting that defects in protamine translational regulation may contribute to protamine deficiency in infertile men (Aoki *et al*., 2006b). Functional evidence is provided by knockout mice demonstrating that a knockout of only one of the two protamine alleles is sufficient to cause male infertility (Cho *et al*., 2001). For further details refer to reviews from Steger (2003) and Oliva (2006).

Applying real-time quantitative RT-PCR (qRT-PCR) in the present study, we aimed at clarifying whether the P1–P2 mRNA ratio represents a reliable biomarker for the

discrimination of fertile and infertile men. We therefore analysed the protamine ratio in both testicular spermatids and ejaculated spermatozoa and compared data with the fertilization rate after ICSI. As sperm DNA integrity is known to be linked to the level of DNA fragmentation and apoptosis, we additionally studied whether amplification of the apoptotic marker Bcl2 is related with male fertility.

Materials and methods

Testicular tissue

After written informed consent, testicular biopsies were obtained from 74 infertile men exhibiting impaired spermatogenesis. In 17 patients with obstructive azoospermia after vasectomy, biopsies were carried out for diagnostic purposes during vasectomy reversal. These patients revealed normal endocrine values and histologically normal spermatogenesis and served as controls (Table I). Although one part of the biopsy was cryopreserved for testicular sperm extraction (TESE), the other part was fixed in Bouiń fixative and embedded in paraffin. For histological evaluation, 5 μm paraffin sections were stained in hematoxylin and eosin and scored, according to Bergmann and Kliesch (1998).

Ejaculates

After written informed consent, ejaculates were obtained from 95 oligozoospermic infertile men and analysed, according to the World Health Organization (WHO, 1992). Ejaculates from volunteers with normozoospermia ($n = 10$) served as controls (Table II). The semen was prepared by migration–sedimentation (Sanchez *et al*., 1996), a method based on the technique of the common swim-up. Native ejaculate was transferred into special tubes around an inner conus and overlayed with medium. The motile sperm migrated actively into the medium and was then sedimented passively into the conus by gravity. After incubation at 37°C for a period of 2–3 h, ∼10% of the native quantity of sperm with a motility of ∼90% could be detected in the medium.

RNA extraction and first strand cDNA synthesis

RNA extraction from testicular tissue was performed as published (Steger *et al*., 2003). Briefly, six paraffin sections were collected in a reaction tube and deparaffinized in 500 μl xylene for 10 min at 53°C. After centrifugation, pellet was resuspended in 200 μl 1 M guanidine thiocyanate, 0.5% sarcosyl, 0.72% β-mercaptoethanol, 20 mM Tris–HCl (pH 7.5). After adding proteinase K to a final concentration of 0.5 μg/μl, samples were digested for 12–16 h at 58°C. Note that digestion with proteinase K is indispensable for the liberation of RNA from crosslined/fixed tissue (Fink *et al*., 2000) and RNA-binding proteins binding within the coding sequence of P1 and P2 mRNA in round spermatids (Steger *et al*., 2002). Subsequently, 20 μl 2 M sodium acetate, 220 μl phenol (pH 4.3) and 60 μl chloroform/isoamylalcohol (24/1) were added. Samples were vortexed and centrifuged for 15 min at 4°C. The aqueous layer was collected, 1 μl glycogen (10 mg/ml) added, and precipitated with 200 μl isopropanol. Samples were frozen for one hour at −20°C and centrifuged for 15 min at 12 000 × g. Pellets were washed with 75% ethanol, air-dried and resuspended in 10 μl RNase-free water.

RNA extraction from ejaculates was performed using the RNA extraction kit RNeasy MINI (Qiagen, Germany).

First strand complementary DNA (cDNA) synthesis was performed using Sensiscript (for testicular tissue) and Omniscript (for ejaculates), according to the manufacturers protocol (Qiagen, Germany).

Table I. Characterization of patients undergoing testicular sperm extraction followed by ICSI.

	Patients	Controls	*P*-value
Number of biopsies	74	17	
Age (years), male partner mean (range)	36.1 (25–53)	34.9 (20–58)	
Age (years), female partner mean (range)	31.7 (18–43)	—	
C_{tP1}, mean ± SEM	33.5 ± 0.23	30.5 ± 0.90	
C_{tP2}, mean ± SEM	35.6 ± 0.16	35.7 ± 0.36	
$\Delta C_{t(P2-P1)}$, mean ± SEM	2.05 ± 0.24	5.48 ± 0.92	0.0038
C_{tBcl2}, mean ± SEM	32.2 ± 1.26	36.6 ± 0.76	
$\Delta C_{t(Bcl2-P1)}$, mean ± SEM	3.4 ± 1.70	−0.6 ± 0.75	0.0115
Fertilization (%)	28.6 ± 2.48	—	
Pregnancy	9	—	

P1, protamine-1; P2, protamine-2; C_t, fractional cycle numbers where the background corrected amplification curves crossed a threshold value; ΔC_t, difference between two C_t values.

Table II. Characterization of patients undergoing ICSI with ejaculated spermatozoa.

	Patients	Controls	*P*-value
Number of ejaculates	95	10	
Age (years), male partner mean (range)	38.4 (26–56)	39.6 (21–65)	
Age (years), female partner mean (range)	34.0 (22–46)	—	
Sperm density ($\times 10^6$) mean (range)	11.6 (0.05–50.1)	34.4 (6.5–69.2)	
C_{tP1}, mean ± SEM	22.7 ± 0.4	22.6 ± 0.70	
C_{tP2}, mean ± SEM	23.7 ± 0.45	22.3 ± 0.82	
$\Delta C_{t(P2-P1)}$, mean ± SEM	0.8 ± 0.09	−0.3 ± 0.12	0.0002
C_{tBcl2}, mean ± SEM	31.2 ± 0.55	34.9 ± 0.86	
$\Delta C_{t(Bcl2-P1)}$, mean ± SEM	−6.7 ± 0.94	−14.2 ± 0.51	0.000007
Fertilization (%)	73.6 ± 2.18	—	
Pregnancy	32	—	

Real-time qRT-PCR

Real-time qRT-PCR was performed using iQ™ SYBR Green Supermix and iCycler (BioRad, Germany). Per sample, 4 μl cDNA were used for amplification of P1, P2 and Bcl2. Cycling conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, and 73°C for 30 s. The following primers were employed (MWG, Germany):

P1: 5′cggagctgccagacacgga3′ (forward; bp 182–200) and 5′ctacatctcggtctgtacctggg3′ (reverse; bp 224–246) resulting in a 65 bp amplification product.

P2: 5′aagacgctcctgcaggcac3′ (forward; bp 344–362) and 5′gccttctgcatgttctctt3′ (reverse; bp 396–414) resulting in a 71 bp amplification product.

Bcl2: 5′acatcgccctgtggatgact3′ (forward; bp 1008–1027) and 5′gggccgtacagttccacaaa3′ (reverse; bp 1085–1104) resulting in a 97 bp amplification product.

Negative controls included samples lacking reverse transcriptase. Furthermore, intron spanning primers were applied to avoid amplification of genomic DNA. All experiments were carried out in duplicate.

Values recorded for quantification were the fractional cycle numbers (C_t) where the background corrected amplification curves crossed a threshold value. The threshold value was set within the log-linear phase of the amplification curves.

Quality of oocytes and embryos

Oocytes were considered as good quality oocytes when they were in metaphasis II stage and had no morphological defects, such as vacuoles, granulation, fragmentation or deformation in various patterns.

To evaluate the embryo quality, we classified the embryos on day 2 into four groups, according to Steer *et al*. (1992). (A) no fragmentation, equal blastomeres; (B) low fragmentation (<20%); (C) 20–50% fragmentation; (D) high fragmentation (>50%). The number of blastomeres and the fragmentation grade ($A = 4$, $B = 3$, $C = 2$, $D = 1$) were added to a score. Pregnancy was defined as a serum βHCG level >30 mIU/ml 2 weeks after puncture, combined with the detection of a gestational sac.

Statistical analysis

Data were analysed using R (R Development Core Team, 2007). C_t values of technical replicates were averaged and averages were used for further calculations. Relative expression levels were expressed by ΔC_t values which represent a measure of the log-ratio of the transcript abundances in the samples. The log-ratio of P1 and P2 is given by $\Delta C_t = C_{tP2} - C_{tP1}$. The log-ratio of Bcl2 and P2 is given by $\Delta C_t = C_{tP2} - C_{tBcl2}$. The normal distribution of C_t and ΔC_t values was checked with normal-QQ plots. Differences of the mean values between groups were tested with the Welsh *t*-test (two-sided). Linear correlations were tested using the Pearson coefficient of correlation. All tests were performed at a confidence level of 95%. All values are presented as means ± SEM. Power analysis was performed with the software G-Power using the 'compromise analysis' for *t*-tests on the difference of sample means and correlations (Faul and Erdfelder, 1992). The power of the statistical tests at $\alpha = 0.05$ were >80%. The power of the two-sided two-sample Welsh *t*-test do detect at least 'strong' relative effects (Cohen's $d = 1$). In the mean, ΔC_t between patients and controls was 84% for ejaculates with $n_1 = 10$; $n_2 = 95$, and 94% for testicular tissue with $n_1 = 17$; $n_2 = 74$. The power of the correlation tests for $r \geq 0.3$ was 91% ($n = 95$) and 85% ($n = 74$).

Results

Testicular tissue

Real-time qRT-PCR for P1, P2 and Bcl2 was performed for both testes per man. As no statistically significant difference could be observed between right and left testes, the mean value was used for further calculation. The mean of P1 in infertile men ($C_t = 33.5 \pm 0.23$) is lower than in the control group ($C_t = 30.5 \pm 0.90$), whereas the mean of P2 is almost identical in infertile men ($C_t = 35.6 \pm 0.16$) and in the control group ($C_t = 35.7 \pm 0.36$). The P1–P2 ratio is ∼1: 3.2 in the control group and 1:4 in infertile patients. The difference of the ratios between controls and infertile men is statistically significant ($P = 0.0038$) (Fig. 1a) and larger for P2 than for P1. In addition, infertile men exhibit more than 10-fold the amount of Bcl2 mRNA when compared with controls ($P = 0.0155$) (Fig. 1b). Furthermore, the Bcl2 content reveals a positive linear correlation ($P = 0.0250$) with the P1–P2 ratio (Fig. 2a).

TESE ($n = 74$) in combination with ICSI (max three cycles) produced nine pregnancies (12.1%), the fertilization rate was 28.6 ± 2.48%. Neither the fertilization rate nor the pregnancy rate revealed a significant correlation with the P1–P2 ratios. In addition, no significant correlation could be demonstrated between the pregnancy rate and the quality of oocytes and embryos. Results are summarized in Table I.

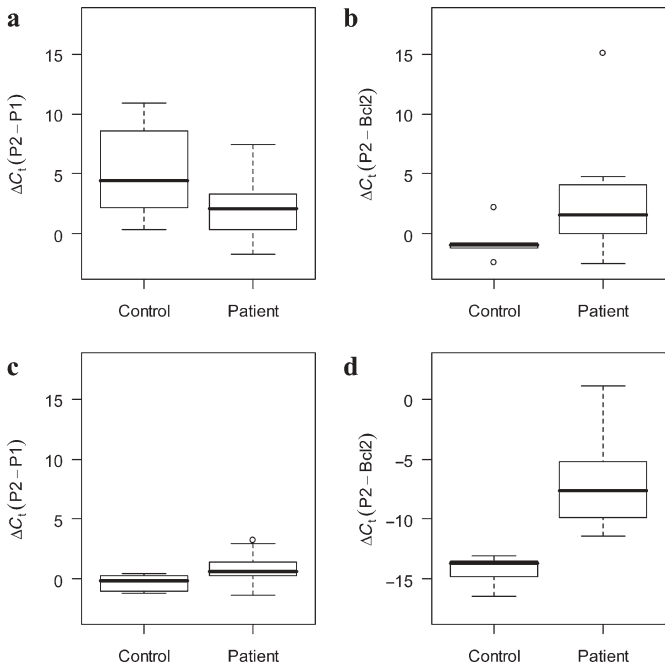

Figure 1: (**a**) Protamine-1 to protamine-2 log-ratio (ΔC_t(P2–P1)) of controls and patients in testicular biopsies ($P = 0.0038$). (**b**) Log-concentration of Bcl2 normalized to protamine-2 (ΔC_t(P2–Bcl2)) of controls and patients in testicular biopsies ($P = 0.0115$). (**c**) Protamine-1 to protamine-2 log-ratio (ΔC_t(P2–P1)) of controls and patients in ejaculates ($P = 0.0002$). (**d**) Log-concentration of Bcl2 normalized to protamine-2 (ΔC_t(P2–Bcl2)) of controls and patients in ejaculates ($P = 7.0 \times 10^{-6}$). Welsh two sample *t*-test. Data are mean ± SEM. ΔC_t, difference between C_t values

Ejaculates

The P1–P2 ratio is ∼1:1 in the control group and 1:1.7 in infertile men. The difference is statistically significant ($P = 0.0002$) (Fig. 1c). No correlation could be found between the absolute sperm density and the P1–P2 ratio or the amount of Bcl2 mRNA. However, the amount of Bcl2 in infertile men is more than 10-fold the amount of Bcl2 in the control group ($P = 7.0 \times 10^{-6}$) (Fig. 1d). In addition, the Bcl2 content revealed a positive linear correlation ($P = 0.0003$) with the P1–P2 ratio (Fig. 2b).

ICSI (1 cycle) with ejaculates ($n = 95$) resulted in 32 pregnancies (32.5%), the ferilization rate was 73.6 ± 2.18%. Similar to TESE, neither the fertilization rate nor the pregnancy rate revealed a significant relation with the P1–P2 ratio. In addition, no significant correlation could be demonstrated between the pregnancy rate and the quality of oocytes and embryos. Results are summarized in Table II.

Discussion

Spermatozoa from normozoospermic men contain similar amounts of P1 and P2 (Balhorn *et al*., 1988). An aberrant protamine ratio has been demonstrated in infertile men (Balhorn *et al*., 1988; Belokopytova *et al*., 1993). In previous studies, we demonstrated a significant correlation between the protamine ratio and successful fertilization using *in situ* hybridization (Steger *et al*., 2001; 65 patients; Mitchell *et al*., 2005;

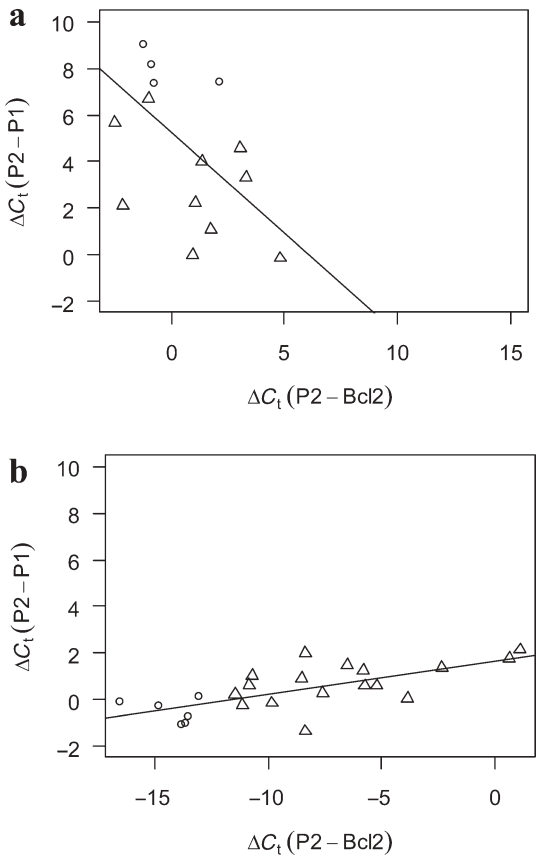

Figure 2: (**a**) Correlation between the protamine-1 to protamine-2 log-ratio (ΔC_t(P2−P1)) and normalized Bcl2 log-concentration (ΔC_t(P2−Bcl2)) in testicular biopsies ($R^2 = 0.3320$, $P = 0.0250$). (**b**) Correlation between the protamine-1 to protamine-2 log-ratio (ΔC_t(P2−P1)) and normalized Bcl2 log-concentration (ΔC_t(P2−Bcl2)) in ejaculates ($R^2 = 0.4636$, $P = 0.0003$). Pearson correlation test. Circles represent controls, triangles patients

41 patients) and qRT-PCR (Steger *et al*., 2003; 57 patients) on testicular biopsies, as well as Western blot analysis (Nasr-Esfahani *et al*., 2004; 71 patients) on ejaculated spermatozoa. Applying qRT-PCR, in the present study, we comparatively analysed the protamine ratio in testicular biopsies and ejaculates from fertile and infertile men. The testicular samples were different from those used in our previous studies (Steger *et al*., 2001, 2003).

We were able to demonstrate a significant difference in the P1–P2 ratio between infertile (1:4 in testicular biopsies and 1:1.7 in ejaculates) and fertile (1:3.2 in testicular biopsies and 1:1 in ejaculates) men. Both P1 and P2 change between fertile and infertile men but P2 changes more than P1, suggesting different mRNA stabilities for the two molecules. P1 and P2 may have different functions, as (1) P2, unlike P1, represents a zinc finger protein revealing a Cys2–His2 motif (Bianchi *et al*., 1992) and (2) P1 is invariably present in all mammals, while P2 is expressed only in some mammals (Corzett *et al*., 2002) suggesting a more basic and conserved function for P1 and an accessory function for P2.

As genes for protamines are solely transcribed in round spermatids (Steger *et al*., 2000) and stored as silent mRNAs for later translation in elongating spermatids in which transcription is no longer active (Steger, 1999, 2001; Aoki *et al*., 2006b), it is well justified to consider altered mRNA levels as a potential origin of altered protein levels. Owing to the fact that each spermatid contains ∼20,000 protamine transcripts (Braun *et al*., 1989) which may be stored as silent mRNAs for up to 7 days (Hecht, 1998), RNA extraction from routinely Bouin-fixed and paraffin-embedded testicular biopsies followed by qRT-PCR represents a suitable time- and tissue-saving procedure for the study of quantitative gene expression in haploid male germ cells. This method has already been reported to represent a valuable tool for RNA extraction from archive material (Godfrey *et al*., 2000).

In the present study, the amount of P2 mRNA in testicular spermatids was almost identical in patients and controls, while the amount of P1 was lower in infertile men compared with the control group: although this difference was not significant, data were similar to that of other analysed patients (Steger *et al*., 2003). By contrast, the protamine ratio exhibited a highly significant difference between patients and controls. However, no significant correlation could be demonstrated with the fertilization and pregnancy rates. This is in contrast with IVF rates that have been reported to be significantly reduced in patients with abnormally low and high P1–P2 protein ratios (Aoki *et al*., 2006c). However, success of ICSI—unlike IVF—highly depends on the selection of the spermatozoon, as sperm of a given semen sample is known to exhibit a significant intra-sample heterogeneity of protamine content (Aoki *et al*., 2006a). Since the protamine ratio obtained by counting the percentage of protamine-positive round spermatids has been demonstrated to exhibit a significant correlation with successful fertilization (Steger *et al*. 2001), this may represent a more suitable predictive marker for the estimation of successful fertilization than the total amount of protamines obtained by qRT-PCR.

Ejaculated spermatozoa from fertile men contain equal amounts of P1 and P2, both at the protein level (Balhorn *et al*., 1988; Belokopytova *et al*., 1993) and at the mRNA level (this study). Interestingly, in testicular spermatids, we demonstrated an increased P1–P2 mRNA ratio of 1:3.2. Infertile men exhibited an aberrant protamine ratio, namely 1:1.7 in ejaculated spermatozoa and 1:4 in testicular spermatids. Abnormal protamine expression may be the result of an abnormal functioning of a regulator of transcription, translation or post-translational modification that affects not only protamines, but also a broad range of genes involved in spermatogenesis. Therefore, protamine expression may act as a checkpoint during spermiogenesis and abnormal protamine expression may lead to an increased level of apoptosis.

It is known that P1 and P2 haploinsufficient mice exhibit damaged DNA (Cho *et al*., 2003). When applying ICSI, it was possible to activate oocytes, however, only a few could progress to the blastocyst stage. A similar phenomenon has been described in infertile patients (Tesarik *et al*., 2004). There is evidence that links high DNA fragmentation indexes with diminished sperm DNA integrity (Aoki *et al*., 2005), decreased protamine content (Nasr-Esfahani *et al*., 2005) and lower IVF and ICSI rates (Evenson and Wixon, 2006). Although Oosterhuis *et al*. (2000)reported DNA strand breaks and apoptotic marker annexin V in ejaculated

spermatozoa, Sakkas *et al*. (1999)suggested that DNA strand breaks and apoptotic markers did not coexist in mature spermatozoon, as ejaculated spermatozoa with apoptotic markers appeared to have escaped programmed cell death in a process called abortive apoptosis. Therefore, it will be important to distinguish between cells that show high levels of DNA strand breaks and cells that are positive for apoptotic markers.

In recent years, the Bcl2/Bax system has been implicated in the regulation of apoptosis in various physiological and pathological states of cells (reviewed in Koji and Hishikawa, 2003). Although Bcl2 blocks apoptosis, Bax promotes it. In the present study, we concentrated on Bcl2, as Bax have been demonstrated in all germ cells, whereas Bcl2 was present solely in pachytene spermatocytes and spermatids (Damavandi *et al*., 2002). We have shown that infertile men exhibit more than 10-fold the amount of Bcl2 mRNA in testicular spermatids and ejaculated spermatozoa when compared to the appropriate control group. Furthermore, a linear correlation with the protamine ratio was obvious. Investigations on a possible relation between apoptosis and male fertility in mice concentrate on the heterodimeric partner of Bcl2. Bax-deficient male mice have been demonstrated to be infertile due to an accumulation of premeiotic germ cells and a lack of adult spermatids in the seminiferous tubules (Knudson *et al*., 1995).

In conclusion, we found significantly aberrant protamine mRNA ratios and a higher Bcl2 mRNA content in both testicular spermatids and ejaculated spermatozoa of infertile men, identifying these molecules as suitable predictive biomarkers to discriminate between fertile and infertile men.

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