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Evaluation of protamines 1 and 2 transcript contents in spermatozoa from asthenozoospermic men

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Abstract: During mammalian spermatogenesis, the chromatin structure undergoes substantial condensation. The key role in this process is played by protamines 1 and 2 (PRM1, PRM2). We attempted to compare the levels of PRM1 and PRM2 transcripts in mature spermatozoa of normospermic and asthenozoospermic men. Human ejaculates from normozoospermic (n=70) and asthenozoospermic (n=100) donors were purified by centrifugation through discontinuous Percoll density gradient. RNA was isolated from spermatozoa according to the Chomczyński and Sacchi method, treated with DNase I, and reverse-transcribed into cDNA. Using reverse transcription and real-time quantitative polymerase chain reaction analysis, we found a reduction in the levels of PRM1 and PRM2 transcripts in spermatozoa from asthenozoospermic men, as compared to controls ($P<0.001$). Our findings indicate that a reduction in contents of PRM1 and PRM2 transcripts in spermatozoa may be linked with asthenozoospermia.

Key words: Spermatozoa - mRNA - Protamines - Asthenozoospermia.

Introduction

Protamine 1 and 2 (PRM1, PRM2) are small, arginine-rich nuclear proteins that are involved in packing DNA in spermatozoa [1]. The amino acid sequence of both protamines was first determined by Ammer *et al.* (1986). The *PRM1* and *PRM2* genes are localized on chromosome 16p13.3. PRM1 is synthesized as a mature protein product, which is composed of 50 amino acid residues. PRM2 mRNA is translated as a precursor protein consisting of 103 amino acids, which undergoes proteolytic cleavage to form the 50 amino acid mature protein [2]. The PRM1 and PRM2 are transcribed in the post-meiotic haploid spermatid, during early stages of spermiogenesis [3]. PRM1 and PRM2 mRNAs have been found in round spermatids, whereas the proteins are completely absent at this stage [4,5]. The translation of PRM1 and PRM2 transcripts is initiated and completed by the elongation stage of spermatid development [6-8].

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During mammalian spermiogenesis, the structure of chromatin is permanentlly modified [9]. The first step of this process takes place in haploid round spermatids and includes replacement of somatic histones by transition proteins 1 and 2 (TNP1, TNP2). In the phase of elongated spermatids, TNP1 and TNP2 are replaced by protamines. After binding of protamines to nuclear sperm chromatin, the process of gene transcription is completely inactivated [9].

Abnormal spermatozoal PRM1/PRM2 protein ratio can be contributed to abnormal chromatin condensation and increased DNA strand breaks, which result in male infertility [9,10]. Sperm chromatin compaction is crucial to protect sperm chromatin during transport through the epididymis and female reproductive tract. Abnormal chromatin remodelling during spermiogenesis results in chromatin that is prone to denaturation.

Miyagawa *et al.* (2005) found a relationship between increased number of DNA strand breaks and abnormalities of tail midpiece containing mitochondria [11]. This finding indicates that an increased number of DNA strand breaks may initiate the apoptotic signalling pathway, inducing inactivation of mitochondria and immotility of spermatozoa [11]. Therefore, we attempted to analyze the difference between

Table 1. Oligonucleotide sequences used for RQ-PCR analysis.

Transcript	Sequence (5'-3' direction)	Position	ENST number www. ensembl.org/	Exons	Product Size (bp)
PRM1	CGGTGAGCTGCTGCCCAACT GCCTAAGTTCGACTGACCTG	183-203 316-336	00000312511	1, 2	153 bp
PRM2	GGATCCACAGCCGCCAGCATCGCT GCATGTTCTCTTCCTGGTTCTGCA	416-440 496-520	00000241808	3, 4	104 bp
GAPDH	CTGCACCACCAACTGCTT TTCTGGGTGGCAGTGATG	555-574 642-659	00000105679	7, 8	105 bp

Table 2. Semen parameters of normozoospermic and asthenozoospermic men.

Seminal parameters	Normozoospermic (n=70)		Asthenozoospermic (n=100)		*P value
	Median (range)	Mean (± SD)	Median (range)	Mean (± SD)	
Sperm concentration (10^6/ml)	63 (32-109)	62 ± 30	41 (21-58)	42 ± 16	0.002
Grade a + b motility (%)	55 (43-65)	54 ± 21	25 (15-40)	23 ± 12	0.002
Total count (10^6)	280 (110-550)	274 ± 45	130 (70-285)	121 ± 22	0.001
Normal sperm morphology (%)	20 (10-35)	17 ± 5	10 (6-26)	8 ± 3	0.042

*P value was calculated using Fisher exact test.

PRM1 and PRM2 transcript presence in spermatozoa of normozoospermic and asthenozoospermic men.

The mRNAs found in human mature spermatozoa represent remnant transcripts, which survive the condensation of the spermatid nucleus and stay in human ejaculated spermatozoa [12-14]. These transcript remnants of mature sperm cells may correlate to gene expression during spermatogenesis.

Using reverse transcription and real-time quantitative PCR (RQ-PCR) analysis, we evaluated the number of PRM1 and PRM2 mRNAs in Percoll purified spermatozoa from semen samples of normozoospermic and asthenozoospermic donors.

Materials and methods

Sperm purification method. Human ejaculates were obtained from 70 healthy volunteers of proven fertility and normal semen quality as assessed by WHO criteria (1999), and from100 asthenozoospermic men (Table 2). The main criterion for classification of asthenozoospermic men was low sperm motility [15]. The semen parameters, including motility, were assessed by computer-assisted semen analysis (CASA) system [16]. Spermatozoa were then purified by centrifugation through discontinuous Percoll (Amersham Biosciences, UK) density gradient (80:40, vol/vol). The purity of the spermatozoa was examined by using an optical microscope equipped with 100 x oil objective, and spermatozoa contaminated by somatic or round cells were discarded. These separated spermatozoa were subsequently used to isolate RNAs, which were reverse transcribed into cDNA.

RQ-PCR analysis of PRM1 and PRM2 transcript contents in spermatozoa. Each RNA sample was isolated from one million spermatozoa according to Chomczyński and Sacchi (1987), [17]. The purity of the RNA samples was verified spectrophotometrically at 260 and 280 nm. RNA was treated with DNase I (Promega Co. Madison, USA) and reverse-transcribed into cDNA using random hexamer priming and reverse transcriptase (RT) (Sigma Co. St. Louis, USA). Quantitative analysis of PRM1 and PRM2 cDNA was performed by RQ-PCR SYBR Green I analysis (Light Cycler, Roche Diagnostics GmbH, Mannheim, Germany). The PRM1 and PRM2 cDNAs were amplified using pairs of primers (Table 1, Figure 1). For amplification, 2 µl (corresponding to 10^5 spermatozoal RNA) of cDNA was added to 18 µl of PCR mix containing HotStartTaq DNA polymerase, reaction buffer, dNTP mix, SYBR Green I dye, 2.5 mM MgCl2, and primers (Qiagen Inc. Valencia, Ca, USA). Quantification of copy number was derived from a standard curve of a known amount of synthetic DNA template. Since

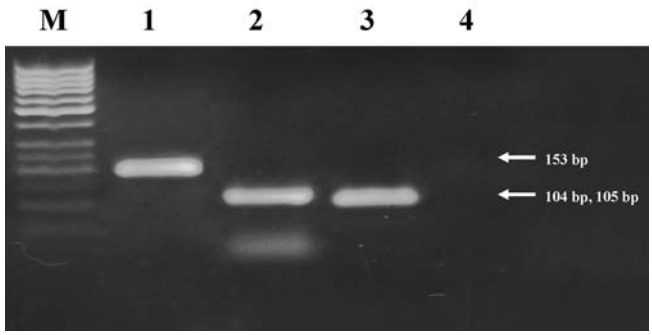

Fig. 1. Picture of agarose gel electrophoresis of RQ-PCR evaluation of spermatozoal PRM1 and PRM2 transcripts. Amplification products of spermatozoal cDNA of PRM1, PRM2, and GAPDH are shown in lanes 1, 2, and 3, respectively. Lanes M and 4 represent molecular weight marker and negative control of amplification, respectively.

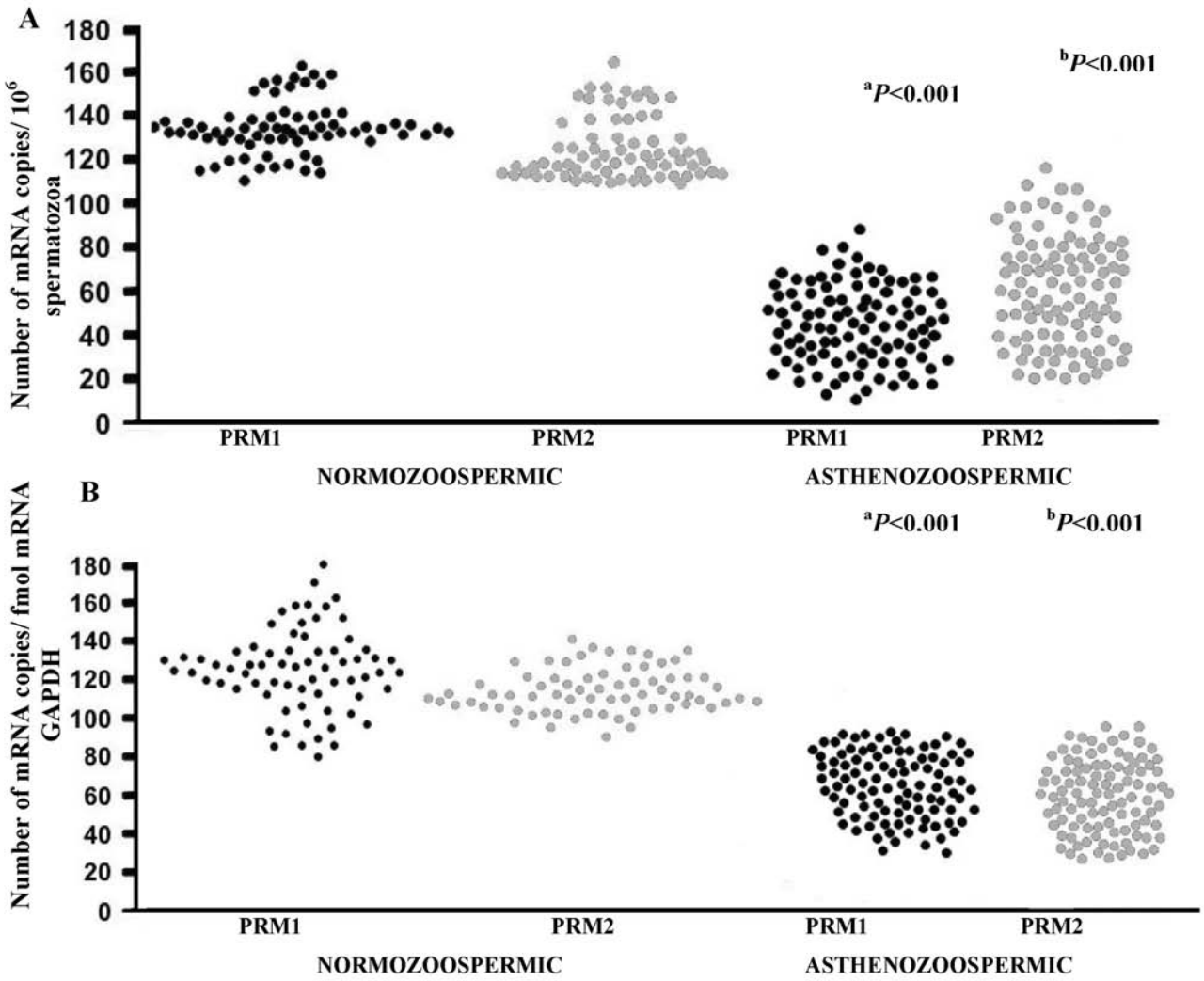

Fig. 2. Number of PRM1 and PRM2 transcript copies in spermatozoa isolated from normozoospermic and asthenozoospermic men. The number of PRM1 and PRM2 transcript copies is presented per 10^6 sperm cells (A) or per fmol of GAPDH (B). ^aP and ^bP represent statistical differences between PRM1 and PRM2 transcript contents in spermatozoa from normospermic and asthenozoospermic donors, respectively.

GAPDH transcript exhibited similar levels in spermatozoa of both normospermic and asthenozoospermic donors, we adjusted the quantity of PRM1 and PRM2 transcripts in each sample to use the same amount of GAPDH [18-20]. The levels of PRM1 and PRM2 mRNAs were expressed by the number of these transcripts per 10^6 spermatozoa or adjusted to fmol of GAPDH transcripts.

Statistical analysis. Each of these experiments was performed at least in triplicate. Results were estimated using Student's t-test and one-way analysis of variance (ANOVA) with Newman-Keule's post-hoc test. P value was determined by Student's t-test, with $p<0.05$ as the level of significance.

Results

Using RQ-PCR analysis, we evaluated the number of PRM1 and PRM2 transcripts in Percoll density gradient isolated spermatozoa from normozoospermic and asthenozoospermic donor ejaculate.

We found a higher content of PRM1 and PRM2 transcripts in spermatozoa of normozoospermic men versus asthenozoospermic men (Fig. 2). The median of PRM1 transcript contents in spermatozoa of normozoospermic and asthenozoospermic males was 138 (range 109-162) and 42 (range 12-86) copies per million sperm cells, respectively ($p<0.001$), (Fig. 2A). The number of PRM2 transcripts in spermatozoa was also higher in normozoospermic donors as compared to asthenozoospermic men. The median of PRM2 mRNA contents in normozoospermic and asthenozoospermic men was 119 (range 102-163) and 63 (range 18-116), ($p<0.001$) copies per million of spermatozoa, respectively (Fig. 2A). We also adjusted PRM1 and PRM2 transcript number to fmol GAPDH mRNA, which confirmed significant differences between normozoospermic and asthenozoospermic men (Fig. 2B).

Discussion

During spermiogenesis, the haploid round spermatids undergo an elongation phase, cytoplasmic droplet rejection, and eventually differentiation into mature spermatozoa [21,22]. There is no de novo synthesis of RNA in human sperm [23]. However, human mature spermatozoa contain a subset population of RNA, which has also been found in human testes. Microarray analysis has revealed that about 3000 different transcripts exist in ejaculated spermatozoa [24-26].

Expression of protamines is essential in spermatogenesis and male fertility. They are needed for proper sperm chromatin compaction, which contributes to resistance of spermatozoal chromatin to denaturation. Male infertility is generally characterised by dysfunctions in processes of penetration, adhesion, and fusion with oocyte. Ahmadi *et al.* (1993), using hamster egg test, has proved that destruction of the protamines, using dithiothreitol as a disulfide-reducing reagent, leads to reduction of sperm binding to oocyte and spermatozoal penetration ability [27]. Distinct levels of spermatozoal PRM1 and PRM2 mRNAs were found in spermatozoa of fertile and infertile men [9]. Moreover, Carrell *et al.* (2001) have shown that PRM2 was absent in 13 of 75 infertile patients, although this protamine was found in all 50 fertile men in their study [28]. They also found that low levels of PRM2 correlated with low sperm counts, low motility, and morphological abnormalities [28]. In contrast, Steger *et al.* (2003) did not find differences in PRM2 transcript levels in spermatozoa isolated from men with impaired spermatogenesis compared with men with normal spermatogenesis [29]. However, they observed significantly decreased PRM1 transcript contents in the group of patients with impaired smermatogenesis [29].

Our results first showed a decrease in both PRM1 and PRM2 transcripts in spermatozoa from asthenozoospermic men compared to the same transcripts in spermatozoa from normozoospermic men.

Protamines play a crucial role in sperm chromatin condensation and protection of the paternal genome from DNA damage [30-32]. Low levels of PRM1 or PRM2 may lead to DNA damage accumulation in sperm, morphological abnormalities, and initiation of apoptotic pathway, inactivating mitochondria and decreasing sperm motility [11].

Our results first described significantly decreased levels of PRM1 and PRM2 mRNAs in spermatozoa from men with asthenozoospermia. We presume that decreased levels of both PRM1 and PRM2 transcripts in spermatozoa from this group of patients may be associated with lower sperm motility and reduction of reproductive ability of these donors. Further studies are required to determine molecular mechanisms responsible for PRM1 or PRM2 transcript reduction in spermatozoa from asthenozoospermic donors.

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