

The Role of Protamine 2 Gene Expression and Caspase 9 Activity in Male Infertility

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Abbreviations and Acronyms

GAPDH = glyceraldehyde-3-phosphate dehydrogenase

PCR = polymerase chain reaction

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Purpose: Approximately 15% of couples are affected by infertility with the man responsible in almost half of the cases. PRMs (protamines) confer a higher order of DNA packaging in sperm than that in somatic cells. Because of the critical roles of PRMs in spermatid differentiation, aberrations in PRM expression or changes in protein structure could be causes of certain types of idiopathic human male infertility. The aim of this study was to give insight into the role of PRM2 gene expression and caspase 9 activity in the pathogenesis of male infertility.

Materials and Methods: The current study included 70 men with idiopathic infertility and 64 fertile men who attended the andrology outpatient clinic at Mansoura University Hospital. Semen sample analyses were done according to WHO recommendations. The acrosome reaction of spermatozoa recovered from each sample was assessed. Samples were separated using discontinuous gradient separation. From each semen sample mature sperm were separated from immature sperm. The resulting samples were divided into 2 parts, including one to determine caspase 9 activity and the other for RNA extraction and reverse transcriptase-polymerase chain reaction of PRM2 gene expression. The polymerase chain reaction product was electrophoresed on 2% agarose gel.

Results: PRM2 gene expression was significantly decreased in immature sperm extracted from the fertile and infertile groups. Caspase 9 activity was significantly increased in immature sperm extracted from both groups.

Conclusions: Low levels of PRM2 may be associated with morphological abnormalities, initiation of the apoptotic pathway and decreasing sperm motility. PRM2 may be an important marker to better understand the key regulatory pathway of spermatogenesis and it may act as a crucial part of fertilization.

Key Words: testis; spermatozoa; infertility, male; protamines; caspase 9

DURING mammalian spermiogenesis PRMs progressively replace somatic histones in a stepwise manner.¹ PRMs are small basic proteins that are widely conserved among species. All mammals have PRM1 while some species, including humans and mice, have PRM2. In sperm nuclei

the DNA-PRM complex compacts, stabilizes and protects the haploid genome.²

Protamines confer a higher order of DNA packaging in sperm than that in somatic cells. The condensed and insoluble nature of the highly condensed sperm chromatin protects

the genetic integrity of the paternal genome during its transport through the male and female reproductive tracts.³

Because of the critical roles of PRMs in spermatid differentiation, aberrations in PRM expression or changes in protein structure could be causes of certain types of idiopathic human male infertility.² Also, Aoki et al suggested that an abnormal spermatozoal PRM1/PRM2 protein ratio may contribute to abnormal chromatin condensation and increased DNA strand breaks, which result in male infertility.⁴

Apoptosis is an important process involved in normal spermatogenesis. However, deregulation of this biological process involves abnormalities in the production of male gametes and male infertility.⁵

Two major pathways are involved in the process of apoptosis in mammalian cells. The extrinsic pathway is characterized by oligomerization of death receptors, such as Fas or tumor necrosis factor, followed by activation of caspase 8 and caspase 3. The intrinsic pathway involves activation of procaspase 9, which in turn activates caspase 3.⁶ The extrinsic and intrinsic pathways have been proved to participate in germ cell apoptosis during spermatogenesis.⁷

In the current study we aimed to evaluate the association between PRM2 transcript contents and caspase 9 activity in mature and immature spermatozoa, and investigate their roles in fertilization ability.

SUBJECTS AND METHODS

Subjects

The current study was performed in 70 men with idiopathic infertility who attended the andrology outpatient clinic at Mansoura University Hospital. Complete general and local genitalia examinations were done to exclude signs of hypogonadism. The genitalia were also examined to exclude varicocele, cryptorchidism and congenital anomalies. Select men had normal serum levels of growth hormone, follicle-stimulating hormone, luteinizing hormone, testosterone, estradiol, prolactin and thyroid hormones to exclude an endocrine cause of infertility such as hyperprolactinemia or increased follicle-stimulating hormone. Study exclusion criteria included leukospermia, frank pyospermia, hemospermia, chronic urinary tract infection, history of intake of medications or tonics, azoospermia and age greater than 45 years to avoid the effects of aging on sperm variables. Any participants with a history of chronic illness and long-term medication were also excluded. In addition, the study included 64 fertile men who were healthy with normal semen parameters.

Informed consent was obtained from all study subjects. The study was performed with the approval of the local ethics committee of the Faculty of Medicine, Mansoura University, in compliance with the 2008 Helsinki Declaration.

Semen samples were obtained from all subjects after 3 days of sexual abstinence. Samples were allowed to liquefy

completely for 15 to 30 minutes at 37C. After complete liquefaction computer assisted semen analysis was performed using an Autosperm device (FertiPro, Beernem, Belgium).⁸ Sperm morphology was evaluated by phase contrast microscopy and sperm Mac stain (FertiPro). Semen samples were assessed according to WHO recommendations.⁹

Peroxidase positive white blood cells were detected by peroxidase stain.¹⁰ Seminal plasma was obtained by centrifugation of the semen sample and analyzed for α -glucosidase activity by the method described by Guerin et al.¹¹ The acrosome reaction of spermatozoa recovered from each sample was assessed before and after stimulation with the calcium ionophore A23187 by the *Pisum sativum* (Sigma®) fluorescence method with simultaneous vitality staining using Hoechst 33258.

From each semen sample spermatozoa were separated into mature and immature by Sil-Select gradient (FertiPro). Separated samples were washed and resuspended in modified human tubal fluid-HEPES medium with 0.3% human serum albumin. The purified spermatozoa were used to assess motility, concentration and acrosome reaction.

To ensure the reliability and purity of specimens after separation each fragment was examined morphologically. Motility and concentration were assessed and the acrosome reaction was done. Strict criteria were used after staining all specimens with sperm Mac stain.

Each separated sample was then divided into 2 parts, including 1 for RNA isolation and the other to assay caspase 9 activity colorimetrically using a kit.¹²

Sperm Pellet Total RNA Isolation

Total RNA was extracted from the sperm pellet according to the method of Das et al¹³ using TriFast™ Reagent (No. 7930b) and a RNA extraction kit (No. 74104, Qiagen®) according to manufacturer instructions.

Semiquantitative reverse transcriptase-PCR of extracted RNA was performed with the Maxima First Strand cDNA Synthesis Kit (No. K1641, Thermo Fisher Scientific, Grand Island, New York) according to manufacturer instructions. PCR Master Mix (2X, No. RR310A, Takara, Tokyo, Japan) was used. Gene specific primers were obtained from Biologio, Nijmegen, The Netherlands.

The sequence of the oligonucleotide primers of the PRM2 was 5'-GGATCCACAGCCGCCATCCCCGT-3' (forward primer) and 5'-GCATGTTCTCTCCTGGTCTGCA-3' (reverse primer) located at 416 to 440 and 496 to 520 of PRM2, respectively. The amplified gene product of PRM2 was 104 bp.¹⁴ GAPDH served as the housekeeping gene. The GAPDH primer sequences were 5'-AATCCCATCACCATCTCC-3' (forward primer) and 5'-CATCACGCCACAGTTTCC-3' (reverse primer) with a resulting 382 bp amplification product.¹⁵

Thermal cycling reaction was performed using a Model FTC3102D thermal cycler (No. TC-321, Techne®) using the program 40 cycles of denaturation at 95C for 30 seconds, annealing at 56C for 30 seconds and extension at 72C for 30 seconds with final extension at 72C for 10 minutes.¹⁴

Amplified PCR Product Detection

PCR products were subjected to 2% agarose gel electrophoresis, stained with ethidium bromide and visualized

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