Expression of the HSPA2 gene in ejaculated spermatozoa from adolescents with and without varicocele

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Objective: To evaluate mRNA expression of the HSPA2 gene in ejaculated spermatozoa in adolescents with and without varicocele.

Design: Controlled prospective study.

Setting: Patients in an academic research environment.

Patients: Adolescent patients with clinical diagnosed bilateral varicocele grades II and III, and adolescent patients without varicocele.

Intervention: Reverse-transcription–polymerase chain reaction (RT-PCR) analysis of HSPA2 gene expression in adolescents with and without varicocele.

Main Outcome Measure: Comparative RT-PCR expression analysis of HSPA2 gene mRNA, compared to the housekeeping β-actin gene.

Results: Sperm from adolescents with varicocele and oligozoospermia had significantly lower levels of HSPA2 gene expression than both adolescents without varicocele (controls) and adolescents with varicocele and normal sperm concentration (P<.05). The latter group had a nonsignificant increase in gene expression compared to the control group.

Conclusions: This is the first report on HSPA2 gene expression in ejaculated spermatozoa from adolescents and its relationship with varicocele pathology. Results demonstrated that HSPA2 expression was down-regulated in adolescents with varicocele and oligozoospermia compared to controls. There was a higher, albeit nonsignificant, gene expression in adolescents with varicocele and normal sperm concentration than in controls. We speculate that expression levels of this gene might be used as a molecular marker for the acquisition of thermal tolerance in ejaculated spermatozoa. (Fertil Steril 2006;86:1659–63. ©2006 by American Society for Reproductive Medicine.)

Key Words: Varicocele, heat stress, HSPA2, sperm, infertility

Spermatogenesis is a complex process in which spermatogonia differentiate into mature spermatozoa, and it involves profound structural and biochemical changes of the germ cell. It is well known that cellular output during male gametogenesis is lower in situations of stress, such as heat stress. Scrotal temperature is physiologically maintained at a lower level than body temperature, and two systems play a key role in this thermal regulation: the scrotal system, controlled by the dartos and cremaster muscles, and the countercurrent system, which allows for heat exchange between arterial and venous blood (1). Varicocele compromises the latter system, hampering cooling of the arterial blood and thus maintaining scrotal temperatures closer to body temperature (2,3). Gonadal hyperthermia, 2.5°C above body temperature on average (4), has a direct effect on germ cells (e.g., changes in metabolism, in Sertoli cell function, and in DNA synthesis), leading to apoptosis during specific stages of spermatogenesis. Spermatocytes in the pachytene stage and spermatids in stages I–IV and XII–XIV of the spermatogenic cycle are susceptible to apoptosis, a fact also observed in an animal model of cryptorchidism (5). The presence of varicocele in adolescents is associated with sperm dysfunction, as demonstrated by an impaired sperm-zona pellucida binding capacity under in vitro conditions (6).

Several mechanisms might be responsible for germ-cell death by thermal aggression, including a reduced synthesis of DNA, RNA, and proteins, and a reduced flow in the capillary blood that supplies these cells, which has an indirect effect on the proteins synthesized by Leydig and Sertoli cells (2). However, in gonadal hyperthermia, the molecular
mechanisms of gene expression and cell death-activating events are obscure (3).

Prokaryote and eukaryote organisms have developed sophisticated strategies to adapt to environmental changes, responding to an exogenous stress such as exposure to high temperatures by synthesizing a group of highly conserved cellular proteins, the heat-shock proteins (HSPs) (7). Heat-shock protein A2 (HSPA2), a member of the 70-kDa family, is a molecular chaperone that assists in the folding, transport, and assembly of proteins in the cytoplasm, mitochondria, and endoplasmic reticulum (8). HSPs have a protective action on cellular autoregulation in response to heat (9) and on the mechanism of homeostasis, providing a balance between protein synthesis and degradation (10). In a knockout animal model, lack of the HSP-70 gene, a homologue to the human HSPA2 gene, led to a significant increase in apoptosis (11). According to Marmar (12), the effect of HSPs in patients with varicocele has not been studied, and further research could provide useful information.

Human semen contains multiple mRNA species originating from early stages of spermatogenesis (13), including a wide range of somatic and germinative transcripts. Thus, the study of these mRNA species could be useful in identifying genes linked to the infertility phenotype (14), representing a noninvasive tool for the evaluation of genetic disorders and reproductive anomalies (12).

The objective of this study was to evaluate the expression of the HSPA2 gene in sperm from adolescents with and without varicocele.

MATERIALS AND METHODS

Subjects

Institutional review board approval was obtained from the São Paulo Federal University Ethics Committee. In a prospective controlled study, we investigated 30 adolescents with bilateral grade II or III varicocele (study group), and 15 adolescents without varicocele (control group). These adolescents did not present with or have a history of any systemic illnesses, cryptorchidism, orchitis, epididymitis, urethritis, or testicular atrophy. All adolescents were examined by the same urologist in a warm room (23–25°C) in the standing position. Varicoceles were classified as follows: grade I, difficult to observe but easily palpable during Valsalva maneuver; grade II, visible and palpable without a Valsalva maneuver; and grade III, easily visible through the scrotal skin, and greatly exacerbated during a Valsalva maneuver. According to the results of the semen analysis, we divided the adolescents into three groups: [1] VN, adolescents with varicocele and normal sperm concentration; [2] VO, oligozoospermic adolescents with varicocele (<20 × 10⁶ sperm/mL); and [3] CN, control (without varicocele and with normal sperm concentration).

Semen Samples

Semen samples were collected by masturbation in an area adjacent to the laboratory after 2–4 days of sexual abstinence. After semen liquefaction, a standard semen analysis was performed according to World Health Organization (15) guidelines, and sperm morphology was evaluated by strict criteria after Diff-Quik staining. Semen samples were then subjected to a Percoll density-gradient centrifugation to recover the fraction of sperm with high motility. Briefly, 1 mL of semen was layered on discontinuous two-layer (45% and 90%) Percoll gradients (catalogue no. 170891-01, GE Healthcare, Amersham Place, England), and centrifuged at 600 × g for 20 minutes in 15-mL conical tubes. The medium used to dilute the Percoll was human tubal fluid (HTF) (catalogue no. 98270, Irvine, Santa Ana, CA), supplemented with 10% bovine serum albumine (A-6003, Sigma, St. Louis, MO).

Spermatozoa collected from the bottom layer (90% layer) were resuspended in HTF medium and centrifuged at 600 × g for 10 minutes. The resulting sperm pellet was immediately flash-frozen in liquid nitrogen. Samples were stored at −80°C until RNA extraction.

Total RNA Preparation and Reverse-Transcription–Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted from the sperm pellets using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA). First-strand complementary DNA (cDNA) synthesis from total RNA was catalyzed by Superscript II RT (Invitrogen Corporation), using oligo(dT) 12–18 according to the manufacturer’s protocol. Polymerase chain reaction was performed with 3 μL of cDNA preparation, using open reading-frame sequence-specific primers made on the basis of gene sequences identified with a BLAST search for β-actin: sense, 5'-CGT GAC ATT AAG GAG AAG CTG TGC-3'; antisense, 5'-CTC AGG AGG AGC AAT GAT CTT GAT-3'. The synthetic oligonucleotide primers (343- base pair [bp] fragment) of HSPA2 were: sense, 5'-TTG TTG GAA GTC TTT GGT ATA-3'; and antisense, 5'-CAT TTG CAT TTA TGC ATT TGT-3' (16).

Pilot tests were performed to determine the appropriate amount of RNA to be used, thus optimizing the reverse-transcription reaction. We used 5 μg of RNA from each sample, for a total of 45 samples (30 from the varicocele group, and 15 from the control group). Contaminating DNA was eliminated with the DNase I enzyme reaction buffer (Invitrogen Corporation). After tests to optimize the technique, including selection of the best reagent concentrations and annealing temperature, the following standard conditions were used: 10 minutes at 94°C for DNA denaturation, followed by 36 cycles (1 minute at 94°C, 1 minute at 57°C, and 1 minute at 72°C), and a final extension for 10 minutes at 72°C, using a Master Mix kit (Eppendorf AG, Barkhausenweg, Germany).
The cDNA amplification was assessed on 2% agarose gels stained with SYBR Green I (excitation maximum, 497 nm; emission maximum, 520 nm) nucleic acid gel stain in dimethyl sulfoxide (Invitrogen Corporation), which emits a blue fluorescence. After this step, the gel was analyzed with the scanner system STORM (GE Healthcare). Fluorescence made it possible to estimate the amount of cDNA (semiquantified) associated with the genes under study. The presence of the expected band was established by confirming the size in base pairs, using as our standard the PHIS RFDNA plasmid digested with the restriction enzyme Hae III. The cDNA amplifications of each sample were assessed for the β-actin and HSP-70 genes. The samples were first analyzed for β-actin, which characterizes the presence of total RNA in the material under study, and then for HSPA2. The HSPA2–β-actin ratio (absorbance unit at 520 nm) was assessed in patients with and without varicocele through reading with STORM, and later we performed an analysis with ImageQuant software (GE Healthcare).

**Statistical Analysis**
Statistical analysis was performed using one-way analysis of variance and Tukey’s Honestly Significant Difference (HSD) for post hoc analysis. P<.05 was considered significant.

**RESULTS**
Adolescents with and without varicocele did not differ in age or in right or left testicular volume (Table 1). Twenty-two patients had a left grade II varicocele and a right grade I (n = 18) or II (n = 10) varicocele. Eight adolescents had a left grade III and right grade I (n = 5) or II (n = 3) varicocele. However, in the varicocele group, regardless of the degree of varicocele, there was a difference in sperm concentration that defined two subgroups: [1], oligozoospermic (<20 × 10⁶ sperm/mL); and [2] VN, normal sperm concentration (>20 × 10⁶ sperm/mL). In contrast, all subjects in the control group presented a normal sperm concentration. Sperm morphology for subjects in the VO group was lower than in controls. No differences in progressive sperm motility were observed (Table 2).

Semiquantitative analysis for the HSPA2 gene expression demonstrated that there was a significantly lower expression of the HSPA2 gene in oligozoospermic patients with varicocele (VO) than in the group with varicocele and normal sperm concentration (VN) (P<.05) (Table 2, Fig. 1).

**DISCUSSION**
The importance of precise thermal regulation in the testis is proven by the fact that even small alterations in temperature, as caused by nonphysiologic factors such as varicocele, affect male fertility. Heat stress compromises spermatogenesis and leads to loss of cellular function (17). It is well known that all organisms from prokaryotic bacteria to mammals, including humans, make use of different strategies in an attempt to overcome this state, enhancing the expression of genes involved in cellular protection such as HSP genes (18). The presence of HSPA2 is correlated during spermatogenesis with maturity, function, and fertility (19). It also participates in the synaptonemal complex function during meiosis in male germ cells, and is linked to mechanisms that inhibit apoptosis (11).

Our group has been interested in the study of varicocele in adolescence and its effect on sperm functions. We demonstrated that adolescents with varicocele (grades II and III) have an impaired functional capacity to bind to the homologous zona pellucida under in vitro conditions in the hemizona assay (6). In the murine model, sperm quality was also shown to be altered following heat-shock by a reduction of progressive sperm motility and a significantly lower IVF rate of oocytes (20). The effect of HSPs in patients with varico-

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td><strong>Age and right and left testicular volume in sperm from adolescents with varicocele and oligozoospermia (VO), adolescents with varicocele and normal sperm concentration (VN), and adolescents without varicocele and normal sperm concentration (control).</strong></td>
</tr>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>Characteristic</td>
</tr>
<tr>
<td>Age (y)</td>
</tr>
<tr>
<td>(16.6, 19.4)</td>
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<tr>
<td>Right testicular volume (mL)</td>
</tr>
<tr>
<td>(16.9, 19.7)</td>
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<tr>
<td>Left testicular volume (mL)</td>
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<td>(15.6, 18.6)</td>
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Note: Values are means ± SD (95% confidence interval). All significance levels: P<.05.
Semen analysis and HSP70-2 gene-expression results in sperm from adolescents with varicocele and oligozoospermia (VO), adolescents with varicocele and normal sperm concentration (VN), and adolescents without varicocele and normal sperm concentration (control).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>VO (n = 15)</th>
<th>VN (n = 15)</th>
<th>Control (n = 15)</th>
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<tbody>
<tr>
<td>Concentration (10^6/mL)</td>
<td>10.9 ± 6.6a</td>
<td>112.9 ± 76.4b</td>
<td>191.4 ± 98.5b</td>
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<td></td>
<td>(7.3, 14.6)</td>
<td>(70.6, 155.3)</td>
<td>(136.9, 246.1)</td>
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<td>Motility (%)</td>
<td>63.7 ± 14.8</td>
<td>67.6 ± 11.6</td>
<td>69.6 ± 6.3</td>
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<td></td>
<td>(55.5, 71.9)</td>
<td>(61.1, 74.1)</td>
<td>(66.1, 73.2)</td>
</tr>
<tr>
<td>Morphology (%)</td>
<td>5.3 ± 2.6a</td>
<td>9.1 ± 5.4b</td>
<td>9.8 ± 4.8b</td>
</tr>
<tr>
<td></td>
<td>(3.9, 6.8)</td>
<td>(6.1, 12.2)</td>
<td>(7.2, 12.5)</td>
</tr>
<tr>
<td>HSPA2 expression (absorbance units)</td>
<td>0.70 ± 0.18a</td>
<td>2.47 ± 2.61b</td>
<td>2.06 ± 2.52b</td>
</tr>
<tr>
<td></td>
<td>(0.60, 0.81)</td>
<td>(1.03, 3.93)</td>
<td>(0.66, 3.46)</td>
</tr>
</tbody>
</table>

Note: Values are means ± SD (95% confidence interval). a, b Different letters in same row: P<.05.


Individual representative gel of semiquantitative RT-PCR analysis of the expression of HSPA2 and β-actin (housekeeping) genes in sperm pellets from one patient of each group: normozoospermic adolescents with varicocele (VN), oligozoospermic adolescents with varicocele (VO), and normozoospermic adolescents without varicocele (C). Base pairs are listed to the right of the figure.

![Individual representative gel of semiquantitative RT-PCR analysis](image)

In this study, subjects with varicocele who showed a higher expression level of HSPA2 both possessed higher sperm concentrations and presented with higher rates of morphologically normal sperm cells than did oligozoospermic subjects. It is important to note that sperm morphology did not differ between normozoospermic adolescents with varicocele and the control group (P > 0.05). Once again, up-regulation of the HSPA2 gene might have conferred the sperm cells additional protection, allowing for spermatogenic development without compromising gamete morphology, which in turn rendered the sperm cells more able to undergo acrosome reaction, and thus there was maintenance of sperm function.

Human ejaculated spermatozoa contain multiple mRNA species carried over from earlier stages in spermatogenesis. To date, gene-specific RT-PCR in situ hybridization has detected transcripts for β-actin and HSPs 70 and 90 (14). Semiquantitative RT-PCR analysis in sperm pellets allows for HSPA2 gene expression analysis. Our results provide further evidence for the concept that ejaculated spermatozoa might provide a useful noninvasive tool in molecular investigations, thus contributing to a better understanding of clinical manifestations of the male infertility phenotype (14).

In conclusion, this study demonstrated that HSPA2 gene expression was down-regulated in sperm from adolescents with varicocele and normal sperm concentrations, compared with adolescents with varicocele and normal sperm concentration. We speculate that expression levels of the HSPA2 gene may be used as a candidate molecular marker for the acquisition of thermal tolerance and heat-induced damage in spermatozoa.

REFERENCES