Seminal antibodies to human 60kd heat shock protein (HSP 60) in male partners of subfertile couples*

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BACKGROUND: Heat shock proteins (HSP) are essential mammalian and bacterial stress proteins. At the cellular level, they act as chaperones, have important regulatory functions, and are considered to be an essential factor for reproduction. Scarcely information exists on the role of sensitization to HSP and the potential role in the aetiology of male infertility. METHODS: The potential association of immunoglobulin (Ig)A antibodies (Ab) to the human 60 kDa heat shock protein (HSP 60) with several parameters of subclinical male genital tract infection/inflammation and with semen quality and sperm fertilizing capacity was analysed in a prospective study. IgA Ab to human HSP 60 were determined in seminal plasma of 202 randomly chosen male partners of subfertile couples with a median duration of infertility of 4 years (range 1–15 years), who were asymptomatic for genital tract infection. After medical history and clinical examination, a comprehensive evaluation of semen quality, in aliquots of the same ejaculates used for HSP Ab determination, included: sperm analysis; local antisperm antibody (ASA) screening; standardized sperm–cervical mucus (CM) penetration testing; immunocytochemical round cell differentiation to determine seminal leukocyte counts; evaluation of complement fraction C3 and of some pro-inflammatory cytokines; and microbial screening. Subsequent fertility was recorded after 6 months. RESULTS: The presence of HSP 60 IgA Ab in seminal fluid (total positive 6.9%) was significantly associated with leukocytospermia, the presence of C3, and also with high interleukin (IL) levels in seminal plasma. HSP 60 Ab were not related to the bacterial colonization of ejaculates. There was no association of seminal IgA Ab to human HSP 60 with semen quality, determined with microscopical semen analysis, nor with local IgG- or IgA-class ASA. There was no relationship with sperm intrinsic motility and duration of motility in the sperm CM-penetration test, nor with sperm fertilizing capacity. CONCLUSIONS: The combined presence of IgA Ab to human 60 kDa HSP, leukocytes and other established infection/inflammation markers in semen might suggest a potential role of the immune response to heat shock proteins (HSP) in cases of silent male genital tract infection, but the results do not indicate a marked relationship of HSP 60 Ab in seminal fluid with standard parameters of semen quality.

Key words: 60 kDa heat shock protein (HSP 60)/complement/leukocytes/male fertility/seminal infection markers

Introduction

Heat shock proteins (HSP) are essential mammalian and bacterial stress proteins. They help to preserve cell survival under adverse environmental conditions. During mammalian embryo development, HSP exhibit specific expression patterns. At the cellular level, HSP function as chaperones and important regulators of differentiation, cell division and apoptosis. Thus the presence of HSP is believed to be a factor essential for mammalian reproduction (Neuer et al., 2000).

In addition to these beneficial and physiological properties of HSP, several pathological conditions have been associated with the presence of HSP. Members of the 60 kDa HSP family (HSP 60) in particular have been recognized as immunodominant antigens of many microbial pathogens, including Chlamydia trachomatis. Chlamydial infection is one of the most frequent sexually transmitted diseases (STD) with enormous consequences for subsequent fertility and adverse pregnancy outcome (Paavonen and Eggert-Kruse, 1999).

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Like eukaryotic cells, microbial pathogens express a constitutive level of HSP required for the maintenance of essential housekeeping functions. During infection the microbial stress protein synthesis is enhanced and the increased amount of microbial HSP at sites of an infection can contribute to immunodominance of the microbial HSP. HSP released from infectious organisms or infected host cells can induce cytokine release and provoke an immune response (Tabona et al., 1998).

Continuous bacterial HSP expression has also been associated with the severity and persistence of infectious/inflammatory disease (LaVerda et al., 2000). Since the amino acid homology between many microbial HSP (e.g. chlamydial HSP 60) and the human 60 kD HSP is high (Morrison et al., 1992), the development of an autoimmune response to the human HSP 60 in susceptible individuals with chronic infections has been suggested (Yi et al., 1993; Domeika et al., 1998; Witkin et al., 1998). A prolonged inflammatory response leading to the induction of antiserum antibodies (ASA), and subsequent impairment of fertility also has been proposed for microbial HSP (Munoz et al., 1996; Witkin et al., 1996a,b; Jeremias et al., 1997). Previous studies have described that, in women, the detection of antichlamydial HSP IgA Ab in the cervix was correlated with adverse IVF treatment outcome (Spandorfer et al., 1999), and, in males, with the development of chronic non-gonococcal urethritis (Horner et al., 1997).

However, for clinical conditions only scarce information exists on the role of sensitization to HSP and the possible influence in the aetiology of male infertility. Thus the aim of this prospective study was to evaluate the possible significance of human HSP 60 Ab as potential indicators of stress response in asymptomatic males under infertility investigation and to correlate these findings with established markers of genital tract infection/inflammation and with clinically relevant parameters of male fertility.

**Materials and methods**

**Patients**

A total of 202 men (male partners of subfertile partnerships) were enrolled in this investigation. Couples were unselected for causes and different factors of infertility, but males with azoospermia were excluded. The median duration of infertility was 4 (range 1–15) years. The median age of the male patients was 34 (range 22–53) years. All men as well as their female partners were without symptoms of genital tract infection. During the time of the study, none of the patients was treated with antibiotics, corticosteroids or antiphlogistics.

**Basic infertility investigation and andrological examination**

A detailed medical history was obtained and physical examinations were performed on both partners. Investigation for female infertility factors (detailed hormonal analyses in the early follicular and the luteal phase, examination of tubal patency and of the uterine and cervical factor) was carried out as described previously (Eggert-Kruse et al., 1989, 1991, 1997). Ejaculates were obtained in hospital after at least 5 days of sexual abstinence, and were examined directly after liquefaction (at room temperature).

Standard semen analysis was performed according to World Health Organization (WHO) criteria (World Health Organization, 1992), and included determination of sperm count, progressive motility directly after liquefaction and additionally after 4 h, morphology, viability, pH, and volume. The seminal plasma (SP) concentrations of fructose and of α-glucosidase were determined with commercial kits (Boehringer/Roche Diagnostics, Mannheim, Germany).

All other parameters of semen or SP which were analysed in this study [apart from postcoital testing (PCT) results] were determined in aliquots of the same ejaculates.

**Detection of antibodies (Ab) to human 60 kD heat shock protein (HSP 60)**

Seminal plasma was examined for IgA Ab to human HSP 60 by means of an enzyme-linked immunosorbent assay (ELISA). Briefly, recombinant human HSP 60 (SPA 806; StressGen, Victoria, B.C., Canada) was diluted to 10 µl/ml in 0.1 mmol/l carbonate buffer, pH 9.8 and 0.1 ml was added separately to wells of a microtiter plate. After an overnight incubation at 4°C the wells were washed four times with 0.1% phosphate buffered saline (PBS)-Tween. Aliquots (0.1 ml) of seminal fluid diluted 1:4 were added to the wells and the plate floated in a 37°C water bath for 60 min. The wells were then washed and incubated with a 1:200 dilution of alkaline phosphate (AP)-conjugated goat Ab to human IgA. Following an additional 37°C 60 min incubation, the wells were washed as above and the colourless AP substrate, p-nitrophenylphosphate in 10% diethanolamine buffer was added. After 30–60 min incubation at room temperature the appearance of a yellow colour in the wells was quantitated at 405 nm. Known positive and negative samples were assayed in parallel to the test specimens. Inter-and intra-assay variation was <10%. A positive sample was defined as one yielding an optical density (OD) value that is at least two standard deviations (SD) above the mean value obtained with a panel of samples from healthy subjects of proven fertility and without history of genital tract infections.

All seminal plasma samples were processed blindly and without knowledge of the clinical diagnosis of the patients, the variables of semen quality and the outcome of infection screening. To examine the relationship with other variables, the outcome of ELISA reading after several incubation periods was taken into consideration, e.g. after 33 min, 53 min or longer intervals. Values of OD after 33 min ranged from 0.062–0.91 with a of median 0.16. Samples with an OD >0.4 after this time were defined as ‘HSP Ab positive’ and were compared with HSP Ab negative semen specimens. ELISA results after different incubation intervals correlated significantly (r >0.9; P < 0.0001). None of the samples that tested negative after 33 min was positive after later time intervals.

In additional statistical analyses (concerning all variables examined in this study), several other cut-offs were used (e.g. OD >0.2 or >0.6) to compare ejaculates exhibiting ‘high’ HSP IgA Ab levels with samples with ‘low’ Ab concentration, but this did not give any additional relevant information.

**Potential parameters of subclinical genital tract infection/inflammation**

**Leukocytes**

Round cells in semen were differentiated in leukocytes and cells of the germ cell line using an immunocytochemical method with monoclonal Ab and a streptavidin-biotin system which has been described previously (Eggert-Kruse et al., 1992a). The mean of triplicate counting was used for analysis. Human peripheral leukocytes, obtained from a healthy donor and separated with a Ficoll-technique, were used as positive controls. Positive and negative [phosphate-buffered saline (PBS) pH 7.4] controls were included in all of the test series.
Samples with $\geq 1 \times 10^6$/ml leukocytes were considered as leukocytospermic, according to the WHO definition (World Health Organization, 1992).

**Cytokine determination**

Commercial kits were used for detection of interleukin (IL)-6 and IL-8 in SP (Quantikine® for IL-8 and Quantikine HS® for IL-6; R&D Systems, Minneapolis, MN, USA). Briefly, these solid phase ELISA with a quantitative sandwich technique use microtitre plates which are pre-coated with mAb specific for IL-6 or -8. After reaction, the resulting colour reaction is proportional to the amount of IL bound and is measured at 450 nm, concentrations are read on a standard curve. Testing was performed according to the manufacturer’s instructions. Negative and positive controls were included in each of the test series. All tests were done in duplicate, the mean was taken for analysis. Intra-and inter-assay variations were <10%. Cut-offs used for analyses were $\geq 30$ pg/ml to define high, and $\geq 100$ pg/ml to define very high SP concentrations of IL-6. With regard to IL-8, thresholds were set at $\geq 1.5$ ng/ml, $\geq 2$ ng/ml, and at $\geq 3$ ng/ml to define high, and $\geq 5$ ng/ml to define very high IL-8 levels in seminal fluid, respectively.

**Complement fraction C3**

The complement fraction C3 was determined using radial immunodiffusion (Nor-Patigen C3, Behring, Marburg, Germany). Briefly, SP was inoculated into ready-for-use agarose-gel layers containing monospecific antisum against human C3. After incubation, the diameter of precipitation was measured and the concentration was determined using a standard curve. Negative and positive controls were included in each of the test series. When C3 was detectable, the sample was defined as C3 positive (detection level 0.0085 g/l). A further threshold at $\geq 0.1$ g/l was used to define high C3 concentration in seminal fluid. If necessary, SP samples were stored frozen at $-70^\circ$C until use.

**Microbial examination**

To screen semen samples for colonizing micro-organisms, swabs were obtained and inoculated into a universal transport medium (Port-a-Cul Universal®, Becton Dickinson, Heidelberg, Germany), additionally, 10 µl of semen were transferred with a disposable sterile plastic loop into Shepard’s medium for detection of mycoplasmas. Microbial prevalence was identified with standard methods (Institute of Microbiology, University of Heidelberg) as previously described (Eggert-Kruse et al., 1992b). A standard amplification assay (ligase chain reaction, LCR) (LCX®; Abbott, Wiesbaden, Germany) was used to detect *Chlamydia trachomatis* in semen, and in parallel in first void urine specimens. Testing was performed according to the manufacturer’s instructions.

In semen samples obtained the same day, anti-chlamydial Ab of the IgA and IgG-class (Chlam Ab) were determined by means of a commercial chlamydial lipopolysaccharid (LPS-) directed, recombinant enzyme linked immunosorbsent assay (rELISA®, Medac, Hamburg, Germany). This test was also used to examine Chlam Ab of the IgA class in seminal fluid, and was performed according to the manufacturer’s instructions.

Furthermore, chlamydial serology was performed on a routine basis to determine Chlam IgG Ab in same-day serum samples using an immunofluorescence (IF) assay (Virgo® *Chlamydia trachomatis* IgG IF test; Spiapparelli Biosystems Inc. Columbia, IL, USA). Testing included positive and negative controls, a serum titre of $\geq 1:256$ was considered strong positive (Eggert-Kruse et al., 1997).

**Evaluation of sperm functional capacity**

*Sperm–cervical mucus penetration test (SCMPT)*

As a parameter of sperm function, the ability to penetrate the cervical mucus (CM) barrier was evaluated by means of the standardized in-vitro sperm–CM penetration test (SCMPT), which was performed as described previously (Eggert-Kruse et al., 1989). Briefly, the penetration of sperm within capillaries, filled with fresh samples of CM, obtained from patients’ female partners under hormonally controlled conditions, was observed microscopically after 30 min, 2 h, and 6 h incubation. Penetration density, migration distance, quality and duration of motility were examined, graded and summarized in a cumulative score, which served to select two groups with inadequate (score $<6$ after 6 h) and adequate sperm penetration ability.

**Postcoital testing (PCT)**

Sperm–CM interaction *in vivo* was assessed with a PCT 8 to 12 h after intercourse (within 1 month related to HSP Ab determination in semen). To control the endocrine influence on the CM properties, only results of PCT obtained after pretreatment with estrogens (for at least 7 days) were taken into consideration. The number of sperm with forward progression in CM was counted using first the low power field (LPF;$\times 100$) and then the high power field (HPF, magnification $\times 400$), the mean of 20 visual fields was taken. PCT was regarded as reduced when $<2$ sperm of highly progressive motility/HPF were counted in CM. For further evaluation, couples with excellent ($\geq 7$ motile sperm/HPF) PCT outcome, and also those with negative PCT, were compared with the other patients.

**Determination of antisperm antibodies (ASA)**

For screening of antisperm Ab in semen samples the direct mixed antiglobulin reaction (MAR) was used. MAR was performed in parallel with IgG and IgA coated erythrocytes and specific antiserum based on the method of Jager et al. (Jager et al., 1978, Eggert-Kruse et al., 1991). Reading was done in triplicate, the mean was taken. A percentage of $\geq 30$% of motile sperm involved in the mixed agglutinates was considered MAR positive, further analyses were performed with MAR $\geq 10$%, and MAR $\geq 60$% as additional cut-offs.

**Subsequent fertility**

Subsequent pregnancy rate (in-vivo conditions of conception in the majority of couples) was recorded 6 months after HSP IgA Ab testing in semen, and was controlled for major parameters of female infertility such as tubal, uterine, and endocrine factors.

**Statistical analysis**

Data were processed using the statistical analysis system (SAS). Spearman-rank correlation, $\chi^2$ analysis, or Fisher’s two-tailed exact test were used. The level of significance was set at $P < 0.05$.

**Results**

**Relationship with general parameters and results of andrological examination**

Seminal plasma samples of 6.9% (14/202) patients were positive for IgA Ab to human HSP 60. The presence of HSP Ab in seminal fluid was not significantly related to patients’ medical history, for example previous genital infection (6.1% of patients), genital trauma, genital surgery such as varicocelectomy (total 9.4% of patients), or a history of maldescensus tests. There was no association of HSP Ab with smoking (36.6% of patients) and drinking habits, and general stress factors. Known allergies (as indicated by the patients on a questionnaire, total 25.7%) were also not related to these Ab.

No significant association of seminal HSP 60 IgA Ab with the outcome of clinical andrological examination was found,
with regard to testicular volume as well as to testicular consistency, or varicocele. None of the HSP Ab positive men had a testicular volume (on either side) of <15 ml. There was no significant correlation of seminal HSP Ab with the body mass index and with the duration of infertility (≥5 years in 32.0%, ≥10 years in 8.1%). Males with HSP Ab in semen were slightly older than the other patients, but differences did not achieve significance (42.9% of HSP positive men were ≥40 years of age, compared with 19.1% of the other men). None of the patients with seminal HSP Ab was <30 years.

**Relationship with semen quality**

Several statistical analyses were performed to evaluate a potential association of HSP 60 IgA Ab in seminal plasma with standard variables of semen quality. As indicated above, semen analysis based on WHO criteria and HSP 60 Ab determination in seminal plasma were performed in the same ejaculates. Overall, no marked association of established parameters of semen quality and the presence of HSP 60 Ab was found. Oligozoospermia (<20×10⁶/ml) was seen in 13.4% of all patients. The prevalence of IgA Ab to HSP 60 in seminal fluid of these men was 7.4% (2/27) compared with 6.9% (12/175) in specimens with higher sperm counts [not significant (NS)]. There were also no significant differences when semen samples with less or more than 40×10⁶ sperm/ml (or with less or more than 60×10⁶ sperm/ml) were compared. Severe asthenozoospermia (<20% progressive motility directly after liquefaction) was diagnosed in 5.2% of the patients. In samples of these men, HSP 60 Ab were detected with slightly higher frequency (20.0%; 2/10) than in seminal plasma of specimens with better sperm motility (HSP 60 Ab in 6.5%; 12/184), but the difference did not reach significance. The prevalence of HSP 60 Ab in samples with <40% progressively motile sperm was 9.1% compared with 6.0% in ejaculates with better motility (NS). There was also no significant association of sperm motility with HSP 60 Ab in seminal fluid, when other thresholds were used, or when motility after 4 h (related to liquefaction) was taken into account. Standard morphology determinants (% normal forms, as well as differentiation in sperm head-, neck-, or tail anomalies) were also not significantly related to the presence of HSP 60 Ab, nor was there an association with sperm viability. In case of high ejaculate volume (≥5 ml), these Ab were slightly less frequent (3.4%) than in the other semen samples (7.8%, NS) and was also not significant when volumes of >4.5, or <1.5 ml were used as thresholds. HSP IgA Ab were more frequent in samples with increased semen pH and were found in 11.8% of specimens with a pH of >7.2 compared with 2.8% in ejaculates with lower pH (P < 0.02), and these differences were more pronounced when a seminal pH value of 7.5 was taken as cut-off. No correlation was found with the seminal fluid concentration of fructose, or of the α-glucosidase as epididymal marker.

The outcome of semen analysis and the comparison of the different variables of semen quality in HSP Ab negative and positive semen samples are presented in Table I.

**Relationship with potential markers of subclinical male genital tract infection/inflammation**

This study demonstrates a significant relationship of HSP 60 IgA Ab with some indicators of subclinical infection or inflammation of the male genital tract. The results are shown in Table II. Although leukocytospermia (>1×10⁹/ml immunocytochemically determined leukocytes) was relatively rare in this asymptomatic population (total 6.3%), significantly more leukocytospermic samples (33.3%) were positive for HSP 60 IgA Ab than the other specimens (4.2%) (P < 0.02).

Furthermore, HSP 60 IgA Ab levels correlated closely with the seminal plasma levels of the pro-inflammatory cytokines IL-6 and IL-8. The correlation coefficient (r) was 0.24 for IL-6 (P < 0.01), and r was 0.26 with regard to IL-8 (P < 0.005). A high concentration of IL-6 (≥30 pg/ml) was detected in 66.7% of the HSP 60 Ab positive samples; this was significantly more frequent than in the other specimens (P < 0.02). A very high concentration of IL-6 (>100 pg/ml) was found in 44.4 compared with 9.4%, respectively. Increased levels of IL-8 in seminal fluid were also much more frequent in HSP 60 positive compared with HSP 60 negative semen samples [high concentrations of IL-8 (≥3 ng/ml) in 55.6% compared with 12.8%, P < 0.001]. Very high concentrations of IL-8 (>5 ng/ml) were determined in 33.3% compared with 3.4% (P < 0.001), respectively. Significant differences were also found when other cut-offs were used (e.g. <1.5 ng/ml for IL-8).

HSP 60 IgA Ab were also significantly associated with the presence of the complement fraction C₃ in seminal fluid of the same ejaculates (see Table II).

**Relationship with microbial findings**

Microbial colonization of ejaculates in these asymptomatic patients was a frequent finding. However, there was no significant relationship of mycoplasmas (Mycoplasma hominis and/or Ureaplasma urealyticum) or other potentially pathogenic bacteria detected in semen cultures with the presence of IgA Ab to the human HSP 60, as can be seen in Table III.

The prevalence of Chlamydia trachomatis, using parallel screening with LCR in both patients’ semen and urine was low (1%). Both patients with direct evidence of genital Chlamydia trachomatis infection tested negative for HSP 60 IgA Ab in their seminal fluid.

With regard to chlamydial serology, IgA Ab to HSP 60 were more frequent in ejaculates of men who also had anti-chlamydial IgA Ab, detected with a LPS directed assay, in seminal fluid (P < 0.001). There was no significant association of seminal HSP 60 Ab with anti-chlamydial IgA or IgG Ab in serum (Table III).

**Relationship with sperm function**

The presence of HSP 60 IgA Ab did not interfere with sperm function as evaluated with the in-vitro SCMPT, using freshly obtained and hormonally standardized CM of patients’ female partners (see Table IV). This could also be demonstrated when CM of female donors was used for the crossed SCMPT, which was performed in parallel, and was confirmed when other thresholds were used to define poor SCMPT results.
Table I. Relationship of HSP 60 Ab in seminal plasma and semen quality

<table>
<thead>
<tr>
<th>Variable of semen analysis</th>
<th>HSP 60 Ab in seminal plasma</th>
<th>Total % (n/n)</th>
<th>( p^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of samples per group (n/n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
<td></td>
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<tr>
<td>Sperm count</td>
<td></td>
<td></td>
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<tr>
<td>Oligozoosperm (&lt;20 × 10^6)/ml</td>
<td>13.3 (25/188)</td>
<td>14.3 (2/14)</td>
<td>13.4 (27/202)</td>
</tr>
<tr>
<td>Reduced (&lt;40 × 10^6)/ml</td>
<td>39.9 (75/188)</td>
<td>21.4 (3/14)</td>
<td>38.6 (78/202)</td>
</tr>
<tr>
<td>Progressive motility</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe asthenozoospermia (&lt;20%)</td>
<td>4.4 (8/180)</td>
<td>14.3 (2/14)</td>
<td>5.2 (10/194)</td>
</tr>
<tr>
<td>Asthenozoospermia (&lt;40%)</td>
<td>38.9 (70/180)</td>
<td>50.0 (7/14)</td>
<td>39.7 (77/194)</td>
</tr>
<tr>
<td>Morphology</td>
<td></td>
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<tr>
<td>Reduced (&lt;60% normal forms)</td>
<td>57.1 (101/177)</td>
<td>42.9 (6/14)</td>
<td>56.0 (107/191)</td>
</tr>
<tr>
<td>Semen pH</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Elevated (&gt;7.5)</td>
<td>2.1 (4/188)</td>
<td>28.6 (4/14)</td>
<td>4.0 (8/202)</td>
</tr>
<tr>
<td>Ejaculate volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased (&gt;5.0 ml)</td>
<td>30.3 (57/188)</td>
<td>15.4 (2/13)</td>
<td>29.4 (59/201)</td>
</tr>
<tr>
<td>Seminal plasma fructose conc. reduced (&lt;1800 µg/l)</td>
<td>37.1 (26/70)</td>
<td>33.3 (1/3)</td>
<td>37.0 (27/73)</td>
</tr>
<tr>
<td>α-glucosidase concentration reduced (&lt;10 mIU/ml)</td>
<td>20.9 (14/67)</td>
<td>25.0 (1/4)</td>
<td>21.1 (15/71)</td>
</tr>
</tbody>
</table>

\( ^d \) Determined in aliquots of the same ejaculates, semen quality variables in HSP 60 Ab negative and in HSP 60 Ab positive samples shown.

\( ^b \) \( \chi^2 \) analysis/Fisher’s two-tailed exact test compared with corresponding other group above (or below) the threshold indicated for each variable.

NS = not significant.

Table II. Relationship of HSP 60 Ab in seminal plasma and parameters of subclinical infection/inflammation of the male genital tract

<table>
<thead>
<tr>
<th>Variable of subclinical infection/inflammation</th>
<th>HSP 60 Ab in seminal plasma</th>
<th>Total % (n/n)</th>
<th>( p^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of samples per group (n/n)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
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<tr>
<td>Leukocytes in semen</td>
<td></td>
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<tr>
<td>Leukocytospermia (≥1 × 10^6)/ml</td>
<td>4.2 (5/118)</td>
<td>33.3 (3/9)</td>
<td>6.3 (8/127)</td>
</tr>
<tr>
<td>Pro-inflammatory cytokines in seminal plasma</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Interleukin (IL)-6</td>
<td></td>
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<tr>
<td>≥30 pg/ml</td>
<td>26.5 (31/117)</td>
<td>66.7 (6/9)</td>
<td>29.4 (37/126)</td>
</tr>
<tr>
<td>≥100 pg/ml</td>
<td>9.4 (11/117)</td>
<td>44.4 (4/9)</td>
<td>11.9 (15/126)</td>
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<tr>
<td>Interleukin (IL)-8</td>
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<tr>
<td>≥3 ng/ml</td>
<td>12.8 (15/117)</td>
<td>55.6 (5/9)</td>
<td>15.9 (20/126)</td>
</tr>
<tr>
<td>≥5 ng/ml</td>
<td>3.4 (4/117)</td>
<td>33.3 (3/9)</td>
<td>5.6 (7/126)</td>
</tr>
<tr>
<td>Seminal plasma complement complement fraction C3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>37.3 (38/102)</td>
<td>77.8 (7/9)</td>
<td>40.5 (45/111)</td>
</tr>
<tr>
<td>High (≥0.01 g/l)</td>
<td>24.5 (25/102)</td>
<td>55.6 (5/9)</td>
<td>27.0 (30/111)</td>
</tr>
</tbody>
</table>

\( ^d \) Determined in aliquots of the same ejaculates, potential parameters of inflammation in HSP 60 Ab negative and in HSP 60 Ab positive samples shown.

\( ^b \) \( \chi^2 \) analysis/Fisher’s two-tailed exact test compared with corresponding other group below the threshold indicated for each variable.

NS = not significant.

Furthermore, no significant influence of HSP 60 IgA Ab on sperm ability to penetrate CM in vivo, as determined with the PCT, was found.

**Relationship with ASA**

Using the direct MAR to screen for local antisperm Ab, 9.3% of semen samples were positive for ASA of the IgG class (MAR ≥30%), and 8.2% for IgA class ASA, respectively. However, the presence of HSP IgA Ab in seminal fluid did not differ significantly between patients with or without seminal antisperm Ab, as can be seen in Table V. There was also no significant relationship of HSP Ab with local ASA, when other thresholds were used, and samples with a MAR ≥60% for IgG and/or IgA ASA were compared with the other specimens.

**Relationship with subsequent fertility**

No significant influence of human HSP 60 IgA Ab on subsequent fertility was noted. Within an observation period of 6 months, pregnancy was achieved by 18.0% of the couples who could be followed (93.6% of the total population): 21.4% (3/14) pregnancies in the HSP 60 Ab positive group, and 17.7% (31/175) in the HSP 60 Ab negative group (NS). In couples with an HSP 60 positive male partner, there were two spontaneous pregnancies, and one pregnancy was achieved.
Table III. Relationship of HSP 60 Ab in seminal plasma and microbial findings

<table>
<thead>
<tr>
<th>Microbial colonization of semen samples</th>
<th>HSP 60 Ab in seminal plasma % of patients per group (n/n)</th>
<th>Total % (n/n)</th>
<th>pb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>mycoplasmasc</td>
<td>12.4 (23/186)</td>
<td>14.3 (2/14)</td>
<td>12.5 (25/200)</td>
</tr>
<tr>
<td>aerobes</td>
<td>41.0 (77/188)</td>
<td>64.3 (9/14)</td>
<td>42.6 (86/202)</td>
</tr>
<tr>
<td>pot. path. aerobes</td>
<td>18.1 (34/188)</td>
<td>35.7 (5/14)</td>
<td>19.3 (39/202)</td>
</tr>
<tr>
<td>anaerobes</td>
<td>9.0 (17/188)</td>
<td>14.3 (2/14)</td>
<td>9.4 (19/202)</td>
</tr>
<tr>
<td>pot. path. species</td>
<td>29.6 (55/188)</td>
<td>42.9 (6/14)</td>
<td>30.5 (61/200)</td>
</tr>
<tr>
<td>Serological findings: Anti-chlamydial antibodies (Chlam Ab)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlam IgA Abd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in seminal plasma positive</td>
<td>12.6 (14/111)</td>
<td>55.6 (5/9)</td>
<td>15.8 (19/120)</td>
</tr>
<tr>
<td>in serum positive</td>
<td>17.7 (21/119)</td>
<td>44.4 (4/9)</td>
<td>19.5 (25/128)</td>
</tr>
<tr>
<td>Chlam IgG Ab I in serum positive</td>
<td>39.5 (47/119)</td>
<td>55.6 (5/9)</td>
<td>40.6 (52/128)</td>
</tr>
<tr>
<td>Chlam IgG II in serum positive</td>
<td>27.8 (52/187)</td>
<td>21.4 (3/14)</td>
<td>27.4 (55/201)</td>
</tr>
<tr>
<td>strong positive (&gt;1:256)</td>
<td>8.0 (15/187)</td>
<td>7.1 (1/14)</td>
<td>8.0 (16/201)</td>
</tr>
</tbody>
</table>

*aDetermined in aliquots of the same ejaculates, microbial and serological findings in patients with HSP 60 Ab negative and in HSP 60 Ab positive samples shown.
*by2 analysis/Fisher’s two-tailed exact test compared with corresponding other group without these micro-organisms in semen or with negative results of serologic testing.
*cMycoplasma hominis and/or Ureaplasma urealyticum.
*dChlam IgG Ab I determined with a recombinant enzyme immunoassay (rELISA®) in same-day serum.
*eChlam IgG Ab II determined with immunofluorescence testing (IF-test), same-day serum titres of >1: 256 considered strong positive.
NS = not significant.

Table IV. Relationship of HSP 60 Ab in seminal plasma with sperm functional capacity

<table>
<thead>
<tr>
<th>HSP 60 Ab in seminal plasma % of patients per group(n/n)</th>
<th>Total % (n/n)</th>
<th>Pab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>Results of sperm-cervical mucus (CM) penetration test in vitrob</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCMTPT with partner’s CM inadaequatec</td>
<td>36.8 (68/185)</td>
<td>28.6 (4/14)</td>
</tr>
<tr>
<td>crossed SCMTPT with donors’ CM inadaequatec</td>
<td>35.1 (65/185)</td>
<td>21.4 (3/14)</td>
</tr>
<tr>
<td>Outcome of PCTd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCT negative</td>
<td>35.4 (63/178)</td>
<td>14.3 (2/14)</td>
</tr>
</tbody>
</table>

*bχ2 analysis/Fisher’s two-tailed exact test compared with corresponding other groups.
*cSCMTPT operated under hormonally standardized conditions with semen aliquots of the same ejaculates.
*dResults of the cumulative penetration index after 6 h in semen samples with HSP 60 Ab negative and positive seminal plasma shown.
*eOnly results of postcoital testing evaluated after estrogen treatment of female partners taken for analysis.
SCMTPT = sperm-cervical mucus penetration test; PCT = post-coital test; NS = not significant.

Discussion
The HSP 60 is produced in response to various stress such as temperature elevation (‘heat shock’), ischaemia, toxic chemicals, metabolic disruption, free oxygen radicals, bacterial and viral infection and inflammation mediators (Young, 1990; Welch, 1992). HSP are remarkable in their evolutionary conservation. Each of the major stress proteins interacts with many different proteins, among them steroid hormone receptors and immunoglobulin heavy chains. The role of HSPs in reproduction is reviewed elsewhere (Neuer et al., 1999).

The relationship of Ab to HSP in women with tubal factor infertility, ectopic pregnancy and reduced IVF success (Claman after IVF treatment. In couples without evidence of HSP 60 Ab in the male’s seminal fluid, 24 pregnancies were achieved under in vivo conditions, six after intra-uterine insemination (IUI) with the partner’s semen, and one pregnancy after IVF treatment. Further statistical analyses were performed after exclusion of couples who were treated with any method of assisted reproduction (in-vivo pregnancy group only), and in several subgroups, e.g. in couples without a tubal or uterine or severe endocrine disorders of patients’ partners, or with combined female infertility factors. No significant relationship of seminal HSP 60 Ab with subsequent fertility was found in any subgroup.
et al., 1996, 1997; Sziller et al., 1998; Spandorfer et al., 1999), reported previously, suggests clinical relevance of these stress proteins for female fertility. These chaperones may be involved in intra-sperm protein transport. However, previous investigations provided only scarce information about the significance of HSPs for semen quality and male fertility (Miller et al., 1992; Dix, 1997; Werner et al., 1997).

The present prospective study indicates that expression of IgA Ab in seminal plasma to the human HSP 60 is closely associated with subclinical male genital tract infection/inflammation as diagnosed with clinically established markers, for example leukocytes and some pro-inflammatory cytokines.

All males were without symptoms of genital tract infection and were not selected because of semen quality variables or immunological factors. The prevalence of HSP 60 Ab was comparable with the findings of others in serum of male patients (Withkin et al., 1998) and slightly lower than a rate of 14.1% in seminal fluid samples of men with normal count and motility (Munoz et al., 1996). In this study, the assays for HSP 60 Ab determination were performed in aliquots from the same ejaculates that were also used to examine other parameters such as standard sperm analysis, ASA, sperm–CM penetration testing, and infection screening. This allows adequate comparison.

Testing for semen quality was performed under standardized conditions; e.g. all ejaculates were obtained in the hospital, in the early morning, and after a period of sexual abstinence of at least 5 days. None of the men received medication, such as antibiotics or corticosteroids, which might interfere with the results.

The clinical relevance of subclinical infection or inflammation of the male genital tract is under considerable debate (Comhaire et al., 1980; Eggert-Kruse et al., 1992a, 1996a; Aitken and Baker, 1995; Kurpisz and Alexander, 1995; Munoz and Withkin, 1995; Withkin et al., 1996a,b). Impairment of semen quality caused by accessory gland infection or inflammation involves many different mechanisms (Comhaire et al., 1999). In asymptomatic patients, silent infection can be diagnosed by laboratory parameters only.

Leukocytes are considered as an established infection and inflammation marker although the role of seminal leukocytes for male fertility has been the subject of considerable controversy during recent years (Barratt et al., 1990; Tomlinson et al., 1993; Aitken and Baker, 1995). The use of mAb in an immunocytological approach allows clear differentiation of round cells to determine leukocyte counts. A number of certain subpopulations and by localization of genital tract inflammation.

Furthermore, a significant association of HSP 60 Ab with increased levels of pro-inflammatory interleukins was found here. Cytokines play a pivotal role as mediators of numerous physiological and pathological processes, particularly in the initiation of the immuno-inflammatory cascade (Rees, 1992; Rutanen, 1993; Bookfor et al., 1994; Henderson et al., 1996). An adverse effect on sperm motility and on membrane properties has been suggested, potentially mediated by increased oxidative stress (Buch et al., 1994; Rajasekaran et al., 1995). Cytokine elevation may represent part of a non-specific acute-phase response or may be due to specific interactions of micro-organisms (or other stimuli) and the immune system. Recently, IL-8 has been proposed to correlate with the activity of certain virus infections and to be involved in virus dissemination (Craig et al., 1997; Grundy et al., 1998). There was a wide inter-individual range for IL levels.

### Table V. Relationship of HSP 60 Ab in seminal plasma and and local antisperm antibodies (ASA)\(^3\)

<table>
<thead>
<tr>
<th>HSP 60 Ab in seminal plasma</th>
<th>Total % (n/n)</th>
<th>(p^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of samples per group (n/n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local ASA in semen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAR(^c) IgG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slightly elevated ((&gt;10%))</td>
<td>11.2% (20/179)</td>
<td>14.3% (2/14)</td>
</tr>
<tr>
<td>Positive ((&gt;30%))</td>
<td>8.9% (16/179)</td>
<td>14.3% (2/14)</td>
</tr>
<tr>
<td>MAR(^c) IgA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive ((&gt;30%))</td>
<td>7.6% (13/170)</td>
<td>14.3% (2/14)</td>
</tr>
</tbody>
</table>

\(^3\) Determined in aliquots of the same ejaculates, frequency of local ASA in HSP 60 Ab negative and in HSP 60 Ab positive samples shown.

\(^b\) Fisher’s two-tailed exact test compared with corresponding other group below the threshold indicated.

\(^c\) MAR = mixed antiglobulin reaction, performed with coated erythrocytes.

NS = not significant.
in this study. Interleukin 8 concentration in seminal fluid was much higher than that of IL-6, and both IL-8 and IL-6 were closely related to IgA Ab to the human HSP 60. However, immunoresponse to HSP 60 did not necessarily indicate increased IL concentrations in the same ejaculates, which might indicate stimulation of these systems by different mechanisms and/or a different time course of events.

The relationship of HSP 60 Ab with established inflammatory parameters was confirmed in this study, when other biochemical assays, such as the C3 determination with radial immunodiffusion, were used. Factors of the C3 complement component have opsonic power, and are involved in the system of humoral immunity. C3 measurement is considered as a sensitive parameter for identification of inflammatory processes in the prostate and adnexae (Blenk and Hofstetter, 1991). On the other hand, the presence of IgA Ab to the human HSP 60 was not related to the microbial colonization of the semen samples. In the clinical setting, there is much controversy about the consequences of positive semen cultures in asymptomatic subfertile males. This surely depends on the pathogenicity of the species identified in semen (or urethral swabs). For example, *Chlamydia trachomatis* is very important for subsequent fertility: sexual transmission would have severe sequelae e.g. for tubal function in patients’ female partners (Paavonen and Eggert-Kruse, 1999). These micro-organisms were a rare finding in this study despite the sensitive amplification methods used for detection. The association of chlamydial prevalence with population characteristics is well known; patients of this study surely represented a low-risk population (e.g. with regard to age, social class, promiscuity and presence of other STDs). Findings are in accordance with low prevalences in the endocervix of large groups of asymptomatic subfertile women (Eggert-Kruse et al., 1997, 2001). Much higher positive rates are seen in symptomatic male and female patients attending genito-urinary medicine clinics (Horner et al., 1997).

The role of *Chlamydia trachomatis* with regard to male factor infertility is under debate. Chlamydial infection, as a main cause of urethritis and epididymiditis, could potentially exert a considerable influence on male reproductive function by different mechanisms (Witkin et al., 1995; Eggert-Kruse et al., 1996a; Paavonen and Eggert-Kruse, 1999). Intracellular bacteria may induce a stress response in the host cells. A significant relationship of IgA Ab to human HSP 60 with anti-chlamydial IgA Ab in semen samples found here confirms previous reports (Munoz et al., 1996). However, this correlation was restricted to seminal fluid; there was no relationship with anti-chlamydial Ab (of the IgA- as well of the IgG-class) in patients’ same-day serum samples. Findings also contrast with the lack of association with LCR proven *Chlamydia trachomatis* infection. The patients with direct evidence of chlamydial prevalence were negative for HSP 60 Ab in their seminal fluid. It must be kept in mind that chlamydial serology with the assays currently available for routine use only identifies Chlam Ab to the genus level. Therefore the role of genital *Chlamydia trachomatis* infection for induction of an immunoresponse to the human HSP 60 in asymptomatic males has to be confirmed with more specific methods in future investigations before definite conclusions can be drawn.

With regard to other bacteria, which are frequently cultured in ejaculates (e.g. *Escherichia coli*, enterococci, streptococci, Proteus spp.) there was no association with expression of HSP 60 Ab. Potentially pathogenic bacteria were found in a considerable number of ejaculates; low leukocyte counts suggest colonization rather than infection. However, the prevalence of potentially pathogenic anaerobes, including facultative micro-organisms, was relatively low and the considerable influence of transport and culture conditions on these bacteria (Yourassowsky, 1980; Eggert-Kruse et al., 1995) has to be considered. A relationship of HSP 60 Ab with genital *Ureaplasma urealyticum*, as proposed by other authors who used amplification methods instead of culture (Witkin et al., 1998), could not be confirmed.

Colonization of semen samples with potentially pathogenic bacteria is a completely different entity to male genital tract infection. The lack of association of HSP 60 Ab with the seminal micro-organisms found here corresponds with previous studies which did not show a relationship of semen cultures with WBC in ejaculates of asymptomatic men (Eggert-Kruse et al., 1992a). However, results may be different in patients with symptoms of inflammation. Furthermore, bacterial infection is not the only factor that might lead to induction of stress proteins. Environmental toxins, nutritional factors and oxygen radicals may exert a considerable influence. Stress proteins may be stimulated by virus infection *in vitro* (e.g. herpesviruses or adenoviruses), and some viruses directly induce expression of stress protein genes (Young, 1990). It is not clear whether increased levels of HSP facilitate viral proliferation. In most studies viruses are not considered in semen samples, and more research along this line is needed.

Immune responses against stress proteins can be highly cross-reactive and can even involve anti-self reactivity. Therefore the induction of anti-sperm Ab following prolonged expression of HSP 60 might be possible. Sperm Ab in semen, particularly those of the IgA class, are important as they may exert a strong influence on sperm functional quality, e.g. the ability to penetrate CM, and may considerably impair subsequent fertility (Eggert-Kruse et al., 1991; Kremer and Jager 1992; Andreou et al., 1995). Some studies indicate that inflammations such as urethritis, prostatitis, or epididymitis may facilitate the formation of ASA and that molecular mimicry between bacteria and sperm might be a major factor inducing antisperm immunological reactions (Kurpisz and Alexander, 1995). A considerably higher prevalence of ASA, identified with the immunobead test, in males with anti-chlamydial IgA Ab has been reported (Witkin et al., 1995), and it has been suggested that Ab against conserved epitopes on chlamydial HSP 60 may cross-react with those of human HSP 60 and initiate an autoimmune response. The MAR test is a reliable method for detection of seminal ASA and was used to identify sperm directed Ab of the IgG- and IgA-class, in the same ejaculates which were also used for HSP 60 Ab screening. No significant relationship of ASA with an immunoresponse to the human HSP 60 was found.

Patients investigated here were without symptoms of genito-
urinary tract infection, the findings do not exclude a potential induction of HSP Ab formation in men with acute inflammation of the accessory sexual glands. Results are also limited for the one point determination of these HSP directed Ab. Future investigations will have to show the intra-individual variations over a longer time period, and a potential stimulation of human HSP 60 and associated Ab after proven infection of the male genital tract organs.

It is a matter for discussion whether the presence of potential seminal infection/inflammation markers in asymptomatic subfertile individuals is associated with reduced semen quality (Barratt et al., 1990; Tomlinson et al., 1993; Munoz and Witkin, 1995; Rajasekaran et al., 1995; Yanupsolsky et al., 1996). The present investigation did not indicate a close relationship of seminal HSP 60 Ab with semen characteristics with regard to standard parameters such as count, motility or morphology. It has to be considered, however, that severe male factor cases were relatively rare, and that the impact on semen quality might be more pronounced in a group of males selected on the basis of andrological disorders.

On the other hand, it could be demonstrated that the presence of IgA Ab to human HSP 60 in semen also did not impair aspects of sperm function, which are important under in vivo conditions of conception. The standardized in-vitro SCMPT which was used here, has shown to be a reliable indicator of sperm fertilizing capacity after natural intercourse (Eggert-Kruse et al., 1989). The lack of influence of HSP 60 Ab on sperm migration ability was found when CM of patients’ partners, as well as CM of donors in a parallel test setting, was used. It cannot be excluded that the immune response against HSP 60 as an indicator of silent infection might impair other parameters of sperm function, such as zona-binding or acrosome reaction which are not examined in these global assays. However, there was also no association of HSP 60 Ab in semen with couples’ subsequent fertility. Future studies will have to show if the presence of these Ab interferes with fertilization rates under in-vitro conditions. The long-term effects, and the influence of HSP 60 Ab on pregnancy outcome, also remain to be determined.

In summary, the significant association of HSP 60 IgA Ab in seminal fluid with leukocytospermia and other inflammation markers may reflect the role of HSP as acute stress related proteins and deserves further attention. Future efforts are necessary to analyse factors which influence HSP 60 expression and the HSP directed immunoresponsere under physiological and pathological conditions to improve our understanding of male genital tract infection and the clinical consequences of silent inflammation for reproductive function.

References


Seminal antibodies to HSP 60 in subfertile men


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