Induction of messenger RNA for the 70 kDa heat shock protein in HeLa cells and the human endocervix following exposure to semen: implications for antisperm antibody production and susceptibility to sexually transmitted infections

J. Jeremias¹, S. S. David², M. Toth¹ and S. S. Witkin¹,³

¹Division of Immunology and Infectious Diseases, Department of Obstetrics and Gynecology, Cornell University Medical College and ²Department of Obstetrics and Gynecology, Mount Sinai Medical Center, New York, USA
³To whom correspondence should be addressed at: Department of Obstetrics and Gynecology, Cornell University Medical College, 515 East 71st Street, New York, NY 10021, USA

The 70 kDa heat shock protein (HSP70) is induced in cells exposed to chemical or physical stress. HSP70 facilitates cell survival by preventing protein denaturation and incorrect assembly of polypeptides. Induction of HSP70 messenger RNA (mRNA) synthesis also inhibits transcription of genes coding for pro-inflammatory cytokines. We analysed whether HSP70 mRNA was expressed in a cultured human cervical cell line (HeLa cells) following exposure to human semen, or in cells obtained from the endocervix of sexually active women. HeLa cells were co-cultured with a 1:50 dilution of semen from four men, with purified spermatozoa, or with cell-free seminal fluid. Endocervical swabs were obtained at mid-cycle from 53 women. HSP70 mRNA was detected in HeLa cells by a reverse transcriptase-polymerase chain reaction (RT–PCR) and analysis on agarose gels. HSP70 mRNA in cervical cells was measured by RT–PCR followed by hybridization with an HSP70-specific internal probe and detection by ELISA. Cervical IgA antibodies to HSP70 were measured by ELISA. HeLa cell–semen co-culture led in each case to induction of HSP70 mRNA. Cell-free seminal fluid and isolated motile spermatozoa also induced HSP70 mRNA when incubated individually with HeLa cells. Hsp70 mRNA was detected in 28 (52.8%) of 53 endocervical cell samples obtained from women at varying times after sexual intercourse. The percentage of samples expressing HSP70 mRNA was 37.5% at <10 h, 64.3% at 10 h, 70.0% at 11 h and between 36 and 50% at later times after semen exposure. Cervical IgA antibodies to HSP70 were also detected in some women and their occurrence was highly correlated with HSP70 gene transcription (P < 0.0001). The data demonstrate that exposure to semen induces HSP70 mRNA in endocervical cells.

Key words: endocervix/HeLa cells/human semen/sexual intercourse/70 kDa heat shock protein

Introduction

Although human spermatozoa are immunogenic, most sexually active women do not produce antisperm antibodies. Mechanisms have evolved to limit the female’s immune response to the male gamete (Witkin, 1988a). Numerous molecules with immune suppressor activity exist in seminal fluid (James and Hargrave, 1984). Living motile spermatozoa dramatically inhibited immune responses following their inoculation into mice (Hurtenbach and Shearer, 1982) and rabbits (Richards et al., 1984). In vitro, human spermatozoa failed to induce interferon-γ, a product of immune activation, following coculture with peripheral blood mononuclear cells (PBMC) (Witkin, 1988b). In addition, spermatozoa from different men were shown to inhibit the ability of PBMC to proliferate in response to a microbial antigen and a failure of this sperm-mediated suppression was associated with antisperm antibodies in the female partners (Witkin, 1989).

A number of investigations have demonstrated that induction of transcription of the gene coding for the inducible 70 kDa heat shock protein (HSP70) led to an inhibition of transcription of genes coding for the pro-inflammatory cytokines interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) (Schmidt and Abdulla, 1988; Snyder et al., 1992; Hall, 1994; Cahill et al., 1996). Heat shock proteins are members of highly conserved protein families consisting of both constitutive and inducible components. Constitutively synthesized heat shock proteins function as molecular chaperones, aid in antigen presentation, and regulate steroid receptor function (Burel et al., 1992). Inducible heat shock proteins prevent protein denaturation and incorrect polypeptide aggregation during exposure to physiochemical insults: elevated temperature, activated oxygen and nitrogen intermediates, inflammatory mediators or infection (Kantengwa et al., 1991).

Seminal fluid is rich in prostaglandins, polyamines, zinc, proteases and other enzymes which may initiate a stress response and thereby induce transcription of HSP70 in endocervical cells. A direct interaction between sperm and endocervical cells may also induce a stress response. Spermatozoa are capable of associating with, and even penetrating into, somatic cells (Bendich et al., 1974). During coitus the pH in the vagina changes from 4.0–4.5 to about 7.0. Since an acidic pH suppresses the synthesis of HSP70 (Hang and Fox, 1994), neutralization of the vaginal pH by semen would further create conditions permissive for the synthesis of HSP70 at this site. Therefore, it was of interest to determine whether semen induced HSP70 mRNA in cells of the female genital tract. Existence of such induction would suggest an additional mechanism limiting immunity to spermatozoa: prevention of pro-inflammatory cytokine synthesis and T lymphocyte activation secondary to initiation of HSP70 gene transcription. In this communication we demonstrate that human semen, as well as cell-free seminal fluid and motile spermatozoa, induced...
HSP70 mRNA transcription in a cell line derived from human cervical cells. HSP70 mRNA was also identified in cells of the endocervix from sexually active women.

Materials and methods

Cell culture
HeLa cells (American Type Culture Collection, Rockville, MD, USA), were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 0.05 µg/ml gentamicin. Cells were maintained in a 37°C, 5% CO₂/95% air atmosphere.

Processing of semen
Semen samples were obtained by masturbation. Following liquefaction, motile spermatozoa were isolated by overlaying the semen samples with an equal volume of sterile warm (37°C) phosphate-buffered saline (PBS), incubating at 37°C for 60 min and collecting the upper layer. The remaining semen was centrifuged and cell-free seminal fluid was filter-sterilized with a 0.2 µm syringe filter.

HeLa/semen experiments
HeLa cells were grown in 24-well flat bottom plates until confluent after which the monolayers were washed with Hanks’ balanced salt solution (HBSS). Unfractionated semen, seminal fluid, or motile sperm, diluted as indicated in the individual experiments, were added in triplicate to each well. After a 24 h incubation in a 37°C, 5% CO₂ incubator, the wells were washed three times to remove semen components; 0.5 ml of 0.25% trypsin-1 mM EDTA was added to each culture, like wells were pooled and washed twice with HBSS. The collected cells were pelleted by centrifugation at 6500 r.p.m. and RNA extracted as described below. Viability was determined on an aliquot of each cell culture by vital dye exclusion (0.4% trypan blue).

Subjects
The study population consisted of 53 reproductive age married women. All subjects had been tested for aerobic and anaerobic organisms within the past 12 months and treated if culture positive. All were retested for Chlamydia trachomatis by the AmpliMed polymerase chain reaction (Roche Diagnostics, Branchbrook, NJ, USA) at the time of sample collection; none was positive. None of the women was using contraception at the time of the study.

Cervical sample preparation
Cervical samples were obtained from all women at mid-cycle by inserting a Dacron swab into the endocervix, twirling the swab and then removing it into a tube containing 0.5 ml PBS. Samples were vortexed and liquid was extracted from the swab using a sterile Pasteur pipette. Cells were pelleted by centrifugation and the supernatant removed and frozen at –80°C until utilized in the determination of anti-HSP70 antibodies. The cell pellet was washed three times and RNA was immediately extracted as described below.

RNA isolation
Cell pellets were resuspended in 0.2 ml 10 mM Tris–HCl pH 7.5, containing 0.15 M NaCl, 1.5 mM MgCl₂, 0.65% Nonidet P-40 detergent and 1 µl RNAguard ribonuclease inhibitor (Pharmacia, Piskatway, NJ, USA) to lyse the cells and release the nucleic acid. Following centrifugation for 5 min at 6500 r.p.m. the supernatant was removed and mixed with an equal volume of 7 M urea, 1% sodium dodecyl sulphate, 0.35 M NaCl, 10 mM EDTA, and 10 mM Tris–HCl. RNA was extracted by addition of an equal volume of 50:50:1 phenol:chloroform:isoamyl alcohol. The samples were vortexed and centrifuged at 12 500 r.p.m. for 4 min at 4°C. The RNA underwent a double extraction procedure to ensure RNA purity. RNA was precipitated at –20°C overnight in 100% ethanol and 3 M sodium acetate, washed with 70% ethanol, dried by desiccation and resuspended in diethyl pyrocarbonate-treated water. Samples were stored at –80°C until reverse transcription and polymerase chain reaction (PCR) were performed.

Deoxyribonuclease treatment and reverse transcription of RNA
Prior to reverse transcription all RNA samples were treated for 15 min with 1 unit of amplification grade deoxyribonuclease (DNase I, Gibco BRL. Life Technologies, Grand Island, NY, USA) to destroy any contaminating DNA. DNase activity was removed by the addition of 2 mM EDTA and heating for 10 min at 65°C.

Reverse transcription of RNA to complementary DNA was performed in the presence of 600 units of murine Maloney leukaemia virus reverse transcriptase (Gibco BRL) in 50 mM Tris–HCl, 75 mM KCl, 3 mM MgCl₂, 100 µg/ml bovine serum albumin, 0.5 mM each dATP, dTTP, dCTP, and dGTP (Promega, Madison, WI, USA), 1330 units/ml RNasin ribonuclease inhibitor (Promega) and 50 µg/ml oligo dT₁₅ primer. RNA samples were incubated for 60 min at 37°C, heated at 95°C for 5 min to inactivate the reverse transcriptase and stored at –80°C until PCR was performed.

Polymerase chain reaction
Aliquots of complementary DNA were combined with reaction buffer (10 mM Tris–HCl, 1.5 mM MgCl₂, 50 mM KCl, 200 µM of each dATP, dCTP, dGTP, 190 µM dTTP, and 10 µM digoxigenin-11-2',3'-deoxyuridine-5'-triphosphate; dig-11-dUTP, Boehringer Mannheim, Indianapolis, IN, USA), 0.15 µM HSP70 oligonucleotide primer pairs for the inductive form of HSP70 (StressGen, STM-506), and 1.25 units of Taq DNA polymerase in a total volume of 50 µl. Samples were subjected to 1 cycle at 95°C for 3 min, 48°C for 30 s and 72°C for 1 min 30 s followed by 28 cycles of 95°C for 15 s, 55°C for 30 s, 72°C for 1 min 30 s, followed by a 5 min extension at 72°C. PCR products were subjected to electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light.

As a control to guarantee that complementary DNA synthesis occurred in our samples, each sample was examined for the presence of β-actin complementary DNA. Complementary DNA samples were amplified, using the same reaction conditions as above, in the presence of 6 pmol of human β-actin specific primers: reverse 5'-CGT CAC GCC ACC TTC TAC AAT GAG CTG CG-3' (Oswel DNA Service, Southampton, UK).

Quantitation of amplified PCR products by enzyme-linked immuno- sorbent assay (ELISA)
To increase sensitivity and specificity of mRNA detection from the human endocervical-derived samples, amplified PCR products were detected by ELISA following hybridization with an HSP70-specific internal probe. Aliquots (10 µl) of digoxigenin amplified PCR products were denatured at 25°C for 10 min and hybridized with 7.5 pmol/ml of a biotinylated HSP70-specific oligonucleotide probe: biotin 5'-GCA AGG TGG AGA TCA TCG CCA ACC ACC AGC-3' (Oswel DNA Service). Digoxigenin-labelled biotin PCR complexes were then incubated in a streptavidin-coated microtitre plate for 3 h at 51.1°C and the bound product was detected using peroxidase conjugated anti-digoxigenin antibody (Boehringer Mannheim) and the colorimetric substrate ABTS. Wells containing no complementary DNA, and negative for PCR amplification products were used as negative.
ELISA controls. Known concentrations of human DNA (0.2–60 ng/ml) were amplified by PCR to derive a standard curve.

**Detection of IgA antibodies to HSP70 in cervical supernatants**

Recombinant HSP70 (StressGen) was diluted to 10 µg/ml in 0.1 M carbonate buffer, pH 9.8. 0.1 ml added to wells of a microtitre plate and the plate incubated at 4°C for 16 h. The liquid was then removed and the wells washed three times with 0.1 ml PBS containing 0.05% Tween 20 detergent. Cervical samples were thawed, diluted 1:5 in PBS–Tween and 0.1 ml added to individual wells. Following a 60 min incubation in a 37°C water bath the wells were washed three times as above and 0.1 ml of a 1:50 dilution in PBS–Tween buffer of alkaline phosphatase (AP)-conjugated goat antibody to human IgA (Kirkegaard and Perry, Gaithersburg, MD, USA) was added to the wells. After an additional 60 min at 37°C, the wells were again washed three times and the AP substrate, ρ-nitrophenylphosphate in 10% diethanolamine buffer, pH 9.8, was added. The development of a yellow colour in the wells was quantitated at 405 nm after 30 min with a microtitre plate reader. Known positive and negative samples were always tested in parallel to the test samples. A sample was scored as positive for IgA antibodies to the HSP70 if the final absorbance was at least two standard deviations above the mean absorbance value obtained with cervical samples from 25 healthy reproductive age women not exposed to semen for >7 days (a value ≥0.227).

**Statistics**

Differences between groups were analysed for statistical significance using Fisher’s exact test.

**Results**

**Induction of HSP70 mRNA in HeLa cells by semen and semen components**

Whole semen from four different men when added to HeLa cell cultures at a final concentration of 1:50 induced the expression of HSP70 mRNA (Figure 1). HeLa cell viability was 80–93% at the termination of these incubations. Lower dilutions of semen were cytotoxic to HeLa cell cultures, as determined by trypan blue exclusion. No detectable HSP70 mRNA was detected in semen alone; HeLa cells incubated in the absence of semen yielded at most a low level of HSP70 mRNA. Equivalent concentrations of β-actin mRNA were detected in all HeLa cell cultures.

To clarify which semen constituents were inducing HSP70 mRNA, cell-free seminal fluid and motile sperm from a semen donor were isolated and individually incubated with HeLa cells. At concentrations as low as 1:100 filter-sterilized seminal fluid induced HSP70 mRNA. Cell viability was always >80%. No HSP70 mRNA was detected in these HeLa cultures in the absence of seminal fluid or from seminal fluid alone (Figure 2).

HeLa cells cultured with motile sperm at a HeLa:sperm ratio of 1:10 or 1:2 resulted in induction of HSP70 mRNA. HeLa cell viability was consistently >80% under these conditions. Hsp70 mRNA was not detected in these HeLa cultures in the absence of sperm or from cultures of motile sperm that contained no HeLa cells (Figure 3).

**Hsp70 mRNA in endocervical cells of sexually active women**

To ascertain whether HSP70 was induced in the cervix following exposure to semen, endocervical cells obtained from women at different times after sexual intercourse were examined for HSP70 mRNA by reverse transcriptase PCR/ELISA. Hsp70 mRNA was detected in 28 of 53 (52.8%) samples that were positive for β-actin mRNA. The relation between time since exposure to semen and the prevalence and concentration of HSP70 mRNA is shown in Table I. Among women exposed...
Table II. Relation between 70 kDa heat shock protein (HSP70) messenger RNA induction and detection of IgA antibodies to HSP70 in the endocervix

<table>
<thead>
<tr>
<th>HSP70 mRNA</th>
<th>No. of subjects</th>
<th>No. with IgA anti-HSP70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>10</td>
<td>8 (80.0%)</td>
</tr>
<tr>
<td>Absent</td>
<td>26</td>
<td>2 (7.7%)</td>
</tr>
</tbody>
</table>

*P = 0.0001 (Fisher’s exact test).

**Discussion**

Human semen was shown to induce HSP70 gene transcription in endocervix-derived cells both *in vivo* and *in vitro*. Cell-free seminal fluid as well as motile spermatozoa were able to trigger this response. The cell type(s) in the endocervix that produced HSP70 remains to be determined.

HSP70 has been identified in human spermatozoa and in seminal fluid (Miller et al., 1992). However, the detection of HSP70 mRNA in HeLa cell cultures following removal of seminal fluid and non-adherent semen-derived cells by washing and the inability to detect HSP70 mRNA in spermatozoa or semen utilized for these experiments, strongly suggests that semen-derived HSP70 mRNA was not being measured. Identification of HSP70 mRNA in cervical cells obtained from women after sexual intercourse parallels the *in vitro* observations with cultured cells. The correlation between HSP70 mRNA and cervical IgA antibody to HSP70 further suggests that HSP70 gene transcription was induced in cervical cells by semen and that the resulting protein stimulated a localized immune response. In pilot experiments utilizing peripheral blood mononuclear cells co-incubated with human semen in *vitro*, HSP70 mRNA was first detectable 11 h after culture initiation and reached peak levels by 18 h. This time course parallels our *in vivo* observations (Table I).

Whether HSP70 in semen also contributed to the cervical anti-HSP70 IgA response remains to be determined. The semen samples utilized were not tested for HSP70 protein. In addition, although none of the semen samples utilized for the HeLa cell experiments contained HSP70 mRNA, semen from male partners of the women utilized for this study were not available for this testing.

Heat shock proteins, especially HSP70, are intimately associated with the life cycles of a multitude of RNA and DNA viruses. HSP70 may be needed at specific stages to promote the transcription of viral genes, assembly of viral polypeptides and/or virus infectivity (Santoro, 1994). Studies have also demonstrated that transcription of endogenous human immunodeficiency virus provirus may be initiated simultaneously with HSP70 gene transcription due to similarities in the DNA sequences in the respective promoter regions of the two genes (Stanley et al., 1990). Initiation of HSP70 transcription thus might facilitate the reactivation of latent viral infections as well as augment viral replication and transmission from an infected donor to a non-infected recipient by promoting enhanced viral production.

Whether semen-induced HSP70 is beneficial in inhibiting immune responses to spermatozoa or harmful in facilitating viral infectivity or inhibiting immune activation in response to semen <10 h previously, 37.5% had endocervical HSP70 mRNA. This percentage increased to 64.3% at 10 h after intercourse, 70.0% at 11 h after intercourse and decreased to 40–50% at longer time intervals.

**IgA antibodies to HSP70 in the endocervix**

The supernatant fraction of endocervical swab material, available from 36 of the subjects, was examined for IgA antibodies to HSP70. Samples from 10 women (27.8%) were positive (Table II). The presence of cervical anti-HSP70 IgA was associated with detection of cervical HSP70 mRNA (P < 0.0001). This antibody was present in eight of 10 (80%) women with, and in only two of 26 (7.7%) women without, detectable HSP70 mRNA. The prevalence of IgA antibodies to HSP70 varied with time since last semen exposure. IgA anti-HSP70 was identified in the cervices of five of seven (71.4%) women who had sexual intercourse >48 h prior to sample collection, in four of 23 (17.4%) exposed to semen from 10 to 16 h earlier, and in one of eight (12.5%) women who had intercourse <10 h prior to sample collection.

<table>
<thead>
<tr>
<th>Time since intercourse (h)</th>
<th>No. of subjects</th>
<th>No. positive (%)</th>
<th>Concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10</td>
<td>8</td>
<td>3 (37.5)</td>
<td>0.1–1.2</td>
</tr>
<tr>
<td>10</td>
<td>14</td>
<td>9 (64.3)</td>
<td>0.2–7.9</td>
</tr>
<tr>
<td>11</td>
<td>10</td>
<td>7 (70.0)</td>
<td>0.2–1.5</td>
</tr>
<tr>
<td>12–16</td>
<td>11</td>
<td>4 (36.4)</td>
<td>0.2–1.9</td>
</tr>
<tr>
<td>&gt;48</td>
<td>10</td>
<td>5 (50.0)</td>
<td>0.2–4.2</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>28 (52.8%)</td>
<td></td>
</tr>
</tbody>
</table>

All samples were positive for messenger RNA to β-actin.
to other pathogens that might be present in a particular ejaculate may depend on a number of factors. The genetic capacity of individual women’s cells to transcribe, translate and degrade HSP70 mRNA under various conditions is undoubtedly variable. In addition, the relative concentration of innate and acquired components of individual semen samples which may induce HSP70 gene expression may differ. The presence or absence of virally infected cells or microbial pathogens in the ejaculate or in the female genital tract will also influence the consequences of HSP70 mRNA transcription. The observed variation between women in HSP70 mRNA detection and concentration at various times after sexual intercourse may be related to individual differences in these factors.

The effect of sexual intercourse and concomitant variables on the expression of cytokines that selectively activate cell-mediated (TH1) or humoral (TH2) immune responses are currently under investigation. Whether HSP70 is induced in females who use various methods of birth control or whose male partners use condoms during sexual intercourse is also being explored.

Acknowledgement

We thank Dr Andreas Neuer for fruitful discussions and a critical review of the manuscript and Prof. Tom Brown of Oswel DNA Service for design of the DNA probes. David Cho provided expert technical assistance. This work was supported by NIH grant HD33194.

References


Received on February 7, 1997; accepted on June 4, 1997