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Retraction

Retracted: Induction of Heat Shock Protein Expression in Cervical Epithelial Cells by Human Semen

Infectious Diseases in Obstetrics and Gynecology

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Infectious Diseases in Obstetrics and Gynecology has retracted the article titled "Induction of Heat Shock Protein Expression in Cervical Epithelial Cells by Human Semen" [1]. The article was found to report the same results, without citation, as the following published article: J. Jeremias, S. S. David, M. Toth, S. S. Witkin, "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," Human Reproduction (1997) 12 (9): 1915–1919. DOI: https://doi.org/10.1093/humrep/12.9.1915 [2]. The last author does not approve of retraction and the other authors could not be contacted.

References

[1] J. C. Jeremias, A. M. Bongiovanni, and S. S. Witkin, "Induction of heat shock protein expression in cervical epithelial cells by human semen," *Infectious Diseases in Obstetrics and Gynecology*, vol. 7, no. 1-2, pp. 17–22, 1999.

[2] J. Jeremias, S. S. David, M. Toth, and S. S. Witkin, "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," *Human Reproduction*, vol. 12, no. 9, pp. 1915–1919, 1997.

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Induction of Heat Shock Protein Expression in Cervical Epithelial Cells by Human Semen

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ABSTRACT

Objective: **The 70kD heat shock protein (Hsp70), induced when cells are subjected to environmental stress, prevents the denaturation and incorrect folding of polypeptides and may expedite replication and transmission of DNA and RNA viruses. We analyzed whether messenger RNA (mRNA) for Hsp70 was expressed following exposure of a cultured human cervical cell line (HeLa cells) to human semen or in cervical cells from sexually active women.**

Study Design: **HeLa cells were co-cultured with a 1:50 dilution of semen from four men or with purified spermatozoa or cell-free seminal fluid. Endocervical swabs were acquired at mid-cycle from 53 women. Heat shock protein 70 mRNA was detected by a reverse transcriptase-polymerase chain reaction utilizing specific primer pairs and analysis on agarose gels. In cervical cells Hsp70 mRNA was measured identically followed by hybridization with an Hsp70-specific internal probe and detection by enzyme-linked immunosorbent assay (ELISA). Cervical immunoglobulin A (IgA) antibodies to the human Hsp70 were determined by ELISA.**

Results: **HeLa cell-semen co-culture resulted in the induction of Hsp70 mRNA. In addition, cell-free seminal plasma and motile sperm incubated individually with HeLa cells also induced this mRNA. Heat shock protein 70 mRNA was detected in 28 (52.8%) of 53 endocervical samples obtained from women at various time points following intercourse. The percentage of samples expressing this mRNA was 37.5% at less than 10 hours, 64.3% at 10 hours, 70% at 11 hours, and between 36% and 50% at later times after semen exposure. The detection of cervical IgA antibodies to the Hsp70 was highly associated with Hsp70 gene transcription.**

Conclusion: **Human semen induces transcription of Hsp70 in cervical epithelial cells. Infect. Dis. Obstet. Gynecol. 7:17–22, 1999.** © 1999 Wiley-Liss, Inc.

KEY WORDS

semen; human; heat shock proteins; cervical cells; Hsp70

Heat shock proteins (Hsps) are universal constituents of all prokaryotic and eukaryotic cells. Constitutively synthesized Hsps function as molecular chaperones, aid in antigen presentation, and regulate steroid receptor function.[1] Hsps may also be induced following exposure to a wide variety of physio-chemical insults: temperature variation, steroids, amino acid analogues, heavy metals, and oxidative damage.[2] Mammalian cells produce Hsps in response to infection by viral or bacterial pathogens, and this response has been indicated to exert a cytoprotective effect during disease pathogenesis. A number of investigators have demonstrated that induction of transcription of the gene coding for the inducible heat shock protein 70 (Hsp70) led to an inhibition of transcription of genes coding for the pro-inflammatory cytokines interleukin-1 and tumor necrosis factor-α.[3–6] Tran-

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scriptional induction of Hsps requires activation of a heat shock factor, a transregulatory protein that attaches to specific promoter elements located upstream of several Hsp genes.

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. Contact between sperm and endocervical cells may also bring about a stress response. Spermatozoa are capable of associating with, and even penetrating into, somatic cells.[7] During coitus, the pH in the vagina changes from 4.0–4.5 to approximately 7.0. Since an acidic pH suppresses the synthesis of Hsp70,[8] neutralization of the pH in the vagina by semen would create a milieu, at this site, acceptable for the synthesis of Hsp70. Therefore, it was of interest to determine whether semen induced Hsp70 messenger RNA (mRNA) in cells of the female genital tract. The actuality of such induction would suggest an additional mechanism limiting immunity to spermatozoa: prevention of proinflammatory 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. The Hsp70 mRNA was also identified in cells of the endocervix from sexually active women after intercourse.

MATERIALS AND METHODS

Cell Culture

Human cervical (HeLa) cells, obtained from American Type Culture Collection (ATCC, Rockville, MD), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 0.05µg/mL gentamicin. Cells were maintained in a 37°C, 5% CO_2/95% 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), incubated at 37°C for 60 minutes, and collecting the upper PBS layer. The remaining semen was centrifuged, and cell-free seminal fluid was sterilized with a 0.2 µm syringe filter.

HeLa/Semen Experiments

Cells were grown in 24 well flat bottom plates until confluent, after which the monolayers were washed with Hank's Balanced Salt Solution (HBSS). Unfractionated semen, seminal fluid, or motile sperm, diluted as indicated in the individual experiments, was added to each well in duplicate. After an overnight incubation in a 37°C, 5% CO_2 incubator, 0.5 mL of 0.25% Trypsin-1mM edetic acid (EDTA) was added to each culture and like wells were pooled and washed twice with HBSS. The collected cells were pelleted by centrifugation at 6500 rpm 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 was comprised of 53 married women of reproductive age. All subjects had been tested for aerobic and anaerobic organisms within the past 12 months and treated if culture positive. All were tested for *Chlamydia trachomatis* by the Amplicor polymerase chain reaction (PCR; Roche Diagnostics, Branchberg, NJ) at the time of sample collection, and none were positive. At the time of the study, none of the women were using contraception.

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 used for the determination of anti-Hsp70 antibodies. The cell pellet was washed three times with PBS, 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 $MgCl_2$, 0.65% Nonidet P-40 detergent, and 1 µL RNAguard ribonuclease inhibitor (Pharmacia, Piskataway, NJ), to lyse the cells and release the

nucleic acid. Following centrifugation for 5 min. at 6500 rpm, the supernatant was removed, admixed with an equal volume of 7 M urea, 1% sodium dodecyl sulfate, 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 rpm 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 −20°C 70% ethanol, dried by desiccation, and resuspended in diethyl pyrocarbonate-treated water. Samples were stored at −80°C until reverse transcription (RT) and PCR were performed.

Deoxyribonuclease Treatment and Reverse Transcription of RNA

Prior to RT of the RNA, samples were treated for 15 min. with 1 unit of amplification grade Deoxyribonuclease (DNase 1, Gibco BRL Life Technologies, Grand Island, NY) 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 (cDNA) was performed in the presence of 60 units murine Maloney leukemia virus reverse transcriptase (Gibco BRL) in 50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 100 g/mL bovine serum albumin, 0.5 mM each dATP, dCTP, dGTP, and dTTP (Promega, Madison, WI), 1330 U/mL RNasin ribonuclease inhibitor (Promega) and 50 µg/mL oligo dT_{15} 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 cDNA were combined with reaction buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 200 µM each of dATP, dCTP, and dGTP, and 190 µM dTTP and 10 µM digoxigenin-11-2′-deoxyuridine-5′-triphosphate [dig-11-dUTP, Boehringer Mannheim, Indianapolis, IN]), 0.15µM Hsp70 oligonucleotide primer pairs for the inducible Hsp70 (StressGen, Victoria, B.C., Canada), and 1.25 units of Taq DNA polymerase in a total volume of 50 µL. Samples were subjected to one cycle at 95°C for 3 min., 48°C for 30 sec., 72°C for 1 min., 30 sec. followed by 28 cycles of 95°C for 15 sec., 55°C for 30 sec., 72°C for 1 min., 30 sec., followed by a 5 min. extension cycle at 72°C. The PCR products were subjected to electrophoresis on 1.7% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light.

As a control to guarantee that cDNA synthesis occurred in our samples, each sample was examined for the presence of β-actin cDNA. Samples of cDNA were amplified, using the same reaction conditions as above, in the presence of 6 pmol of human β-actin specific primers: reverse 5′-CGT CAT ACT CCT GCT TGC TGA TCC AAT CTGC-3′ and forward 5′-ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3′ (Oswel DNA Service, Southhampton, England).

Quantitation of Amplified PCR Products by Enzyme-Linked Immunosorbent Assay

Amplified PCR products were detected by enzyme-linked immunosorbent assay (ELISA) following hybridization with an Hsp70-specific internal probe, to increase sensitivity and specificity of mRNA detection from the human endocervical-derived samples. Aliquots (10µL) 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 ACG ACC AGG-3′ (Oswel DNA Service). Digoxigenin-labeled biotin PCR complexes were then incubated in a streptavidin-coated microtitre plate for 3 hr. at 51.1°C, and the bound product was detected using peroxidase conjugated anti-digoxigenin antibody (Boehringer Mannheim) and the colorimetric substrate ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulfate (6)] diammonium salt). Wells containing no cDNA and negative 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 Immunoglobulin A 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 hr. The liquid was then

TABLE 1. Messenger RNA for Hsp70 in endocervical cells obtained at various times after sexual intercourse[a]

	Time since intercourse (hr)					
	<10	10	11	12–16	>48	Total
No. of subjects	8	14	10	11	10	53
No. positive (%)	3 (37.5)	9 (64.3)	7 (70)	4 (36.4)	5 (50)	28 (52.8)
Concentration (ng/ml)	0.1–1.2	0.2–7.9	0.2–1.5	0.2–1.9	0.2–4.2	

[a]All samples were positive for mRNA for β-actin.

removed and the wells washed three times with 0.1 mL PBS containing 0.5% Tween 20 detergent. Thawed cervical samples were 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 1:500 dilution in PBS-Tween buffer of alkaline phosphatase (AP)-conjugated goat antibody to human immunoglobulin A (IgA; Kirkegaard and Perry, Gaithersburg, MD) was added to the wells. After an additional 60 min. at 37°C, the wells were again washed three times and the AP substrate, p-nitrophenylphosphate in 10% diethanolamine buffer, pH 9.8, was added. The development of a yellow color 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 considered positive for IgA antibodies to 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 female controls not exposed to semen for more than 7 days (a value $\geq$ 0.227).

RESULTS

Induction of Hsp70 mRNA in HeLa Cells by Semen and Semen Components

HeLa cells cultured in the presence of whole semen from different men, at a final concentration of 1:50, induced the expression of Hsp70 mRNA. A 1:50 dilution of semen was not cytotoxic to HeLa cell cultures, as determined by trypan blue exclusion. No detectable Hsp70 mRNA could be detected in semen alone or in HeLa cells incubated in the absence of semen. Equivalent concentrations of β-actin mRNA were detected in all HeLa cell cultures but not in semen or semen components

The ability of cell-free seminal fluid and motile sperm from the same semen donor to induce Hsp70 mRNA from HeLa cells was examined. At three different concentrations, filter-sterilized seminal fluid induced Hsp70 mRNA. No Hsp70 mRNA was detected in HeLa cultures in the absence of seminal fluid or from seminal fluid alone. The lack of a dose response in Hsp70 mRNA induction was probably a result of seminal fluid cytotoxicity on HeLa cells at the higher concentrations. The cell viability of HeLa cell cultures with a 1:50 dilution of semen, seminal fluid, or motile sperm was more than 90%.

HeLa cells cultured with motile sperm at a HeLa:sperm ratio of 1:10 or 1:2 resulted in induction of Hsp70 mRNA. Heat shock protein 70-specific mRNA was not detected in HeLa cultures in the absence of sperm or from cultures of motile sperm that contained no HeLa cells.

Induction of Hsp70 mRNA in Endocervical Cells Following Sexual Intercourse

Endocervical cells obtained from women at different times after sexual intercourse were examined for Hsp70 mRNA by RT-PCR ELISA. The Hsp70 mRNA was detected in 28 (52.8%) of 53 samples that were positive for β-actin RNA. The relation between time since exposure to semen and the prevalence and concentration of Hsp70 mRNA is shown in Table 1. Among women exposed to semen less than 10 hours prior to sample collection, 37.5% had endocervical Hsp70 mRNA. This percentage increased to 64.3% at 10 hours after intercourse, 70% at 11 hours after intercourse, and decreased to 40–50% at longer time intervals (Table 1).

Induction of IgA Antibodies to Hsp70 in the Endocervix Following Sexual Intercourse

The supernatant fraction of endocervical swab material, available from 36 of the subjects, was examined for IgA antibodies to the human Hsp70. Samples from 10 women (27.8%) were positive.

TABLE 2. Induction of cervical IgA antibodies to Hsp70 and relation to mRNA induction

No. with IgA anti-Hsp70 (%)	No. of samples	Hsp70 mRNA
8 (80)	10	Positive
2 (7.7)	27	Negative

$P = 0.0001$ (Fisher exact test).

The presence of cervical anti-Hsp70 IgA correlated with detection of cervical Hsp70 mRNA ($P < 0.0001$); this antibody was present in eight (80%) of 10 women with detectable Hsp70 mRNA and in only two (7.7%) of 26 women without detectable Hsp70 mRNA (Table 2). The prevalence of IgA antibodies to Hsp70 increased with the time since last semen exposure ($P = 0.008$). Immunoglobulin A anti-Hsp70 was identified in the cervices of five (71.4%) of seven women who had sexual intercourse greater than 48 hours prior to sample collection, in four (17.4%) of 23 women exposed to semen from 10 to 16 hours earlier, and in one (12.5%) of eight women who had intercourse less than 10 hours before sample collection (Table 3). An increased production of antibodies in women previously sensitized to Hsp70 following re-exposure to newly synthesized Hsp70 protein after intercourse is consistent with the induction of a secondary immune response.

TABLE 3. Presence of IgA antibodies to Hsp70 versus time since semen exposure

	Time since intercourse (hr)		
	>48	10–16	<10
No. of women with anti-Hsp70 IgA present (%)	5 (71.4)	4 (17.4)	1 (12.5)
Total no. of women	7	23	8

$P = 0.008$.

DISCUSSION

Hsp 70 gene transcription was induced in vivo and in vitro in endocervix-derived cells following exposure to semen. Cell-free seminal fluid and motile sperm were also capable of initiating this response. The cells that actually produce Hsp70 mRNA have not yet been identified.

The presence of Hsp70 has been identified in human sperm and seminal fluid. However, in all our experiments, all nonadherent semen components were removed by washing, and we were unable to detect Hsp70 mRNA in spermatozoa. Therefore, we are confident that the source of Hsp70 was the HeLa cells. 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 antibodies to Hsp70 in cervical cells following exposure to semen further suggests that Hsp70 gene transcription and the subsequent production of Hsp70 protein stimulated a localized immune response.

There is a possibility that the Hsp70 in semen could have contributed to the cervical anti-Hsp70 IgA response. However, none of the semen samples used in the HeLa cell experiments contained Hsp70 mRNA. Notwithstanding, the semen samples from the male partners of the women in our study were not available and, therefore, were not assayed for Hsp70 protein.

Heat shock proteins accumulate within virally infected cells and appear to play a significant role in some viral infections. Hsp 70 specifically is intimately associated with the life cycles of numerous RNA and DNA viruses. Hsp 70 may be required to promote the transcription of viral genes, assembly of viral polypeptides, and/or virus infectivity.[9,10] During vaccinia infection, Hsp70 mRNA directly associates with intracellular vaccinia proteins and may act as a chaperonin in the assembly of this virus.[11] Induction of Hsps by elevated temperature upregulates virus production from cell lines chronically infected with human immunodeficiency virus (HIV).[12] Hsp induction by heat or chemicals activated the long terminal repeat (LTR) of HIV. In HeLa cells transfected with the HIV-1 LTR, Hsp induction by heat induced the transcription of the HIV-1 LTR and the Hsp70 heat shock promoter.[13] Elevations in HIV reverse transcriptase were observed for 72 hours following heat shock in infected lymphocytic and monocytic cell lines. Cells allowed to recover after heat shock first showed a lag in release of viral particles, followed by accelerated viral release in stressed cells. Physiological levels of heat shock were not able to induce virus production from cells in vitro. However, these temperatures were able to act synergistically with interleukin-6 and granulocyte macrophage-colony stimulating factor to enhance virus production.[13] Viral activation in response to environmental stress has

also been identified for cytomegalovirus (CMV).[14] A DNA sequence present in the enhancer element of CMV, similar to a decameric sequence in the NF-κB binding site of the HIV LTR, resembled the heat shock element core consensus regulatory sequence.[14,15] This suggested that either Hsps may activate CMV genes or that Hsp genes and CMV genes respond to similar stimuli. 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 noninfected recipient by promoting enhanced viral production.

Since viruses such as HIV and CMV are sexually transmitted, it was of interest to determine whether semen and its components could activate Hsp transcription in cervical cells. Such expression might facilitate the reactivation of latent viral infections as well as augment viral transmission from an infected donor to a noninfected recipient.

Whether semen-induced Hsp70 is beneficial in inhibiting immune responses to spermatozoa, harmful in facilitating viral infectivity, or inhibits immune activation in response to other sexually transmitted infections may depend on a number of factors. The capacity of individual women's cells to transcribe, translate, and degrade Hsp70 mRNA is under genetic control. In addition, the components of individual semen samples to induce Hsp70 expression may differ. The presence of virally infected cells or microbial pathogens in the female genital tract or in the ejaculate may influence the consequences of Hsp70 transcription. The observed difference between women at various times after intercourse may be related to these factors.

The effect of semen on Hsp70 transcription in additional epithelial and nonepithelial cell lines is currently under investigation. The effect of sexual intercourse on cytokine expression and whether Hsp70 expression differs in women using various forms of birth control is also being explored.

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