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Original Article

Prediction of an interactive binding between HSPA2 and TLR 4 in spermatozoa under pathophysiological condition

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ABSTRACT

HSPA2, a testis-specific protein belonging to the HSP70 family, is ubiquitously found in the testes and spermatozoa. It plays a pivotal role in the spermatogenesis process, and associated with human sperm-egg recognition. During various stress conditions, ligand-receptor binding between extracellular HSP70 and TLR 4 has been well established in many cells, except spermatozoa. Bacteriospermia-associated leukocytospermia (LCS/BS)-induced immune infertility is a pathophysiological condition of the male reproductive system mediated by antisperm antibody (ASA) production and TLR 4 signaling. In this study, the mechanisms involved in the interaction between HSPA2 and TLR 4 in the spermatozoa were revealed. Based on previously published data which revealed the identification of several types of seminal bacteria from the LCS/BS patients attending Kar Clinic and Hospitals Pvt. Ltd., semen samples containing *Escherichia coli* infection were selected for this particular study. The immunocytochemistry study showed co-localization of HSPA2 with TLR 4 on the spermatozoa surface confirming a plausible interaction between them. The HSPA2-TLR 4 interaction was further strengthened by multiple pathway analytical tools such as Cytoscape PSICQUIC client service and STRING database version 11.0. Upon CLUSTAL Ω analysis, DnaK and HSPA2 protein sequences showed 47% identity. Using IEDB tool it was revealed that thirty-eight self-peptides of *E. coli* DnaK protein shared homology with HSPA2. This was further confirmed by the strong binding affinity between MHC class-II DRB allele and DnaK/HSPA2 as evidenced by PATCHDOCK software. Altogether, a true interaction between HSPA2 and TLR 4 signaling on the spermatozoa surface was deciphered *via* dual mechanisms: (1) the direct interaction due to LCS/BS-induced immune infertility; (2) and the indirect one where, HSPA2 is the mimicked version of DnaK protein isolated from *E. coli*. These findings indicate the existence of a ligand-receptor relationship between HSPA2 and TLR 4 on the spermatozoa surface during immune infertility.

KEYWORDS: ASA, auto-antigens, DnaK, LCS/BS, HSPA2, immune infertility, TLR 4.

ABBREVIATIONS

ASA- Antisperm Antibody, HSPA2- Heat Shock Protein 70-A2, IEDB- Immune Epitope Database, TLR- Toll-like Receptor, LCS- Leukocytospermia, BS- Bacteriospermia.

INTRODUCTION

Heat shock protein 70 (HSP70) family members are canonical chaperones present in the cells. These are basically responsible for proper folding of newly synthesized proteins including refolding of misfolded and aggregated proteins [1]. They also carry out other non-classical functions such as translocation of membrane bound, secretory and

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signal transduction proteins [2]. Other than normal physiological functions, HSP70 are secreted in acute stress (temperature change, chemical or physical stress, release of free oxygen radicals, heavy metals, ethanol) and diseased conditions (viral and bacterial infections, ischaemia). Therefore, it can act as a biomarker for varied physiological and pathological conditions [3-6]. HSP70 proteins can also act as immunomodulants. They are highly involved in the induction of immune signals in response to external stimuli, thereby binding with the cell surface receptors [7]. Extracellular HSPs act as danger activated molecular patterns (DAMPs) [8]. In these conditions, it is quite predictable that HSPs might have chances to interact with the pathogen recognition receptors (PRRs) such as toll-like receptors (TLRs) to promote inflammatory responses [9]. It has been reported that HSP70 utilizes TLR 4 to instigate proinflammatory cytokine secretion in a $CD14^+$-dependent manner [8, 10, 11]. A study has shown upregulation of TLR 4 by HSP70 through a p38 kinase-mediated pathway in monocytes [12]. It has also been reported that HSP70 peptide interacts with the natural killer (NK) cells *via* CD94, NK cell receptors [13]. Membrane-bound HSP70 activates NK cells whereas, the secreted HSP70 from bacteria and humans can act as ligands to TLR 4 on antigen presenting cells (APCs) [10]. Several studies have shown the binding ability of HSP70 family members with a set of T-cell population, thereby associating both the innate and adaptive immune responses [14-16]. Moreover, HSP70 can also bind to the (i) scavenger receptor (SR) family members SREC-I and FEEL-1/CLEVER-1/STABILIN-1; (ii) c-type lectin binding domain (CTLD) and; (iii) the LOX-1 (a member of both the c-type lectin receptors (CLR) and SR) [7]. From these studies, it can be proposed that HSP70 has the tendency to activate immunity by binding to the receptors present on the cell surface. But, what lies in the crux is the ability of other human HSP70 family proteins to interact with TLR 4 during various pathophysiological conditions.

HSPA2 is a predominant testis-specific form of human HSP70, highly expressed during meiosis I as a component of synaptonemal complex and in the mature spermatozoa during spermiogenesis process [17, 18]. Apart from testes, human HSPA2 have been significantly detected in the spermatocytes and spermatids [19]. In the testis, HSPA2 is involved in the recombination mechanisms, DNA repair, histone-protamine replacement and sperm maturation [17, 20, 21]. Its complete absence may lead to azoospermia and decreased expression is associated with oligozoospermia ultimately leading to chromosomal aneuploidies, DNA damage, morphological anomalies, cytoplasmic retention, lack in histone-protamine replacement, decreased zona binding sites and hyaluronic acid receptors, enhanced infertility and unsuccessful pregnancy following IVF [4, 18, 21-30]. These studies were basically conducted in different species, including humans. HSPA2 plays a vital role in spermatogenesis process including sperm maturity, function and fertility mechanisms [31]. Studies have shown that it is mainly associated with human sperm-egg recognition [32]. HSPA2 aberrant expression has been reported in immature spermatozoa [33, 34], oligospermia [29], and in various infertility cases such as varicocele [29, 30, 35, 36]. Besides, a weak expression of HSPA2 was observed in infertile men as compared to the fertile ones [4]. Additionally, it has also been shown that HSPA2 was completely absent in men with idiopathic infertility [37]. Although extracellular HSP70 interaction with TLR 4 is well established in the form of ligand-receptor binding, such an interaction between HSPA2 and TLR 4 in spermatozoa during bacteriospermia associated leukocytospermia (LCS/BS) condition is unknown.

TLR, an essential PRR sensor of innate immune system, is highly recognized on the sperm surface during bacteria invasion [38, 39]. The presence of *Escherichia coli* in the semen samples of six out of seventeen LCS/BS patients triggered TLR 4 signaling in the spermatozoa as shown in the previous study [40]. *E. coli* being a major uropathogen causing male infertility is involved in the activation of TLR 4-MyD88-independent pathway in the rat testicular cells [41, 42]. It has been reported that HSP70 protein of *E. coli* i.e., DnaK, is constitutively expressed in its cytosol [43] and mediates usual protein folding processes [44]. As DnaK is a homolog of human HSP70 [45] and shares 50-70% amino acid sequence similarity with the other eukaryotic HSP70 protein sequences [46], it has become important to determine the homology between HSPA2 and DnaK protein sequences. Based on the auto-antigen determination strategy

used in our previous studies, it will be interesting to detect if any *E. coli* DnaK-derived self-peptide existed showing homology with the HSPA2 peptide sequence [40, 47]. It is well-known that immune infertility is an autoimmune disease of the male reproductive system involving production of auto-antibodies against sperm antigens (auto-antigens) derived from bacterial proteins [40]. From the above evidences, an interaction between DnaK-mimicked HSPA2 and TLR 4 in the spermatozoa is quite expected. Thus, the current investigation can help us understand the mechanisms behind the interaction between HSPA2 and TLR 4 on the spermatozoa surface in a LCS/BS-induced immune infertility condition.

MATERIALS AND METHODS

Immunocytochemistry staining

This part of the study is in continuation of the work previously published [40] where semen samples were collected and processed for immunocytochemistry with the permission of Institutional Ethical Committee. Spermatozoa were incubated with TLR 4 mouse monoclonal antibody and HSPA2 rabbit monoclonal antibody purchased from ABGENEX (ABM19C4, San Diego, CA, USA), prepared in 1% bovine serum albumin (BSA) in phosphate-buffered saline, 0.1% Tween-20 (PBST) (1:200), for 2 hrs at room temperature. The dilution ratio of spermatozoa and antibody used was 1:1. After washing with PBST, cells were incubated in diluted secondary antibodies (1:2000) i.e., goat anti-mouse IgG (for TLR 4) labeled with FITC (ABGENEX: BA1101), and goat anti-rabbit IgG (for HSPA2) labeled with phycoerythrin (PE, Santa Cruz Biotechnology), prepared in 1% BSA (PBST), for 1 hr at room temperature in dark. Finally, the cells were washed three times in PBST followed by Hoechst staining (Sigma 33342). Images were taken using Olympus fluorescence microscope (Olympus XC10) at 400X magnification.

Pathway prediction

Cytoscape 3.5.1 version was used to predict the pathway for the interaction between HSPA2 (*Uniprot ID: P54652*) and TLR 4 (*Uniprot ID: O00206*). The PSICQUIC client service available along with the Cytoscape tool is strongly supported by 25 major interaction data providers e.g., Uniprot, Reactome, Reactome FIs, STRING, BioGRID, Mentha, IntAct, InnateDB, Apid Interactomes, etc. In addition, the STRING database version 11.0 (*https://string-db.org/*) was used to support the Cytoscape pathway prediction for HSPA2-TLR 4 interaction, thereby using its protein-protein interaction (PPI) statistical significance.

Sequence alignment

CLUSTAL Omega (Ω) multiple sequence alignment (MSA) tool (version 1.2.1) provided by the EMBL-EBI bioinformatics web and programmatic tools framework was used to analyze the protein sequences [48-50]. Hidden Markov Models' (HMMs) profile-profile techniques were used for the Clustal Ω tool to generate protein sequence alignments. These sensitive tools helped to decode remote protein homology and evolutionary relationship between the sequences analyzed. The parameter used for the Clustal Ω alignment includes maximum HMM iterations with a default value of -1. BLOSUM62 matrix was used for pair-wise sequence alignment. All the protein entries were obtained from UniprotKB database reviewed with Swissprot entries. The protein sequences used in this study are mentioned below:

Human HSAP2

>sp|P54652|HSP72_HUMAN Heat shock-related 70 kDa protein 2 OS=Homo sapiens GN=HSPA2 PE=1 SV=1

MSARGPAIGIDLGTTYSCVGVFQHGKVEIIAN
DQGNRTTPSYVAFTDTERLIGDAAKNQVAM
NPTNTIFDAKRLIGRKFEDATVQSDMKHWPF
RVVSEGGKPKVQVEYKGETKTFFPEEISSMV
LTKMKEIAEAYLGGKVHSAVITVPAYFNDSQ
RQATKDAGTITGLNVLRIINEPTAAAIAYGLD
KKGCAGGEKNVLIFDLGGGTFDVSILTIEDGI
FEVKSTAGDTHLGGEDFDNRMVSHLAEEFK
RKHKKDIGPNKRAVRRLRTACERAKRTLSS
STQASIEIDSLYEGVDFYTSITRARFEELNAD
LFRGTLEPVEKALRDAKLDKGQIQEIVLVGG
STRIPKIQKLLQDFFNGKELNKSINPDEAVAY
GAAVQAAILIGDKSENVQDLLLLDVTPLSLG
IETAGGVMTPLIKRNTTIPTKQTQTFTTYSDN
QSSVLVQVYEGERAMTKDNNLLGKFDLTGI
PPAPRGVPQIEVTFDIDANGILNVTAADKST
GKENKITITNDKGRLSKDDIDRMVQEAERY
KSEDEANRDRVAAKNALESYTYNIKQTVED
EKLRGKISEQDKNKILDKCQEVINWLDRNQ

MAEKDEYEHKQKELERVCNPIISKLYQGGPGG
GSGGGGSGASGGPTIEEVD

E. coli DnaK

>sp|P0A6Y8|DNAK_ECOLI Chaperone protein DnaK OS=Escherichia coli (strain K12) OX=83333 GN=dnaK PE=1 SV=2

MGKIIGIDLGTTNSCVAIMDGTTPRVLENAE
GDRTTPSIIAYTQDGETLVGQPAKRQAVTNP
QNTLFAIKRLIGRRFQDEEVQRDVSIMPFKII
AADNGDAWVEVKGQKMAPPQISAEVLKK
MKKTAEDYLGEPVTEAVITVPAYFNDAQRQ
ATKDAGRIAGLEVKRIINEPTAAALAYGLDK
GTGNRTIAVYDLGGGTFDISIIEIDEVDGEKT
FEVLATNGDTHLGGEDFDSRLINYLVEEFKK
DQGIDLRNDPLAMQRLKEAAEKAKIELSSA
QQTDVNLPYITADATGPKHMNIKVTRAKLE
SLVEDLVNRSIEPLKVALQDAGLSVSDIDDV
ILVGGQTRMPMVQKKVAEFFGKEPRKDVNP
DEAVAIGAAVQGGVLTGDVKDVLLLDVTPL
SLGIETMGGVMTTLIAKNTTIPTKHSQVFST
AEDNQSAVTIHVLQGERKRAADNKSLGQFN
LDGINPAPRGMPQIEVTFDIDADGILHVSAK
DKNSGKEQKITIKASSGLNEDEIQKMVRDAE
ANAEADRKFEELVQTRNQGDHLLHSTRKQ
VEEAGDKLPADDKTAIESALTALETALKGE
DKAAIEAKMQELAQVSQKLMEIAQQQHAQ
QQTAGADASANNAKDDDVVDAEFEEVKDKK

>tr|A0A140NFV3|A0A140NFV3_ECOBD Chaperone protein DnaK OS=Escherichia coli (strain B / BL21-DE3) OX=469008 GN=dnaK PE=2 SV=1

MGKIIGIDLGTTNSCVAIMDGTTPRVLENAE
GDRTTPSIIAYTQDGETLVGQPAKRQAVTNP
QNTLFAIKRLIGRRFQDEEVQRDVSIMPFKII
AADNGDAWVEVKGQKMAPPQISAEVLKK
MKKTAEDYLGEPVTEAVITVPAYFNDAQRQ
ATKDAGRIAGLEVKRIINEPTAAALAYGLDK
GTGNRTIAVYDLGGGTFDISIIEIDEVDGEKT
FEVLATNGDTHLGGEDFDSRLINYLVEEFKK
DQGIDLRNDPLAMQRLKEAAEKAKIELSSA
QQTDVNLPYITADATGPKHMNIKVTRAKLE
SLVEDLVNRSIEPLKVALQDAGLSVSDIDDV
ILVGGQTRMPMVQKKVAEFFGKEPRKDVNP
DEAVAIGAAVQGGVLTGDVKDVLLLDVTPL
SLGIETMGGVMTTLIAKNTTIPTKHSQVFST
AEDNQSAVTIHVLQGERKRAADNKSLGQFN
LDGINPAPRGMPQIEVTFDIDADGILHVSAK
DKNSGKEQKITIKASSGLNEDEIQKMVRDAE
ANAEADRKFEELVQTRNQGDHLLHSTRKQ
VEEAGDKLPADDKTAIESALTALETALKGE
DKAAIEAKMQELAQVSQKLMEIAQQQHAQ
QQTAGADASANNAKDDDVVDAEFEEVKDKK

BLAST analysis

NCBI Blastp (protein) or, BLASTP tool version 2.3.1 (*http://blast.ncbi.nlm.nih.gov/*) was used to identify bacterial proteins sharing homology with human proteins of sperm functionality origin [51]. The main criterion to study the protein-protein interaction between the bacteria and human proteins was to reveal the protein of interest i.e., proteins carrying important sperm functions. Thus, T-cell epitope-specific bacterial peptides were targeted for the development of immunocontraception. Bacterial peptides with short input sequences of 15-mer peptide length were blasted against the whole *Homo sapiens* proteome to search for the non-redundant protein sequence database excluding experimental models and uncultured/environmental sample sequences. The general parameters used for this tool were automatically adjusted for short input sequences with a maximum target sequence of 100 and expect threshold value of 10. The 'expect threshold' (*E-value*) used in this study is the statistical significance for reporting stringent chance matches against database sequences. Significance of alignment is inversely proportional to *the E-value* i.e., lower the *E-value*, higher is the significance of amino acid sequence alignment. BLOSUM62 with existence: 11 Extension: 1 and conditional compositional score matrix adjustment were used as scoring parameters.

IEDB-based T-cell epitope mapping tool

Immune Epitope Database (IEDB) recommended/ Consensus/Combinatorial library/NN-align/SMM_ align/Sturniolo/NetMHCIIpan tools of IEDB (*http://tools.immuneepitope.org/mhcii/*) were used to predict pentadecameric (15-mer) peptides binding to MHC class II molecules [52, 53]. MHC class II-bacterial peptide predictions were performed using IEDB recommended prediction method which considers compilation of three methods (Combinatorial library, NN_align and SMM_align) methods. The median percentile rank of these three methods was used to create the rank for consensus method. NetMHCIIpan method was used only when a peptide could not be predicted by any of the

above methods. FASTA sequence of *E. coli* DnaK protein was submitted to determine its binding strength against a set of *HLA-DRB alleles* (*HLA-DRB1*01:01*, *HLA-DRB1*03:01*, *HLA-DRB1*04:01*, *HLA-DRB1*04:05*, *HLA-DRB1*07:01*, *HLA-DRB1*08:02*, *HLA-DRB1*09:01*, *HLA-DRB1*11:01*, *HLA-DRB1*12:01*, *HLA-DRB1*13:02*, *HLA-DRB1*15:01*, *HLA-DRB3* 01:01*, *HLA-DRB3*02:02*, *HLA-DRB4*01:01* and *HLA-DRB5*01:01*) present in the IEDB database. Selection of the bacterial self-peptides with high binding affinity was based on a consensus percentile rank of the top 10% output. Bacterial peptides binding to HLA-DRB alleles with a low percentile rank have stronger binding affinity and hence, are capable to trigger immune response upon infection.

Docking study

PatchDock software with default program 4.0 was used to determine the binding affinities of the ligands HSPA2 and, Dnak with their common receptor TLR 4. The protein data bank identification (*PDB ID*) numbers for HSPA2, DnaK and TLR 4 are *FSV*, *KHO* and *FXI*, respectively. This software was also used to study the HSPA2 (*PDB ID: 3i33*)-MHC Class II DR alleles (*PDB ID: 1H15* & *PDB ID: 1DLH*) binding affinity. The top ten best solutions from the PatchDock results were obtained. Then these binding solutions were further refined by performing the FireDock analysis. The output was based on the global energy which decides the binding energy of the protein interaction. Other forces such as the attractive and repulsive Van der Waal's energy (VdW), atomic contact energy (ACE) and the hydrogen bond (HB) energy bonds contributed towards the global binding energy [54]. We used Research Collaboratory for Structural Bioinformatics Protein Data Bank Identification (RCSB PDB ID) to find PDB IDs of MHC class II alleles, HSPA2, DnaK and TLR 4 proteins to perform various interactive studies.

RESULTS

Co-localization of HSPA2 with TLR 4 in spermatozoa

Upon immunostaining, co-localization of HSPA2 with TLR 4 was observed on the spermatozoa surface (Figure 1). In a LCS/BS condition, the immunologically privileged spermatozoa triggered TLR 4 signals in the sperm head and tail regions. A high expression of HSPA2 was observed in the sperm head and middle piece regions. Thus, HSPA2 and TLR 4 co-localized exactly in the head and middle piece regions of spermatozoa (Figure 1).

Interaction between HSPA2 and TLR 4

Figure 2 depicts the pathway analysis between HSPA2 and TLR 4 by using the Cytoscape PSICQUIC client service and STRING database version 11.0. It was observed that HSPA2 interacted with TLR 4 *via* 2 different channels including protein molecules HSPABP2/STUB1 (E3 ubiquitin- protein ligase) and HSPA8 (Figure 2a). This data was supported by the results obtained from the STRING database as depicted in Figure 2b. The HSPA2-TLR 4 interaction using STRING database was represented by the number of nodes, 4; number of edges, 5; average node degree, 2.5; average local clustering coefficient, 0.833 and; expected number of edges, 1. A very high statistical significance with a PPI enrichment *p-value* of 0.00361 confirmed the strong interaction between HSPA2 and TLR 4.

Binding affinity

Figure 3 shows a strong binding affinity between DnaK (*PDB ID: KHO*) and TLR 4 (*PDB ID: FXI*) upon performing ligand-receptor docking study. The attractive global energy, attractive VdW, ACE and the HB energy for this binding were found to be -5.82, -16.79, 7.06 and -2.12. The transformation values after ligand refinement were -2.205131, -0.192770, 0.161174, -31.127636, -11.294695 and -65.176582. Similarly, a strong binding affinity was found between HSPA2 (*PDB ID: FSV*) and TLR 4 (*PDB ID: FXI*) proteins (Figure 3). The attractive global energy, attractive VdW, ACE and the HB energy for this binding were found to be -7.55, -25.46, 1.19 and -2.09. The transformation values after ligand refinement were 3.080862, -0.344162, 0.007777, -37.146214, 16.364790 and 86.395988.

DnaK-mimicked HSPA2 amino acid sequence

IEDB database followed by BLASTp tool were used to derive *E. coli*-specific DnaK (*UniprotKB ID: P0A6Y8*) self-peptides in a similar manner as described in the previous study [47]. Thirty-eight putative self-peptides derived from *E. coli* DnaK protein showed homology with the human HSPA2 amino acid sequences (Table 1).

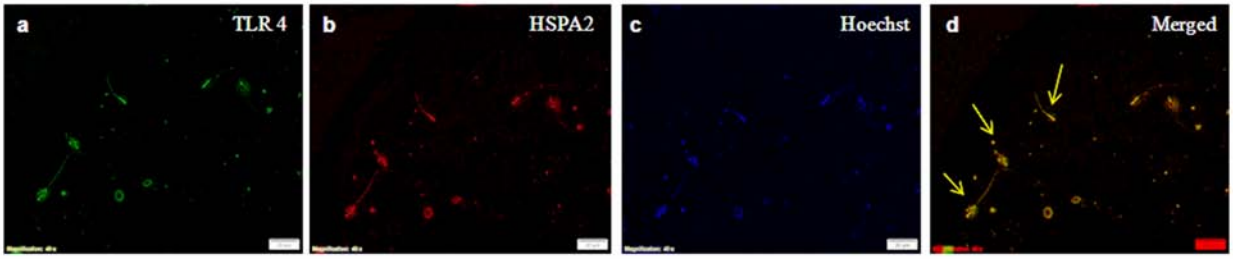

Figure 1. HSPA2 co-localizes with TLR 4 in the spermatozoa surface. (a) TLR 4 was stained with FITC (*green*); **(b)** HSPA2 was stained with Phycoerythrin (*red*) and; **(c)** nucleus was stained with Hoechst 33324 (*blue*). Merged figure represents HSPA2-TLR 4 co-localization in spermatozoa (d). Images were taken using Olympus fluorescence microscope (Olympus XC10) at 400X magnification. *Yellow arrows indicate the HSPA2-TLR 4 co-localization. Abbreviation: LCS/BS: Bacteriospermia-associated Leukocytospermia*.

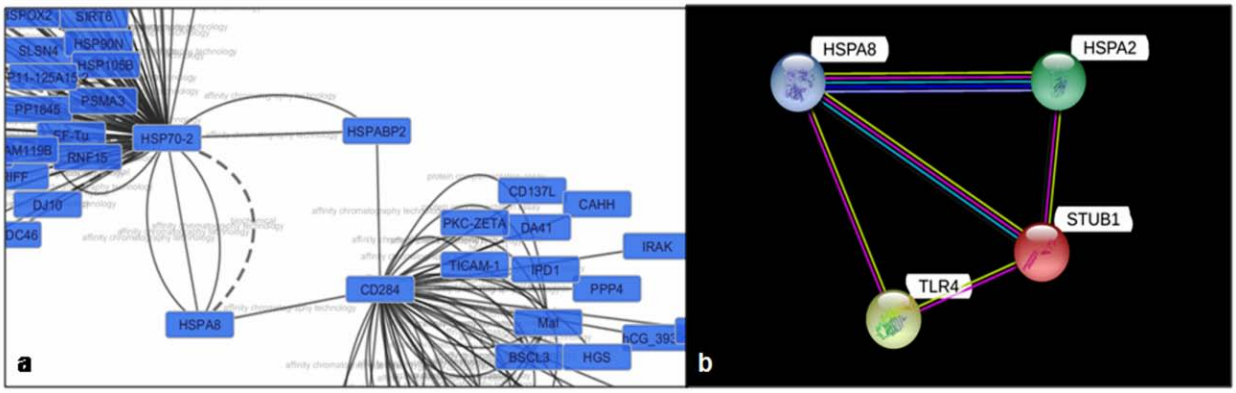

Figure 2. HSPA2 interacts with TLR 4. (a) Cytoscape pathway analytical tool PSICQUIC client service showed that testis-specific HSP70 i.e., HSPA2 interacts with TLR 4 *via* HSPABP2/STUB1 (STIP1 homology and U-Box containing protein 1) and HSPA8. **(b)** STRING pathway showed HSPA2 interaction with TLR 4 *via* HSPA8 and HSPABP2 based on curated, experimental and text mining database.

Table 1. List of self-peptides derived from *E. coli* DnaK protein showing resemblance with HSPA2.

DnaK peptides	***Self-peptides***	***HSPA2 peptides***
AIKRLIGRRFQDEEV	KRLIGR	KRLIGRKFND
FAIKRLIGRRFQDEE		FDAKRLIGRKFED
IKRLIGRRFQDEEVQ		KRLIGRKFEDATVQ
PQNTLFAIKRLIGRR		PQNTVFDAKRLIGR
TLFAIKRLIGRRFQD		TIFDAKRLIGRKFED
QNTLFAIKRLIGRRF	KRLIGRK	QNTVFDAKRLIGRKF
LFAIKRLIGRRFQDE	KRLIGRKF	FDAKRLIGRRFDD
NTLFAIKRLIGRRFQ		NTVFDAKRLIGRRF
VITVPAYFNDAQRQA	VITVPAYFND	VITVPAYFNDSQRQA
ITVPAYFNDAQRQAT	ITVPAYFND, QRQAT	ITVPAYFNDSQRQAT

Table1 continued..

TVPAYFNDAQRQATK	TVPAYFND, QRQATK	TVPAYFNDSQRQATK
VPAYFNDAQRQATKD	VPAYFND, QRQATKD	VPAYFNDSQRQATKD
PAYFNDAQRQATKDA	PAYFND, QRQATKDA	PAYFNDSQRQATKDA
AYFNDAQRQATKDAG	AYFND, QRQATKDAG	AYFNDSQRQATKDAG
YFNDAQRQATKDAGR	QRQATKDAG	YFNDSQRQATKDAG
GDVKDVLLLDVTPLS	LLLDVTPLS	NVQDLLLLDVTPLS
DVKDVLLLDVTPLSL	LLLDVTPLSL	NVQDLLLLDVTPLSL
DVLLLDVTPLSLGIE	LLLDVTPLSLGIE	DLLLLDVTPLSLGIE
EVKRIINEPTAAALA	RIINEPTAAA	RIINEPTAAAIA
GLEVKRIINEPTAAA		GLNVLRIINEPTAAA
KRIINEPTAAALAYG		RIINEPTAAAIAYG
LEVKRIINEPTAAAL		LNVLRIINEPTAAA
VKRIINEPTAAALAY		RIINEPTAAAIAY
PQIEVTFDIDADGIL	PQIEVTFDIDA	PQIEVTFDIDANGIL
QIEVTFDIDADGILH	QIEVTFDIDA	QIEVTFDIDANGIL
IEVTFDIDADGILHV	IEVTFDIDA	IEVTFDIDANGILNV
EVTFDIDADGILHVS	EVTFDIDA	EVTFDIDANGILNV
VTFDIDADGILHVSA	VTFDIDA	VTFDIDANGILNVTA
TFDIDADGILHVSAK	TFDIDA	TFDIDANGILNVTA
FDIDADGILHVSAKD	FDIDA	FDIDANGILNVTATD
VKDVLLLDVTPLSLG	LLLDVTPLSLG	VQDLLLLDVTPLSLG
KDVLLLDVTPLSLGI	LLLDVTPLSLGI	DLLLLDVTPLSLGI
LLLDVTPLSLGIETM	LLLDVTPLSLGIET	LLLDVTPLSLGIET
VLLLDVTPLSLGIET		LLLDVTPLSLGIET
KEPRKDVNPDEAVAI	NPDEAVA	KELNKSINPDEAVA
TLIAKNTTIPTKHSQ	NTTIPTK	LIKRNTTIPTKQTQ
TTLIAKNTTIPTKHS		TPLIKRNTTIPTKQ
VMTTLIAKNTTIPTK		VMTPLIKRNTTIPTK

Binding affinity of HSPA2 with HLA-DR

In accordance with the results of IEDB binding affinity of DnaK with MHC class II DRB allele, it was important to determine the binding affinity of its homolog HSPA2 with the same MHC molecule. To detect such a binding affinity between HSPA2 and TLR 4, PatchDock study was performed. Figure 4a illustrates a strong binding affinity between the HLA class II allele DRA1/DRB5 (*PDB ID: 1H15*) and HSPA2 (*PDB ID: 3i33*). The attractive global energy, attractive VdW, ACE and the HB energy for this interaction were -14.48, -18.25, 2.42 and -0.66, respectively. Similarly, Figure 4b illustrates the strong binding affinity between the HLA class II allele DR1 (*PDB ID: 1DLH*) and human sperm protein HSPA2 (*PDB ID: 3i33*). The attractive global energy, attractive VdW, ACE and the HB

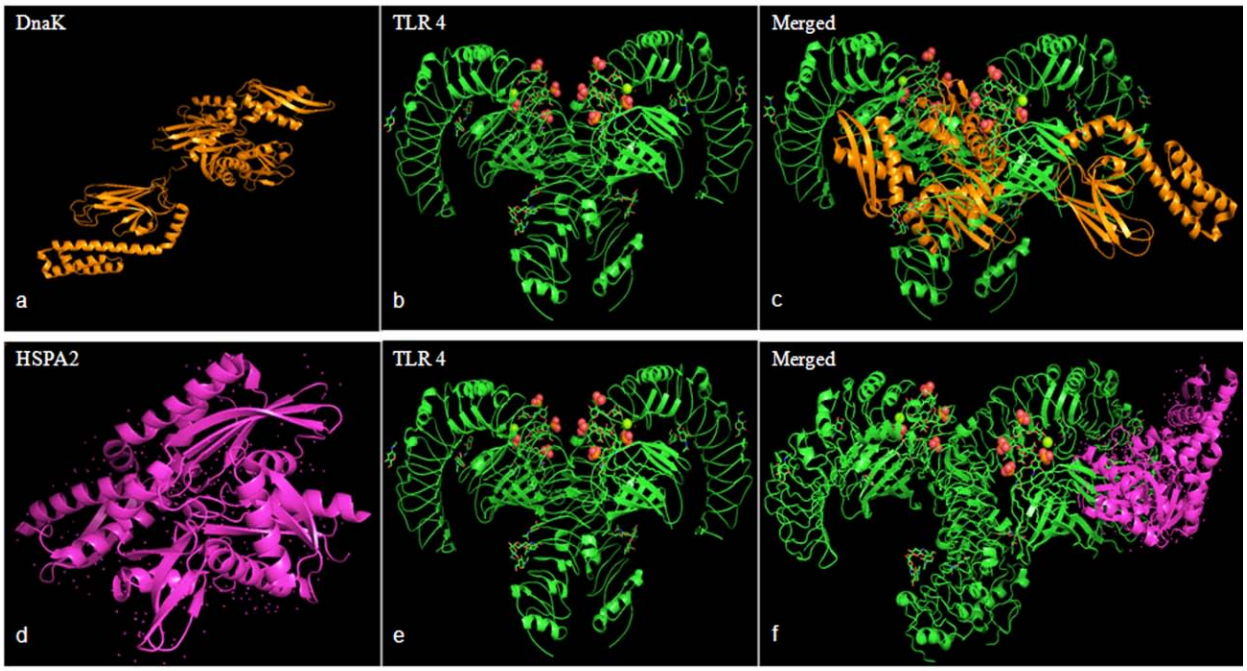

Figure 3. Ligand-receptor docking study. **(a-c)** A strong binding affinity was determined between DnaK (*PDB ID: KHO*) and TLR 4 (*PDB ID: FXI*) upon merging **(d-f)** A strong binding affinity was determined between HSPA2 (*PDB ID: FSV*) and TLR 4 (*PDB ID: FXI*) proteins upon merging. The parameters used for the binding affinity study were attractive global energy, attractive Vander der Waal's energy (VdW), atomic contact energy (ACE) and the hydrogen bond (HB) energy. Color representation of protein structures: *Orange-DnaK*; *Magenta-HSPA2* and; *Green-TLR 4*.

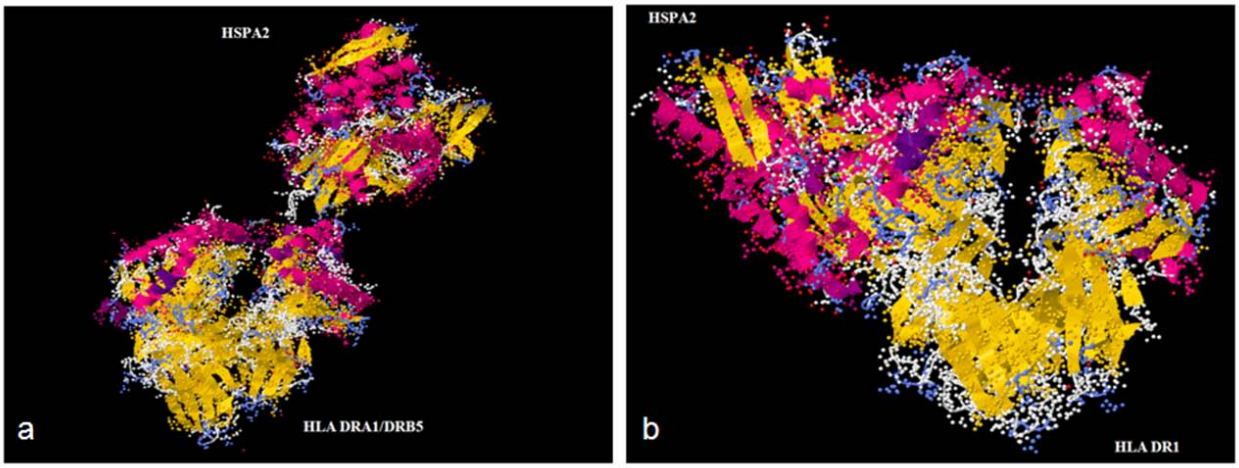

Figure 4. Binding affinity of HSPA2 with HLA-DR alleles. (a) A strong binding affinity between HLA class II allele DRA1/DRB5 (*PDB ID: 1H15*) and HSPA2 (*PDB ID: 3i33*) is illustrated in this figure. **(b)** A strong binding affinity between HLA class II allele DR1 (*PDB ID: 1DLH*) and HSPA2 (*PDB ID: 3i33*) is illustrated. The parameters used for this study were attractive global energy, attractive Vander der Waal's energy (VdW), atomic contact energy (ACE) and the hydrogen bond (HB) energy. Color representation of the secondary structure from protein PDB files was read by using Jmol: *Yellow - β strand*; *Pink - α helix*; *Purple - 3_{10} helix*; *Violet - π helix*; *Blue-(β) turn* and; *White - others*.

energy for this interaction were -2.63, -4.32, -0.32 and -0.26, respectively.

Sequence similarity between DnaK and HSPA2 proteins

Multiple sequence alignment between HSPA2 (*UniproKB ID: P54652*) and DnaK (*UniprotKB ID: P0A6Y8, Strain K12; A0A140NFV, Strain B*) protein sequences was performed according to the norms of the CLUSTAL Ω tool (version 1.2.1). HSPA2 containing 639 amino acid sequences were aligned with 638 amino acid sequences of DnaK protein of *E. coli* origin. HSPA2 and DnaK shared 47% identity as depicted in Figure 5.

DISCUSSION

TLR 4 is profoundly expressed on the spermatozoa surface in a LCS/BS condition. These PRRs symbolize the innate immune response that helps the sperm to develop an early protection against bacteria invasion. A high colony forming unit (CFU) of bacteria in the semen has the greatest possibility to cause sperm DNA damage, morphological alterations and enhanced oxidative stress. In fact, this may lead to prolonged infertility. As pathological stress is induced, the role of stress chaperones such as HSP70 comes into play. HSP70 can be triggered in two situations, either as a protector or, as a death indicator. HSP70 has binding affinity towards various cell membranes, including those of bacteria (DnaK protein), leukocytes (HSP70) and sperm (HSPA2). Various studies have shown that HSP70 acts as a ligand to the TLR 4 receptor on different cells. In order to detect whether an interaction exists on the sperm surface in a LCS/BS condition, immunocytochemistry technique and bioinformatics tools were used. Co-localization of HSPA2 with TLR 4 was clearly visualized in the spermatozoa surface confirming a strong binding affinity between them. Earlier studies have shown that TLR 4 is localized in the spermatozoa head including acrosome and nucleus and, in the tail regions in a LCS sample [38, 40, 55]. Similarly, HSPA2 is localized in the sperm head and acrosomal regions [32, 37, 56]. These findings substantiate the results reported in this study. A high intensity HSPA2 co-localized with TLR 4 on the spermatozoa surface indicates towards a possible existence of ligand-receptor engagement between them which was further supported by PatchDock results.

Several investigations have been made to understand the phenomenon of HSP70-TLR 4 binding on different cells undergoing stress conditions. It is very interesting to find such a relationship in spermatozoa in a LCS/BS condition. Mostly infertile men have been reported with low intensity HSPA2 or, with complete lack of HSPA2 in the sperm head [4, 37]. However, a high intensity HSPA2 along with the TLR 4 expression in the spermatozoa surface indicates a probable binding of extracellular HSP70 with the sperm TLR 4. A 47% amino acid sequence similarity between the HSP70 family members i.e., HSPA2 and DnaK proteins makes it even clearer that probably the HSP70 co-localized with TLR 4 in the spermatozoa surface necessarily might not be the testis-specific HSPA2. It can either be a DnaK protein secreted from the bacteria *E. coli* or, HSP70 protein secreted from the leukocytes. It is known that HSP70 protein comprises of HSP70.1 and HSP70.2/HSPA2 which differ from each other by only two amino acids [5, 57]. Moreover, these studies also suggest that HSP70 is 90% identical with HSP70.1, while HSP70.1 shares 85% similarity with HSP70.2 (HSPA2). DnaK protein, being one of the most sought after *E. coli* HSP70 protein with 80% intraspecies amino acid sequence identity, shares 57% similarity with human HSP70 sequences [45]. It is quite evident that secreted HSP70 binds to TLR 4 on various cells [5, 8, 10-12]. Based on these identity facts that HSP70 resembles HSPA2, a strong HSPA2-TLR 4 binding in the spermatozoa surface was achieved by using PatchDock tool. From the IEDB T-cell epitope mapping study the binding affinity of DnaK protein with MHC class II DRB allele was identified. But the binding affinity of DnaK-derived auto-antigen, HSPA2, with MHC class II allele remains unclear. To prove this point, PatchDock study was conducted confirming a strong binding affinity between HSPA2 and MHC class II DRB allele. The identical amino acid sequences between DnaK and HSPA2 protein sequences increased fair chances of binding with the same cell surface receptor i.e., TLR 4. Although HSPA2-TLR 4 direct binding can occur as a result of secretion of HSPA2 in the LCS/BS pathological condition, binding of *E. coli* DnaK-derived HSPA2

with TLR 4 is also expectable. Therefore, it was necessary to detect the DnaK-TLR 4 binding affinity as a control study. The autoantibodies developed against HSPA2 auto-antigens are expected to develop an autoimmune environment in the semen, ultimately causing immune infertility. Thus, strong binding affinity between HSPA2-TLR 4 is a great example of the association between genital tract infections; LCS/BS-induced immune infertility; innate immunity and; HSPA2 involvement in one package.

Studies have shown that SIGLEC (sialic acid-binding immunoglobulin-like lectin)-5 and -14 immunoreceptors can bind to HSP70 ligands and meanwhile, they are also involved in blocking inflammatory response by interacting with TLRs [58, 59]. Later, it was discovered that SIGLEC-14 enhances TLR signaling and SIGLEC-5 blocks TLR signaling by binding to HSP70 ligands. These results clarified various contradictions associated with the immunoregulatory functions of HSP70 [5]. Despite such a disagreement, HSP70 interaction with TLR 4 on the sperm surface is quite fascinating. Requirement of a pathway involving intermediate/interacting molecules for the intriguing HSPA2-TLR 4 binding is highly recommended. Thus, Cytoscape pathway analytical tool and STRING database were used to determine HSPABP2/STUB1 (STIP1 homology and U-Box containing protein 1) and HSPA8 as the interacting mediators in the HSPA2-TLR 4 interaction. STUB1, otherwise known as CHIP (carboxyl terminus of Hsc70-interacting protein) increases HSP70 induction during stress conditions and brings them back to normal. Basically, it helps in the maintenance of chaperones during stress and recovery as well [60]. STUB1 also binds to HSPA8 and HSP70 at the beginning but later, involves in a negative regulation of the forward reaction [61]. Like HSP70, HSPA8 (HSC70) acts under both normal as well as stress conditions [62, 63]. HSPA8 has low affinity for its substrate proteins when in ATP-bound state [64]. Upon ATP hydrolysis to ADP, HSPA8 undergoes conformational modifications to bind with other substrate proteins such as BAG1 (Bcl2-associated athanogene 1), BAG2, BAG5, HSPH1 (BAG3, BAG4, HSPH1, HSP40, HSP90, HIP (HSC70-interacting protein), hsc70-hsp90 organizing protein (HOP), STUB1, etc., *via* a linker present in its structure [64, 65]. In addition, it also binds with the bacterial lipopolysaccharide (LPS) and triggers LPS-induced inflammatory response such as TNF secretion by monocytes [66, 67]. The association of HSPA8 with the cell surface might help immune cells in cell recognition and act as a cell receptor in case of viral infections [64, 68-73]. Moreover, STRING results further strengthened the HSPA2-TLR 4 interaction based on several curated, experimentally determined and predicted interaction databases. Altogether, these interacting mediators and functional facts accentuate the possibility of a strong interaction between HSPA2 and TLR 4 in the spermatozoa.

The emergence of extracellular HSP70 has significantly increased due to their interaction with the complicated immune system and promotion of intercellular signaling and transport [74]. These proteins are released in stress conditions (including bacterial infections) and then they bind to the adjacent cell surface through cell surface receptors such as TLR 4. Thus, the binding property of the HSP70 to different cell surface receptor instigates physiological functions in immune cells such as leukocytes**.** Depending on the type of cell surface receptor it binds, HSP70 can be either immunoregulatory or, inflammatory in nature. Hence, it has become necessary to understand the complicated immunological properties of HSP70 family members during pathogenesis conditions [75]. The most astonishing fact lies in the mechanism through which these extracellular HSP70s are released. Due to the deficiency of a hydrophobic N-terminus, it is quite impossible for the cells to release HSP70 proteins in their secreted form. A non-canonical secretion approach has been followed by the cells to release HSP70s in its free secreted form. HSP70 is secreted as well as co-secreted from cells with lysosomal effect. Release of HSP70 by different cellular types in lipid vesicles such as ectosomes (cytosolic proteins and exosomes) has been reported previously. Studies have shown that HSP-containing exosomes contribute towards a wide variety of immunological functions based on the protein content of the exosomes, cell origin and cell of interest. DnaK, a

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sp|P54652|HSP72_HUMAN           MSARGPAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTD-TERLIGDAAKNQ     59
sp|P0A6Y8|DNAK_ECOLI            ---MGKIIGIDLGTTNSCVAIMDGTTPRVLENAEGDRTTPSIIAYTQDGETLVGQPAKRQ     57
tr|A0A140NFV3|A0A140NFV3_ECOBD  ---MGKIIGIDLGTTNSCVAIMDGTTPRVLENAEGDRTTPSIIAYTQDGETLVGQPAKRQ     57

sp|P54652|HSP72_HUMAN           VAMNPTNTIFDAKRLIGRKFEDATVQSDMKHWPFRVVSEGGKPKVQVEYKGETKTFFPEE    119
sp|P0A6Y8|DNAK_ECOLI            AVTNPQNTLFAIKRLIGRRFQDEEVQRDVSIMPFKIIAADNGD-AWVEVK--GQKMAPPQ    114
tr|A0A140NFV3|A0A140NFV3_ECOBD  AVTNPQNTLFAIKRLIGRRFQDEEVQRDVSIMPFKIIAADNGD-AWVEVK--GQKMAPPQ    114

sp|P54652|HSP72_HUMAN           ISSMVLTKMKEIAEAYLGKKVHSAVITVPAYFNDSQRQATKDAGTIIGLNVLRIINEPTA    179
sp|P0A6Y8|DNAK_ECOLI            ISAEVLKKMKKTAEDYLGEPVTEAVITVPAYFNDAQRQATKDAGRIAGLEVKRIINEPTA    174
tr|A0A140NFV3|A0A140NFV3_ECOBD  ISAEVLKKMKKTAEDYLGEPVTEAVITVPAYFNDAQRQATKDAGRIAGLEVKRIINEPTA    174

sp|P54652|HSP72_HUMAN           AAIAYGLDKKGCAGEKNVLIFDLGGGTFDVSILTIED----GIFEVKSTAGDTHLGGED    235
sp|P0A6Y8|DNAK_ECOLI            AALAYGLDKG---TGNRTIAVYDLGGGTFDISIIEIDEVDGEKTFEVLATNGDTHLGGED    231
tr|A0A140NFV3|A0A140NFV3_ECOBD  AALAYGLDKG---TGNRTIAVYDLGGGTFDISIIEIDEVDGEKTFEVLATNGDTHLGGED    231

sp|P54652|HSP72_HUMAN           FDNRMVSHLAEEFKRKHKKDIGPNKRAVRRLRTACERAKRTLSSSTQASIEIDSLYE---    292
sp|P0A6Y8|DNAK_ECOLI            FDSRLINYLVEEFKKDQGIDLRNDPLAMQRLKEAAEKAKIELSSAQQTDVNLPYITADAT    291
tr|A0A140NFV3|A0A140NFV3_ECOBD  FDSRLINYLVEEFKKDQGIDLRNDPLAMQRLKEAAEKAKIELSSAQQTDVNLPYITADAT    291

sp|P54652|HSP72_HUMAN           -GVDFYTSITRARFEELNADLFRGTLEPVEKALRDAKLDKGQIQEIVLVGGSTRIPKIQK    351
sp|P0A6Y8|DNAK_ECOLI            GPKHMNIKVTRAKLESLVEDLVNRSIEPLKVALQDAGLSVSDIDDVILVGGQTRMPMVQK    351
tr|A0A140NFV3|A0A140NFV3_ECOBD  GPKHMNIKVTRAKLESLVEDLVNRSIEPLKVALQDAGLSVSDIDDVILVGGQTRMPMVQK    351

sp|P54652|HSP72_HUMAN           LLQDFFNGKELNKSINPDEAVAYGAAVQAAILIGDKSENVQDLLLLDVTPLSLGIETAGG    411
sp|P0A6Y8|DNAK_ECOLI            KVAEFF-GKEPRKDVNPDEAVAIGAAVQGGVLTGD----VKDVLLLDVTPLSLGIETMGG    406
tr|A0A140NFV3|A0A140NFV3_ECOBD  KVAEFF-GKEPRKDVNPDEAVAIGAAVQGGVLTGD----VKDVLLLDVTPLSLGIETMGG    406

sp|P54652|HSP72_HUMAN           VMTPLIKRNTTIPTKQTQTFTTYSDNQSSVLVQVYEGERAMTKDNNLLGKFDLTGIPPAP    471
sp|P0A6Y8|DNAK_ECOLI            VMTTLIAKNTTIPTKHSQVFSTAEDNQSAVTIHVLQGERKRAADNKSLGQFNLDGINPAP    466
tr|A0A140NFV3|A0A140NFV3_ECOBD  VMTTLIAKNTTIPTKHSQVFSTAEDNQSAVTIHVLQGERKRAADNKSLGQFNLDGINPAP    466

sp|P54652|HSP72_HUMAN           RGVPQIEVTFDIDANGILNVTAADKSTGKENKITITNDKGRLSKDDIDRMVQEAERYKSE    531
sp|P0A6Y8|DNAK_ECOLI            RGMPQIEVTFDIDADGILHVSAKDKNSGKEQKITIKASSG-LNEDEIQKMVRDAEANAEA    525
tr|A0A140NFV3|A0A140NFV3_ECOBD  RGMPQIEVTFDIDADGILHVSAKDKNSGKEQKITIKASSG-LNEDEIQKMVRDAEANAEA    525

sp|P54652|HSP72_HUMAN           DEANRDRVAAKNALESYTYNIKQTVEDEKLRGKISEQDKNKILDKCQEVINWLDRNQMAE    591
sp|P0A6Y8|DNAK_ECOLI            DRKFEELVQTRNQGDHLLHSTRKQVEE--AGDKLPADDKTAIESALTALETALK---GED    580
tr|A0A140NFV3|A0A140NFV3_ECOBD  DRKFEELVQTRNQGDHLLHSTRKQVEE--AGDKLPADDKTAIESALTALETALK---GED    580

sp|P54652|HSP72_HUMAN           KDEYEHKQKELERVCNPIISKLYQGGPGGGSGGGSGASGGP-------TIEEVD---  639
sp|P0A6Y8|DNAK_ECOLI            KAAIEAKMQELAQVSQKLMEIAQQQHAQQQTAGADASANNAKDDDVVDAEFEEVKDKK  638
tr|A0A140NFV3|A0A140NFV3_ECOBD  KAAIEAKMQELAQVSQKLMEIAQQQHAQQQTAGADASANNAKDDDVVDAEFEEVKDKK  638
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Figure 5. HSPA2 and DnaK share 47% amino acid sequence identity. This study showed conserved pattern present between DnaK protein of *E. coli* bacteria (*UniprotKB ID*: *P0A6Y8, Strain K12*; *A0A140NFV, Strain B*) and human HSPA2 protein (UniprotKB ID: *P54652*) using multiple sequence alignment tool Clustal Ω. *Cyan color represents the homologous amino acids.*

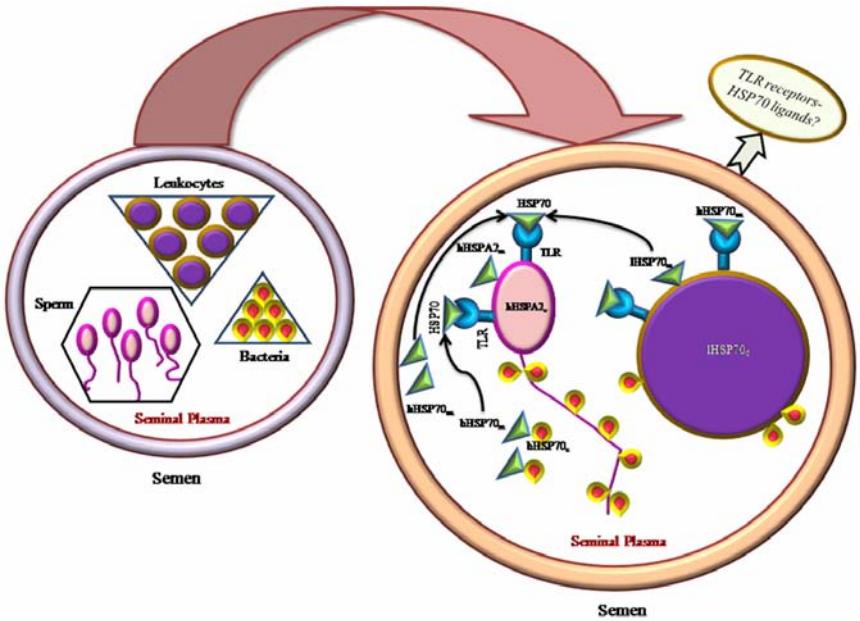

Figure 6. Prediction of a possible HSPA2 ligand-TLR receptor binding on the spermatozoa surface. This figure illustrates a future positive binding that might exist between HSPA2 and TLRs. Purple circle: semen obtained from leukocytospermia/bacteriospermia patient which contains leukocytes (violet circles in triangle); sperm (pink in hexagon) and; bacteria (yellow-red in triangle). The peach circle signifies the possible binding that might occur between extracellular HSP70 family members and TLRs. Extracellular HSPs (HSP70ex) are shown to bind to TLRs on the spermatozoa. These HSP70ex can be either membranous (m) or, cytosolic (c) secreted *via* bacteria (bHSP70m/bHSP70c), leukocytes (lHSP70m) and human sperm (hHSP70m/hHSP70c).

member of the HSP70 family, is secreted or remain bound to the membrane in acute stress conditions as a result of which, bacterial cell can raise stress tolerance and survive in a variety of conditions [57]. The reason behind such a response is the ability of the bacteria to remodel protein complexes and promote phosphorylation dependent signal transduction systems ultimately leading to cell sustenance without affecting the pathogenicity. Secretion of exosomes by cellular plasma membrane is also carried out by bacterial cells. Both gram negative as well as gram positive bacteria bear the ability to secrete biologically active outer membrane vesicles or, OMVs [76-83]. Thus, combining the above facts it can be deciphered that DnaK-containing exosomes in bacteria can lead to immunostimulatory as well as immunosuppressive functions.

CONCLUSION

From the above studies, it can be concluded that there is a possibility of true ligand-receptor binding between HSPA2 and TLR 4 on the spermatozoa surface under varied pathophysiological conditions such as LCS/BS only, or, LCS/BS-induced immune infertility. HSPA2 proteins can be secreted from a wide array of cells both in the free and membrane bound state and, cells undergoing necrosis also release HSPs from their membrane to the cytosol in the form of extracellular particles. Thus, it is obvious that HSPA2 present in the spermatozoa is also released to the exterior upon membrane damage due to induction of oxidative stress during a pathophysiological condition. In a previous study it has been reported that TLR ligands are the ultimate channel for therapeutic vaccine delivery due to their ability to bind covalently to various antigens [84], thereby suggesting a possible HSPA2-TLR interface. Thus, Figure 6 provides an overview of the interaction between extracellular HSP70 and the TLR receptors on the spermatozoa surface indicating a possible ligand-receptor relationship between HSPA2 and TLR 4, in particular.

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AUTHOR CONTRIBUTION

RP designed the study, conducted experiments, performed bioinformatics study, analyzed data and wrote the manuscript. PKJ and LS supervised the study. SK provided clinical samples.

CONFLICT OF INTEREST STATEMENT

No conflict of interest.

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