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# THE ROLE OF HUMAN 70 KD HEAT SHOCK PROTEIN AS RISK FACTOR IN IMMUNE RESPONSE OF RHEUMATOID ARTHRITIS PATIENTS IN THI-QAR PROVINCE, SOUTHERN OF IRAQ

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## ABSTRACT

Heat shock proteins or HSPs are treated under different kinds of stress conditions and act as molecular chaperones for protein molecules. As these proteins were first found in cells that were exposed to high temperature levels, they are called "heat shock proteins". Several risk factors are known to be involved in the pathogenesis of autoimmune rheumatic diseases, including genetic factors, smocking, chronic infections, sex hormones and stress.

Stress is now considered as an important risk factor in the pathogenesis of rheumatoid arthritis by considering the activation of the stress response of the immune system. This study deals with the investigation about the role of heat shock protein 70 in pathogenesis and immune response in RA. The sample of the study includes 145 patients with RA. These patients were tested for four or more of the criteria of the 2010 American College of Rheumatology, the sample of control in the study include 60 persons who were actually health volunteers.

This study shows a high level of statistical significance ($P<0.00$). When we measured concentration of HSP70 on samples between the test group and control group, we understand that there is high level of statistical significance between the test groups ($P<0.05$) based on the gender and this study shows high significant difference between subgroups of patients such as smokers, non-smokers, stressed, non-stressed and the low economic status e.t.c.

**Conclusions**

Some of the environmental factors such as smoking, stress, other chronic infections, duration of disease, age and sex reactive with genetic factors play an important role in the determination of immunological marker in patients with RA.

**KEYWORDS:** Rheumatoid Arthritis, Heat Shock Protein 70, Smoking, Stress, Immune Response

## INTRODUCTION

The heat shock protein 70 family is a set of highly conserved proteins that are induced by a variety of biological stress. The human HSP70 family members include: HSP70, a 70 Kda protein which is strongly induced in all organisms but which is also constitutively expressed in primate cells HSP72, a72 Kda protein which is induced and expressed in protein exclusively under stress conditions ( Ritossa F.,1962).

As newly synthesized proteins emerge from the ribosomes, the substrate binding domain of Hsp70 recognizes the sequences of hydrophobic amino acid residues, and subsequently interacts with them. This spontaneous interaction is reversible, and in the ATP bound state, Hsp70 may freely bind together and thus release peptides. However, the presence of

a peptide in the binding domain stimulates the ATPase activity of Hsp70. These co-chaperones drastically increases the ATPase activity of Hsp70 in the presence of interacting peptides (Bahr *et al.*,1990). By binding firmly to partially synthesized peptide sequences (incomplete proteins), Hsp70 prevents them from aggregating and being rendered nonfunctional. Once the entire protein has completely synthesized, a nucleotide exchange factor (BAG-1 and HspBP1 are among those which have been identified) stimulates the release of ADP and binding of fresh ATPs, thus opening up the binding pocket. The protein is then free to fold on its own, or to be transferred to other chaperones.

For further processing. HOP (the Hsp70/Hsp90 Organizing Protein) can bind to both Hsp70 and Hsp90 simultaneously, and mediates the transfer of peptides from Hsp70 to Hsp90**.** Hsp70 thus also aids in trans membrane transport of proteins, by stabilizing them in a partially folded state.**(**Wegele *et al*., 2004).

Hsp70 proteins can act to shield cells from thermal or oxidative stress. These stress normally act to damage proteins, causing partial unfolding and possible aggregation. By temporarily binding to hydrophobic residues exposed by stress, Hsp70 prevents these partially denatured proteins from aggregating, and allows them to refold.((Albani *et al*., 1995) Low ATP is characterized by a heat shock and sustained binding is seen as aggregation of suppression, suggesting a second mode of binding regulation based on oxidative stress. Hsp70 seems to be able to participate in the disposal of damaged or defective proteins (Satpute *at el* 2009).

Interaction with CHIP (*C*arboxyl-terminus of HSP 70 Interacting Protein) – an E3 ubiquitin ligase, allows Hsp70 to pass proteins to the cell's ubiquitination and proteolysis pathways. Consequently, in addition to improving overall protein integrity, HSP 70 directly inhibits apoptosis (Beere, *et al*., 2000).

There are a number of studies that have confirmed the presence of antibodies against HSPs belonging to the HSP70 (Chukwuocha *et al*., 1999; Yoshida A., 2001), besides other studies which also confirmed the presence of antibodies against serpins (HSP47) (Hattori, 2000) and against HSP60 (Tsoulfa *et al*.,1989) Studies also confirmed the presence of antibodies against HSP70 (Mavropoulos *et al*., 2005). However, previous researches were not limited to the member of the HSP family, they also confirmed the presence of auto-antibodies in HSP90 family which was consistently identified in sera from patients with RA. One of the target antigens for anti-HSP antibodies in RA being human GRP78 (BiP), which also serves as a T-cell target in this disease (Blass, Union *et al*., 2001; Mavropoulos,*et al*., 2005).

The majority of studies on HSP-induced T-cell activation in RA are focused on the HSPs family, including human HSP70 (Li *et al*.,1992;) and HSP70 with HSP90 (Huang *et al*.,2009) The mononuclear cells in the synovial fluid of RA patients frequently show higher responses after stimulation with HSP than those in the peripheral blood (Fischer, 1991). These results apparently offer substantial support to the idea regarding the role of HSP-reactive T-cells in RA synovitis. Some investigations have identified specific epitopes of HSPs involved in the activation of T-cell in human RA, such as regions 1-170 (Celis *et al*., 1997), 241-255 (Li SG, *et al*.,1992) 303-540 (Celis *et al*., 1997), and 451-466 (Gaston *et al*., 1991) a peptide fragment of *Ecoli*HSP70 (Prakken *et al*., 2004 ). It has been proposed that bacterial HSP-induced T-cells may cause arthritis through a cross-reactivity with the homologous human HSP (Li *et al*., 1992; Albani *et al*., 1995; Auger et *al*.,.2002). A few studies have refuted any specific association between the T- cells response against HSP70 and RA (Fischer, *et al*., 1991; Wendling., *et al*; Zou et *al*., 2002).

**Aim of the Study:** The investigation of heat shock proteins 70 (HSP70) in sera of RA patients as the pathogenesis

of autoimmune arthritis.

## MATERIALS AND METHOD

**Subjects** The study groups which has been investigated, includes:

**The Patients Group:** The study included 145 Iraqi RA patients, aged between 5-75 years. Those patients were attending the consultant clinic for Rheumatology in Al-Husain Teaching Hospital from September 2014 to Septembe2015.

The committee of rheumatologists performed the clinical examination under the supervision of staff in the rheumatology unit.

**The Control Group:** Sixty persons apparently healthy persons were included in this study as a healthy control group., who have no history or clinical evidence of RA or any other chronic disease, and no obvious abnormalities observed.

### Blood Samples

Blood samples were collected from patients and controls (five milliliters of venous blood) were drawn by 22G disposable syringe under aseptic technique. Each blood sample was further divided into two parts:

- Three millilitres of blood samples were directly added to a sterile tube containing EDTA for WBC total count, WBC deferential count and phagocytosis processes within 2 hours.
- Two millilitres of blood samples were placed in a sterile plane tube and allowed to clot, then serum was separated by centrifugation at 2500 r.p.m. for nearly15 minutes. The serum was stored at -10 degrees Celsius. These 200 sera (150 RA patients and 60 controls) were used for estimating the concentration of heat shock protein 70 (HSPs 70). (Human Heat Shock Protein 70 (HSP-70)ELISA Kit assay). As sourced from Human company, Germany.

## RESULTS

Measuring concentration of HSP 70 in the samples have been studied in the experiment depending on the results as shown in table 4-8. The table shows a high significant difference between the patients group and the control group. HSP 70 concentration shows significantly higher difference in male group than in females at 0.05 level (2-tailed) =.000, as shown in the table No. 2. We have found a high significant difference between each of the groups (21-40years) and (41-60 ) than in age group (1-20 years, and 60> years ), age group (41-60 years) found significantly less difference than the age group (21-40 years) and the group (1-20 years). Small differences between other age groups are found to be non-significant

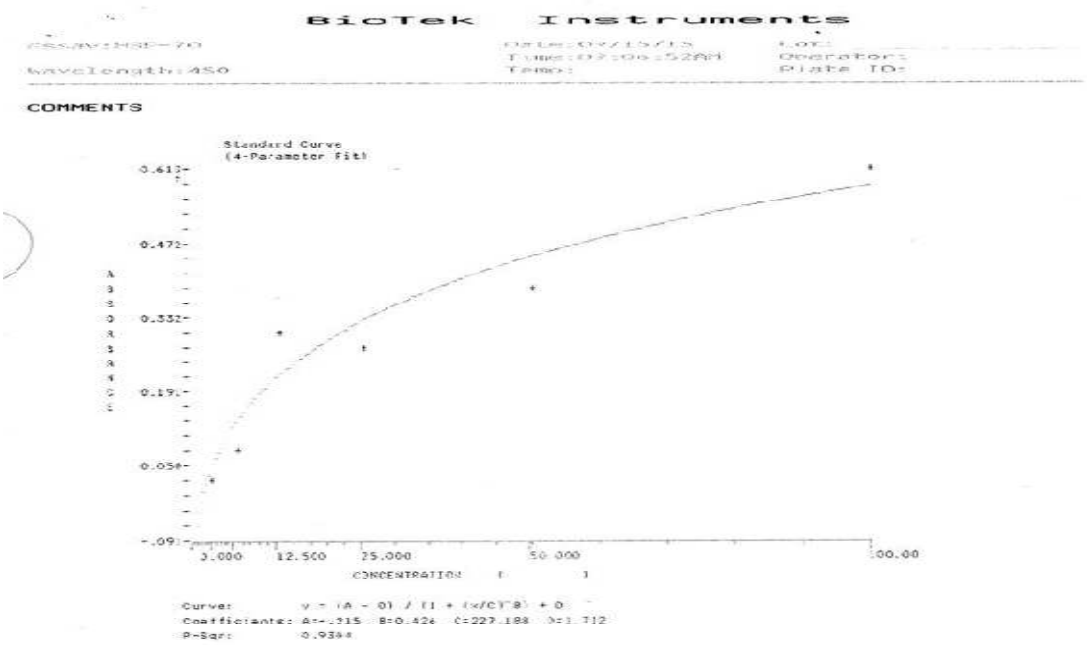


**Figure 1: Curve of Concentration of HSP 70**

**Table1: Evaluation of the Concentration of HSP70 (pg/ml) in the Study Group**

| Groups | No. | % of No. | Mean of HSP70 Concentration | t | df | Sig. |
|---|---|---|---|---|---|---|
| **Patients gruop** | **50** | **83.3%** | **1.022±.33** | **5.136** | **58** | **.000 HS** |
| **control** | **10** | **16.6%** | **0.47±.07** | | | |
| **male** | **33** | **66%** | **1.010±.32** | **-.343** | **48** | **.540 NS** |
| **female** | **17** | **34%** | **1.046±.36** | | | |

HS= High Significant difference (P<0.001).

NS= Non Significant difference (P>0.05).

df :Degree of freedom

According to the statistical analysis in tables 1 and 2 as shown below, the results demonstrated that the concentration of HSP70 in the age group (41-60) was higher than the other age groups by significant difference (P≤0.001).

**Table 2: Demonstrated Concentration of HSP70 (Ng/Ml) According to the Age Groups**

| Groups | No. | % of No. | Mean±SD of HSP70 levels |
|---|---|---|---|
| **(1-20) group age** | **10** | **20%** | **.73 ±.30** |
| **(21-40) group age** | **20** | **40%** | **1.11±.36**** |
| **(41-60) group age** | **10** | **20%** | **1.20±.15**** |
| **>60 group age** | **10** | **20%** | **.95±.25** |

The mean difference is significant at the.05 level.**is significant at 0.001

NS mean non difference is significant (P>0.05).

**Table 3: Shown the Frequency of Concentration of HSP 70 in Patients Group with Mean of Age**

| Age Mean | No. of Cases | Mean* | Std. Deviation | Percentage** |
|---|---|---|---|---|
| 25.00 | 3 | 1.015 | .178 | 6.0% |
| 26.00 | 3 | .939 | .425 | 6.0% |
| 30.00 | 5 | 1.110 | .399 | 10.0% |

| Table 3: Contd., | | | | |
|---|---|---|---|---|
| 32.00 | 3 | .893 | .249 | 6.0% |
| 35.00 | 2 | 1.318 | .126 | 4.0% |
| 38.00 | 3 | .860 | .356 | 6.0% |
| 40.00 | 2 | 1.038 | .187 | 4.0% |
| 42.00 | 2 | 1.387 | .168 | 4.0% |
| 45.00 | 2 | 1.180 | .132 | 4.0% |
| 47.00 | 5 | 1.064 | .340 | 10.0% |
| 50.00 | 6 | .883 | .408 | 12.0% |
| 60.00 | 2 | .734 | .523 | 4.0% |
| 65.00 | 6 | 1.200 | .236 | 12.0% |
| 70.00 | 6 | .823 | .401 | 12.0% |
| **Total** | **50** | **1.015** | **.337** | **100.0%** |

*mean of concentration of HSP70 in sera of RA. Patients.
** percentage of number of cases.

Table 3 depicts the frequency of concentration of HSP 70 in treatment group with mean of age It is found that there is higher level of concentration in patients who are aged about 42 years than lower concentration in patients who are aged about 60 years and we can understand that the concentration is lower as the age progresses beyond 60 years.

**Table 4: shown HSP 70 Concentration between Studied Group According Smoking Factor**

| Name of Group | N | Mean* | Std. Deviation | Percentage ** |
|---|---|---|---|---|
| Smoking patients | 17 | 1.0199 | 0.3396 | 26.6% |
| Patients( non-smoking) | 13 | 0.8327 | 0.3021 | 21.2% |
| Healthy (smoking ) | 14 | 0.4405 | 0.1012 | 25.5% |
| Healthy (nonsmoking ) | 16 | 0.4306 | 0.1163 | 26.6% |
| **Total** | **60** | **0.6753** | **.35015** | **100.0%** |

*mean of concentration of HSP70 in sera of RA. Patients. ** percentage of number of cases.

HSP 70 concentration as measured in samples showed a significantly higher concentration in group 1 (smoking patients ) and with group 3-4 (mean= **1.019±0.339** pg/dl) in comparison to the normal average calculated for two health groups (**0.440** ± **0.1012**) - (**0.4306** ± **0.1163** pg/dl. t-test value =11.225, and 11.491 significance (2-tailed) =.000, 95% confidence interval from( 0.6818 ) to (0.4769) and (0.6911 to.487 )

The mean of HSP70 concentration of patients (non-smoking) samples found significantly higher in groups of about 3-4 samples (0.8327436 pg/dl) in comparison to normal value of healthy individuals(smoking group) is 0.4405745 pg/dl. t- test value = 8.359, significance (2-tailed) =.000 while the mean difference is.39216912

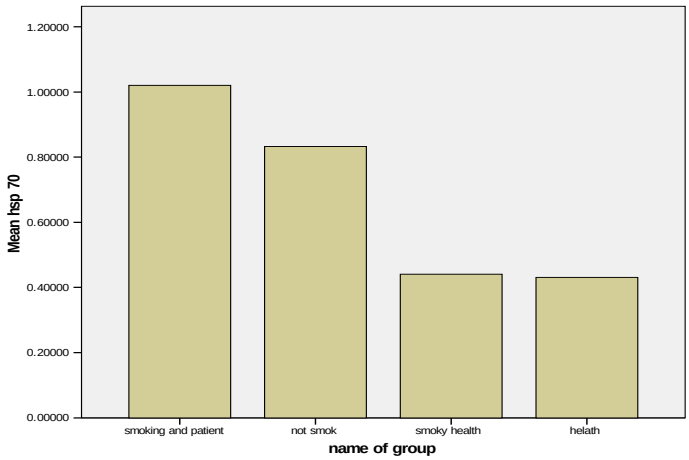


**Figure 2: Shown Comparison of Levels of Concentration of HPS70 According to Smoking Levels**

In the figure above., it can be seen that the concentration of heat shock protein70 in smokers is more than that of other categories.

**Table 5: HSP 70 Concentration According to the Duration of Disease**

| Duration of Disease | No. | Mean of HSP* | Std. Deviation | Percentage** |
|---|---|---|---|---|
| .50 | 2 | 1.335 | .2206 | 4.0% |
| .70 | 2 | 1.3015 | .0346 | 4.0% |
| .80 | 2 | 1.3185 | .1265 | 4.0% |
| .90 | 3 | .9396 | .4255 | 6.0% |
| 1.50 | 2 | 1.2210 | .3903 | 4.0% |
| 2.00 | 12 | 1.1264 | .2449 | 24.0% |
| 3.00 | 12 | .9820 | .3250 | 24.0% |
| 4.00 | 3 | 1.1370 | .2141 | 6.0% |
| 5.00 | 3 | .53266 | .1404 | 6.0% |
| 7.00 | 3 | .90633 | .3709 | 6.0% |
| 10.00 | 6 | .76016 | .3993 | 12.0% |
| **Total** | **50** | **1.01524** | **.3377** | **100.0%** |

*mean of concentration of HSP70 in sera of RA. Patients.

** percentage of number of cases.

Comparative mean between HSP 70 concentrations with duration of disease is found to have a higher level of HSP 70 in boys who is less than one year than in girls who is of 2 years of age and the all-time of disease from 0.5 years to 10 years.

The data regarding the risks involved for RA patients and the related environmental factors, as collected by way of a questionnaire are summarized in the table as shown below.

**Table 6: Suspected major risk factors for RA patients.**

| Risk factor | | Studeing group | | df | Chi-Square | P-value |
|---|---|---|---|---|---|---|
| | | No caese | % of Total N | | | |
| **Stress** | | **63** | **43.4%** | **1** | **2.490** | **.115 NS** |
| **Without Stress** | | **82** | **56.6%** | | | |
| **smoking** | | **50** | **34.5%** | **1** | **13.96** | **0.00 HS** |
| **No Smoking** | | **95** | **65.5%** | | | |
| **Hard work** | | **52** | **35.9%** | **1** | **11.59** | **.001** |
| **Easy work** | | **93** | **64.1%** | | | |
| **Practice exercises** | | **24** | **16.6%** | **1** | **64.89** | **0.00 HS** |
| **Do not practice exercises** | | **121** | **83.4%** | | | |
| **Economic** | **Low** | **15** | **10.3%** | **2** | **150.71** | **0.00 HS** |
| | **Medium** | **118** | **81.4%** | | | |
| | **high** | **12** | **8.3%** | | | |
| **Other chronic diseases** | **Yes one** | **16** | **11.0%** | **2** | **93.77** | **0.00HS** |
| | **no** | **103** | **71.0%** | | | |
| | **More than one** | **26** | **17.9%** | | | |
| **Total** | | **145** | **100.0%** | | | |

**Table 7: HSP 70 Concentration According to the Risk Factors**

| Type of Studied Group | | No of Cases | Mean of HSP Level | Std. Deviation | Percentage of No. Cases |
|---|---|---|---|---|---|
| **Type of work** | Hard work | 27 | 1.031 | .343 | 54.0% |
| | Easy work | 23 | .996 | .337 | 46.0% |

| | | | | | |
|---|---|---|---|---|---|
| | **Total** | **50** | **1.015** | **.337** | **100.0%** |
| **Sport-exercising patients** | yes | 11 | 1.016 | .284 | 22.0% |
| | no | 39 | 1.014 | .354 | 78.0% |
| | Total | 50 | 1.015 | .337 | 100.0% |
| **Economic Status** | low | 4 | 1.026 | .458 | 8.0% |
| | med | 43 | 1.019 | .330 | 86.0% |
| | high | 3 | .939 | .425 | 6.0% |
| | **Total** | **50** | **1.015** | **.337** | **100.0%** |
| **Other Diseases** | yes | 12 | 1.118 | .384 | 24.0% |
| | no | 24 | 1.046 | .284 | 48.0% |
| | lots | 14 | .872 | .358 | 28.0% |
| | **Total** | **50** | **1.015** | **.337** | **100.0%** |
| **Stressful Patients** | stressful | 33 | 1.048 | .354 | 66.0% |
| | Non stressful | 17 | .997 | .310 | 34.0% |
| | **Total** | **50** | **1.015** | **.337** | **100.0%** |

In this table we can see a higher level of significant difference that is said to exist depending on the nature of their work and between different groups of patients for whom the economic strata are low and medium in comparison with those patients whose cost of living is high. The analysis shows a significant difference between patients with one disease in comparison to patients who have more than one disease. All the above factors are statistically significant at level 0.005. P value =0.00

The results shows that there is no significant difference existing between patients who are exercising regularly in comparison to the other group who is not involved in any kind of sports activities.

## DISCUSSIONS

In table 1, the results show that there is no significant difference between the concentrations of heat shock protein in the male category than that of female. But in contrast we can understand that there is a significant difference between the patients group and the healthy group. According to the statistical table No. 2, presence of clear significant difference between the age groups is demonstrated and also is evident that the 41-60 age group, it is proven that there is higher level of concentrations than the rest of the age groups. We can observe in age group 21-40 that there is a clear significant difference in relation to the other categories, which goes with scientific opinion, that higher is the age group, there are possibilities that they will be frequently affected by some disease.

The distribution of patients according to duration of disease explained in table (5) indicates that there is no significant difference between duration of RA by mean is (2.52±2.5 years ) but we found a wide range of difference prevailing between the age group which is a maximum of 10 years and a minimum of 6+ months by range is ( 9.90). So we can show high frequency of disease in occurring in the age group between 2 to 3 years and higher concentration of HSP 70 at less than 6 weeks which means that there is a high concentration in acute phase of RA which causes inflammation.

There was a high level of significant difference (P< 0.00) between the mean duration of RA and the control group. These findings are agreed with Jawaheer *et al*. (2006). It was noted that there was no significant difference of frequency of disease between the female and the male group

This study demonstrated that there is a clear significant difference between the patients group and control group as clarified in the table **1** and this was agreed to in correspondence with: 1. As per the new hypotheses, the extracellular heat shock proteins (Hsp70) may represent an ancestral danger signal of cellular death or lysis-activating innate immunity.

Recent studies demonstrated a dual role for Hsp70 both as a chaperone and as a cytokine providing support for the hypothesis that extracellular Hsp70 is a messenger of stress (Zolta *at el*., 2002) 2- Certain aspects of the role of HSP-reactive T-cells in RA patients needs clarification. Firstly, it calls for a response as to what is the predominant phenotype of the HSP-reactive T-cells in RA based on the T-cell lineage surface markers and function. A few studies (Prakken *et al*.,2004;Zou *et al*.,2002) have addressed this issue, but no clear consensus has been reached at. In this study, it has been demonstrated that there is a clear significant difference between the no of lymphocytes ($P<0.005$) in the patients group and control group as clarified in the table. This was agreed with the public hypotheses about the role of heat shock protein 70 in pathogenesis and immune response in RA (Arend, 1997). Several risk factors are involved in the pathogenesis of autoimmune rheumatic diseases, including genetic factors, smoking, chronic infections, sex hormones, environmental factors and stress. Stress is now considered as an important risk factor in the pathogenesis of autoimmune rheumatic diseases (i.e. rheumatoid arthritis). The suspected major risk factors for RA patients were represented in Tables 6 and 7.

The results demonstrated that the stress factors including major life events (e.g. death of a spouse, severe long-term illness of a spouse etc. And minor life events, such as the progress of pain and fatigue in rheumatoid arthritis and the overall relation with changing mood (depression/anxiety), psychosocial resources (social support,self-efficacy) and burden (social distress, problematic social supports ) as well as the overlapping risk factors for example economic situation, cost of living and habits like smoking, consumption of coffee etc. can be listed as the reasons for the patients with stress conditions ( 43.4%) in comparison to other factors and the disease persisting for a longer period play an important role in the pathogesis of RA. Also we can show that the rate of smokers is (34.5%), and the patient felt that their work is hard (35.9%), (the patients who do not exercise) (83.4%) and the cost of living (81.4%) is medium, and we found 103 pateints (71.0%) who have RA disease only. We have used an immunological marker to measure the response of this group by considering that the activation of the stress response system influences close relationships existing between the hypothalamic-pituitary-adrenal axis, the sympathetic nervous system and the immune system.

The reported changes of the hypothalamic-pituitary-adrenal (HPA) axis, the sympathetic nervous system (SNS) and the immune system (Straub and Cutolo., 2006), might lead to pro-inflammatory reactions during minor stress in RA. It has been shown in RA that interpersonal stress in patients few days prior to the visit were related to an increased number of circulating CD3+ T cells and increased serum levels of soluble IL-2 receptors (Zautra *et al*., 1997). In addition, during the cold pressure test, an enhanced production of IL-6 by peripheral blood cells was observed (Hattor *et al*., 2000 ) and RA patients demonstrated enhanced levels of IL-6 during mental stress test just before the surgery( Blass S, Union A,*et al*.,2001; Mavropoulos,*et al*., 2005). Prior short-term cortisol infusion shows an increased stimulated levels of interleukinIL-6 and tumor necrosis factor (TNF-α) in humans in vivo (Bahr *et al*.,1991).By considering norepinephrine and the SNS, we can conclude that a higher level of stress is the causative factor which in fact induces the immune response in RA patients. The reported activation of the innate immune system by HSPs, as described above, has been hailed as an important characteristic of HSPs with broad biological significance. The induction of pro inflammatory cytokines by Hsp60 and Hsp70 may contribute to the pathogenesis of autoimmune diseases and chronic inflammation. Hsp70 frequently co-localizes with human Hsp70 in macrophages of atherosclerotic plaques (Gaston *et al*., 2002). Induction of pro-inflammatory cytokine release from macrophages by Hsp70 would render a potential mechanism by which Chlamydia infections may promote athero genesis and precipitate acute ischemic events (Bahr *et al*., 1990 ; Gaston *et al*., 2002). Likewise, the activation and maturation of dendritic cells by HSP 70 may be responsible for the HSP 70 -induced tumor immunity by both the innate and adaptive immune responses.

## CONCLUSIONS

Thus it has been proposed that the HSPs, through their cytokine functions, may serve as a "danger signal" to the innate immune system at the site of tissue injury and that HSPs could be the endogenous ligands for theTLR2 and TLR4. In fact, HSPs are considered to be the prototype of endogenous ligands for toll-like receptors. There is a considerable interest to further explore the implications and therapeutic potential of these HSP cytokine effects.

Several risk factors are involved in the pathogenesis of autoimmune rheumatic diseases, including genetic factors, smocking, chronic infections, sex hormones and stress etc., On a concluding note, the result of this study are in line with few studies having refuted any specific information as stated by Fischer, *et al*., 1991; Li SG, *et al*.,1992; Albani S,*et al*., 1995;Auger., *et al*.2002; Zou,*et al*., 2002).

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