HSP70 gene Polymorphism in Pre-eclamptic Women and its Correlation with Serum Levels of HSP70

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ABSTRACT

Objective*: The present study was designed to evaluate the possible association of three different HSP70; HSPA1A, HSPA1B and HSPA1L; gene polymorphisms with pre-eclampsia and the possible correlation with serum levels of HSP70.* ***Method****: The study included 46 women with pre-eclampsia as group I (GI) and twenty two normotensive pregnant controls representing group II (GII). All candidates of the study were subjected to the following: Measurement of human HSP70 concentrations in serum, molecular analysis for PCR amplification of the HSPA1A G (190)C regions, HSPA1B A(1267)G regions, HSPA1L T(2437)C regions.* ***Results****: Serum HSP70 was found to be significantly increased in pre-eclamptic patients when compared to normal pregnancy. HSPA1A (190) GC and HSPA1B (1267) GG genotypes occurred more frequently in pre-eclamptic patients compared to healthy controls ($p < 0.03$). Significant difference was found in the distribution of HSPA1B (1267) AG genotype between the pre-eclamptic and control group ($p < 0.004$). Distribution of HSPA1L T(2437)C gene was found similar in the Pre-eclamptic and control group. Cases with HSPA1A G(190)C had a mean serum level of HSP70 that is 1.4 ng/ml more than those without HSPA1A G(190)C polymorphism. A significant negative association was noted between maternal age and serum Hsp70 concentration in both pre-eclampsia and healthy pregnant women.* ***Conclusion****: Elevated serum HSP70 level in pre-eclamptic patients seems to reflect the burden of oxidative stress taking place im pre-eclampsia. HSP70 polymorphism may affect the serum level as proved by increase in the mean serum level of HSP70 by 1.4 ng/ml in those with HSPA1A G(190)C polymorphism .*

Key words: *Pre-eclampsia, Heat Shock Proteins, Heat Shock Protein 70, Polymorphisms.*

Abbreviations:

HSPs: *Heat shock proteins*

HSP70*: heat shock protein 70*

PE*: Pre-eclampsia*

INTRODUCTION

Pre-eclampsia is a hypertensive disorder unique to pregnancy. The so-called classic triad of pre-eclampsia includes hypertension, proteinuria, and edema. However, there is now general agreement that edema should not be considered as part of the diagnosis of pre-eclampsia. The incidence of pre-eclampsia is 2-10%, depending on the population studied and definitions of pre-eclampsia. However, the incidence rate of pre-eclampsia in the United States is approximately 5-8% of pregnancies[1,2,3].

Women who develop pre-eclampsia are at increased risk for development of pulmonary edema, coagulation defects, hepatic and/or renal failure, seizures, cerebral hemorrhage, blindness, and death. Pre-eclamptic women have 3-4 times increased risk to deliver a small-for-gestational age infant compared with healthy women [1,2,3].

Evidence is accumulating that oxidative stress and lipid peroxidation play a role in the pathogenesis of pre-eclampsia and may account for its clinical manifestations. Maternal serum and placental levels of lipid peroxides are noted to be increased in pre-eclampsia when compared to normal pregnancies[4,5]. Various substances linked to oxidative stress and lipid peroxidation, such as inducible heat shock protein 70 (HSP70) has been shown to be up-regulated during oxidative stress[6].

Heat shock proteins (HSPs) are evolutionarily ancient families of highly conserved molecules. HSPs can be categorized into several families on the basis of their approximate molecular weight (HSP100, HSP90, HSP70, HSP60, as well as the small HSP family)[7]. HSP70 proteins constitute a major group that, even under normal growth, can represent up to 1% of total cellular protein content. Expression of HSP may vary in certain physiologic conditions, such as pregnancy, besides being a response to stressful stimuli as infection, ischemia, free oxygen radicals, rapid growth and differentiation[8]. Some studies have shown changes in the circulating concentrations of HSP70 in blood, serum, or plasma during normal pregnancy, but their results are controversial[7]. Within the cell, Hsp70 is present in midtrimester amniotic fluid as well as trophoblast cells [8].

HSPs are defined as molecular chaperones that regulate intracellular processes to maintain homeostasis during cell proliferation/differentiation. HSPs prevent the incorrect folding and assembly of peptides; transport misfolded or degraded proteins for elimination, and inhibit the induction of apoptosis. HSPs are essential for the maintenance of normal cell function [7].

In humans, there are three genes localized on chromosome 6p21.3, encoding members of the HSP70 class: HSPA1A (HSP73), HSPA1B (HSP72) and HSPA1L (HSP70-hom)[8]. In a previous work, enhanced activation of heat shock transcriptional regulatory factors in genetically hypertensive animals was

demonstrated[9], which they have coupled to genetic polymorphisms of HSP70 gene[10].

Consequently, in the present study we evaluated the possible association of three different HSP70; HSPA1A, HSPA1B and HSPA1L; gene polymorphisms with pre-eclampsia and the possible correlation with serum levels of HSP70.

STUDY SUBJECTS

The present study included 46 women with pre-eclampsia as group I, GI. Group I were selected from 135 preeclamptic women presented to the Antenatal Clinic at King Fahd Hospital of the University, College of Medicine, King Faisal University. The current work included also twenty two normotensive pregnant controls representing group II, GII. Group II were selected from 40 normal pregnancies. In both groups, the gestational age was determined from the last menstrual cycle and was verified with ultrasound scan measurements. Pre-eclampsia was defined as two or more of the following: Proteinuria is defined as >0.5 g urinary protein excretion in 24 hours. Hypertension is defined as an increase of 30 mmHg systolic, an increase of 15 mmHg diastolic blood pressure, compared to values before 20 weeks of pregnancy, or an absolute blood pressure greater than 140/90 mmHg after 20 weeks gestation if the earlier values were unknown, peripheral edema, and subjective symptoms such as visual disturbances, retrosternal pain, and headache [11]. All women had normal blood glucose tolerance screening between 26 and 28 weeks gestation to exclude the presence of gestational diabetes. The obstetric records of all the patients were reviewed after delivery to confirm reversal of hypertension and proteinuria. Informed consent was obtained from all women in the study. The present study implemented as case-control design.

Exclusion criteria:

None of selected patients and control used any medications. Women with history of metabolic or endocrine disorders were excluded from the study (32 cases). Exclusion criteria also included assisted conception (20 cases). Women who had serious non-obstetric conditions; as lupus erythematosus, chronic hypertension before pregnancy, renal disease, type I or type II diabetes mellitus, seizure disorders, malignancies, drug or alcohol abuse; were screened out of participation in the study (30 cases). Pregnant women with multifetal gestation, fetal infection and fetal congenital anomaly were excluded from the study (14 cases). Lipemic, icteric and hemolyzed specimens were also not accepted and their cases excluded from the study (10 samples). Nine cases were excluded for double conditions.

SAMPLES and ASSAY:

All blood samples were collected between 08.00 and 09.30 a.m. after an overnight fasting. Sample from each patient was divided into two tubes. In a plane non-additive red top vacutainer, samples were collected for the specific measurement of serum HSP70 concentrations. These samples were left to clot for 30 min before centrifugation at 3500 rpm for 8 min, and were stored at -70 °C. The second

part of the sample was collected on EDTA anti-coagulant; lavender top vacutainer tubes; for molecular analysis. The samples from individual subjects were measured in one analytical batch. All candidates of the study were subjected to the following:

1- **Measurement of human HSP70 concentrations in serum:** The Stressgen Hsp70 High Sensitivity ELISA kit; Assay Designs, Inc., USA; is a specially optimized assay for the quantification of Hsp70 in serum and plasma samples. The technique is based on the use of sandwich enzyme immunoassay technique with a monoclonal antibody specific for Hsp70 that has been pre-coated onto a microplate utilizing colorimetric measurement of the developed color at 450nm wavelength in proportion to the amount of Hsp70 present.

2- **Molecular Analysis**: Extraction of genomic DNA from 500 µl fresh peripheral blood samples stored at 4°C for less than three days was performed by salt precipitation technique using Wizard® Genomic DNA Purification Kit - Promega®. For PCR amplification of the ***HSPA1A*** **G(190)C** regions of 196bp at 61 °C, Forward 5'-CGA CCT GGG CAC CAC CTA CTC C-3' and Reverse 5'-AAT CAG GCG CTT CGC GTC AAA C-3' primer sequences; ***HSPA1B*** **A(1267)G** regions of 189bp at 61 °C, Forward 5'-ACC CTG GAG CCC GTG GAG AA-3'and Reverse 5'-CAC CCG CCC GCC CCG TAG G-3' primer sequences; ***HSPA1L T(2437)C*** regions of 168bp at 61 °C, Forward 5'-CCT GCT GGG GCG GTT TGA A-3'and Reverse 5'-GGC GGC CCT TGT CAT TGG T-3' primer sequences reported by some authors[12] were used in 50µl reaction mix containing 250ng of DNA, 2mM $MgCl_2$ and 1.5 units of *Taq* DNA polymerase in the presence of 1 µl; 0.2 units betaine monohydrate (Sigma) as it helped in reducing the formation of secondary structure caused by GC-rich regions. Following an initial denaturation step at 94°C for 4min, samples were subjected to 40 cycles of PCR consisting of 94°C for 30sec, followed by annealing at 60°C (HSPA1B) or 61°C (HSPA1A, HSPA1L) for 30sec, with the extension at 72°C for 30sec and with a final extension time of 8min at 72°C in the GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA).

3- RFLP was performed by digestion of the total PCR product purified and eluted in 25µl of HSPA1A G (190)C, 196bp product digested with BsrBI restriction endonuclease (New England Biolabs Inc. UK) at 37°C overnight; HSPA1B A(1267)G, 189bp product digested with PstI restriction endonuclease (New England Biolabs Inc. UK) at 37°C overnight; HSPA1L T(2437)C 168bp product digested with NcoI restriction endonuclease (New England Biolabs Inc. UK) at 37 °C overnight. The digested PCR products were electrophoresed on 3% agarose gel (1×Tris borate EDTA =TBE) and stained with ethidium bromide for three hours at 125 v (85 mA).

4- Routine biochemical investigations were done for cases and control. Investigations included glucose tolerance test, serum urea, serum creatinine, sGOT, and sGPT.

Table I: Specific primer pairs and restriction enzymes used for RFLP analysis:

Polymorphism	Primers (5' – 3')	**Product length (bp)**	**Restriction Enzyme**	**Cleavage products (bp)**
***HSPA1A* G(190)C**	F: 5'-CGA CCT GGG CAC CAC CTA CTC C-3' R: 5'-AAT CAG GCG CTT CGC GTC AAA C-3'	196	*Bsr*BI	118 + 78
***HSPA1B* A(1267)G**	F: 5'-ACC CTG GAG CCC GTG GAG AA-3' R: 5'-CAC CCG CCC GCC CCG TAG G-3'	189	*Pst*I	116 + 73
***HSPA1L* T(2437)C**	F: 5'-CCT GCT GGG GCG GTT TGA A-3' R: 5'-GGC GGC CCT TGT CAT TGG T-3'	168	*Nco*I	112 + 56

STATISTICAL ANALYSIS:

Continuous variables were presented as means ± S.D. Student's t-test was used to compare mean values between the two groups. Differences in the genotypic frequencies between the study and control groups were analyzed by Fisher's exact test. Multiple linear regression analysis was used to examine the association between the three genotypic polymorphism and serum HSP70 level adjusted for the age, gravida numbers, and the gestational age. P-value of <0.05 was considered to be statistically significant. SPSS version 10.0 was used for statistical analysis.

RESULTS

Results of routine laboratory tests and serum HSP70 are shown in table II. Group I had a mean age of 25.26±4.74 years, with a range of 18-39 years, and a mean gestational age of 31.63±2.36 weeks. Twenty women of G I were primigravida. Group II had a mean age of 24.95±2.55 years, with a range of 24-34 years, and a mean gestational age of 30.95±2.5 weeks. Only 12 women of G II were primigravida. On comparing the two studied groups as regard the age and gestational age, non significant difference were observed. Serum urea, creatinine, sGOT, sGPT, and HSP70 were found to be statistically significantly higher in pre-eclamptic patients compared to normal pregnancy.

Table II: t-test of laboratory tests among the two studied groups

Parameters	Pre-eclampsia Mean ± S.D.	Normal Pregnancy Mean ± S.D.	P-value
Age (years)	25.26±4.74	24.95±2.55	0.78
Gestational age (months)	31.63±2.36	30.95±2.5	0.29
Fasting Blood Glucose (mg/dl)	85.4±7.8	84.5±8.2	0.66
Serum Urea (mg/dl)	40.7±3.7	26.3±4.6	‹0.001*
Serum Creatinine (mg/ dl)	1.1±0.2	0.9±0.2	‹0.001*
SGOT (IU/l)	34±4.6	20.4±4.5	‹0.001*
SGPT (IU/l)	33.9±5.5	18.6±5.5	‹0.001*
Serum HSP70 (ng/ml)	1.72±1.23	0.29±0.18	‹0.001*

As shown in table III, HSPA1A (190) GC and HSPA1B (1267) GG genotypes occurred more frequently in pre-eclamptic patients compared to healthy controls ($p<0.03$). Significant difference was found in the distribution of HSPA1B (1267) AG genotype between the pre-eclamptic and control group ($p< 0.004$). Distribution of HSPA1L T(2437)C gene was found similar in the Pre-eclamptic and control group.

Table III: Genotypic frequencies at the HSPA1A G(190)C, HSPA1B A(1267)G and HSPA1LT(2437)C loci in women with Pre-eclampsia (PE) compared to healthy controls

Group	n	HSPA1A G(190)C			HSPA1B A(1267)G			HSPA1L T(2437)C		
		GG	GC	CC	AA	AG	GG	TT	TC	CC
Healthy control	22	0 (0%)	0 (0%)	22 (100%)	0 (0%)	22 (100%)	0 (0%)	0 (0%)	22 (100%)	0 (0%)
PE	46	0 (0%)	4 (8.7%)*	42 (91.3%)	0 (0%)	37 (80.4%)*	9 (19.6%)*	0 (0%)	46 (100%)	0 (0%)

* $p < 0.05$ is significant value.

None significant associations were found between pre-eclampsia and number of gravidity, HSPA1A G(190)C or HSPA1B A(1267)G polymorphism

As shown in table IV, cases with HSPA1A G (190) C had a mean serum level of HSP70 that is 1.4 ng/ml more than those without HSPA1A G (190) C polymorphism. Gravid and gestational ages were also found to be not significantly associated with serum level of HSP70. A significant negative association was noted between maternal age and serum Hsp70 concentration in both pre-eclampsia and healthy pregnant women.

Table IV: Multiple linear regression analysis of the relationship between serum HSP70 and HSPA1A G(190)C polymorphism and other patient parameters

Factor	Coef.	Std. Err	P	[95% Conf. Interval]	
HSPA1A G(190)C	1.439	0.643	0.029*	0.154	2.725
Age	-0.063	0.031	0.046*	-0.127	0.011
Gravida no.	0.105	0.299	0.726	-0.492	0.703
Gestational age	0.066	0.062	0.291	-0.058	0.19

* $p < 0.05$ is significant value.

As shown in table V, on performing the multiple linear regression analysis by introducing the HSPA1B A(1267)G genetic polymorphism, age, gravidity, and gestational age as independent variables on the serum level of HSP70 as a dependent variable, none significant association was found between HSPA1B A(1267)G polymorphism and serum HSP 70. A significant negative association was noted between maternal age and serum Hsp70 concentration.

Table V: Multiple linear regression analysis of the relationship between serum HSP70 and HSPA1B A(1267)G polymorphism and other patient parameters

serum	Coef.	Std. Err	P	[95% Conf. Interval]	
HSPA1B A(1267)G	0.023	0.442	0.959	-0.86	0.905
Age	-0.084	0.032	0.012*	-0.149	-0.019
Gravida no.	0.053	0.310	0.864	-0.566	0.672
Gestational age	0.098	0.063	0.123	-0.027	0.224

* $p < 0.05$ is significant value.

DISCUSSION

Pre-eclampsia is a major cause of maternal and fetal mortality and morbidity. It remains as one of the unsolved problems in obstetrics [(1-4)] as a consequence of an exceptionally complex interaction between a multiplicity of factors that originate in two genetically different individuals (the mother and the fetus). HSPs are a family of stress-induced proteins exhibiting well described functions in cytoprotection preserving cell viability. Recent studies have documented increased expression of HSP70 associated with cerebral, myocardial and renal ischemia[(13-15)]. However, the role of HSPs in placental ischemia, which is a hallmark of pre-eclampsia, has not been completely understood. Furthermore, a significant increase of serum HSP70 in pre-eclamptic patients as compared to normal pregnant women was observed in the current study. The relative hypovolemia, which takes place in pre-eclamptic hemodynamics as compared to normal pregnant women[(16)], could play a role in the etiology of the significant increase in serum HSP70 of pre-eclamptic women. Other factors such as hormones might also influence the induction of HSP70 in pregnancy. In addition, the role of the fetoplacental unit needs to be taken into account in the turnover of HSPs [(17)], which might explain the noted significant increase in serum HSP70 in pre-eclamptic group as compared to normal pregnant cases of the current study due to the placental ischemia occurring in pre-eclampsia[(17)].

The observed significant increase of serum HSP70 in pre-eclamptic patients as compared to normal pregnant women in the current study supported the previous work of

Jirecek et al.[18] **and Fukushima et al.**[19]. In contrast, **Livingston et al.**[20] failed to detect higher levels of HSP70 which may be due to the fact that they analyzed serum samples of severe pre-eclamptic women. In **Livingston's** study standard deviation of serum levels of HSP70 of pre-eclamptic patients was almost 10-fold higher compared to normotensive pregnant patients, suggesting excessive differences in serum HSP70 levels within the pre-eclamptic group. Analysis of different ethnic groups may have also contributed to dissimilar results of serum HSP70 levels in **Livingston's** study. These two interesting notices may be the cause of dissimilarities between **Livingston's** work and the current work.

In other studies circulating levels of HSP70 during normal pregnancy was controversial. **Jirecek et al.** [18] showed decrease in serum HSP70 concentrations with advancing gestation, whereas **Bloshchinskaya and Davidovich** [21] found that HSP70 concentrations in blood plasma tended to increase with advancing gestation. In a more recent and broader study done by **Holdsworth-Carson et al.** [22] in humans, serum HSP70 concentrations had a significant negative association with maternal age which supported the finding of the current work. On the other hand, they[22] noted a significant positive correlation with gestational age which did not go hand in hand with our findings.

The noted difference in these studies may be due to that **Jirecek et al.**[18] determined serum Hsp70 concentrations with an enzyme-linked immunosorbent assay, whereas **Bloshchinskaya and Davidovich** [21] measured Hsp70 levels in blood plasma using an immunoblotting procedure.

In the current study, a significant negative association was noted between maternal age and serum Hsp70 concentration in both pre-eclampsia and healthy pregnant women. This negative association is in agreement with the findings of **Rea et al.**[23] in non-pregnant subjects. They suggested that the ability of cells to respond to stress and synthesize HSPs decreases with increasing age [23]. According to previous work, the source of circulating HSPs in healthy non-pregnant and pregnant subjects, as well as in patients with pathological conditions has not yet been determined and their physiological roles are incompletely understood [17].

The present study found higher frequency of HSPA1A (190) GC and HSPA1B (1267) GG genotypes among our pre-eclamptic patients. This allelic forms a single-nucleotide polymorphism that is used to detect haplotype difference in pre-eclamptic patients. On the other hand, we could not reveal association between the number of gravidity and pre-eclampsia, and between HSPA1A (190) GC polymorphism and pre-eclampsia inspite of a significant association was noted between HSPA1B (1267) AG polymorphism and pre-eclampsia (P value of 0.03), suggesting that HSPA1B A(1267)G gene polymorphisms may affect the incidence of pre-eclampsia.

We assumed that alteration of gene variants may modify HSP70 expression, influencing cellular

defence against oxidative stress in pre-eclampsia. That assumption was supported by our finding of significant increase of serum HSP70 in pre-eclamptic patients when compared with control subjects in the current work. These noted significant increases in serum HSP70 reflected the effect of different genotypes on the alterations of HSP70 production and reflect augmented oxidative stress which occurs during pre-eclampsia[22]. Our findings, which declare that cases with HSPA1A G(190)C had a mean serum level of HSP70 that is 1.4 ng/ml more than those without HSPA1A G(190)C polymorphism, needs further studies.

In conclusion, we found serum Hsp70 concentrations to be significantly higher in pre-eclamptic women compared with healthy pregnant women, which may reflect the burden of oxidative stress taking place in pre-eclampsia. We also demonstrated more frequent occurrence of HSPA1B (1267) GG and HSPA1A (190) GC genotypes in pre-eclamptic patients compared to healthy controls ($p<0.03$) together with a significant difference which was found in the distribution of HSPA1B A(1267)G genotype between the Pre-eclamptic and control group ($p<0.004$). A significant association was noted between HSPA1B A(1267)G polymorphism and pre-eclampsia (P value of 0.03).

We recommend doing a prospective study with chronological measurements of high risk groups of pre-eclampsia throughout pregnancy and continuing until the end of the puerperium to determine changes in serum Hsp70 levels during the course of pre-eclampsia to predict the actual role of serum HSP70 in pre-eclampsia. Also those who are high risk pregnancy and will not develop pre-eclampsia should be followed thoroughly during the work for their serum HSP70 level changes.

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تعدد الأشكال الجينية لجين ب.ص.ح. في مريضات تسمم الحمل و علاقتها بمستوى (ب.ص.ح.٧٠) في المصل

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الهدف: تم تصميم هذه الدراسة لتقييم مدى الارتباط المحتمل لتعدد الأشكال الجينية لثلاثة جينات لبروتين الصدمة الحرارية (ب.ص.ح.) المختلفة كما يلي:(ب.ص.ح.١ ا) (ب.ص.ح.١ ب) (ب.ص.ح.١ل) مع تسمم الحمل والعلاقة المحتملة مع مستويات المصل ل (ب.ص.ح.٧٠).

طريقة البحث: اشتملت الدراسة على عدد ٤٦ امرأة ممن يعانين من مرض تسمم الحمل كمجموعة أولى وعلى عدد ٢٢ امرأة حامل و ضغط دمهم طبيعي يمثلون المجموعة ثانية (ضابطة). وتعرض جميع المشاركات في هذه الدراسة إلى ما يلي: قياس تركيزات HSP70 (ب.ص.ح.٧٠) في المصل، التحليلات الجزيئية لتضخيم تفاعل سلسلة البلمرة لمجموعة (ب.ص.ح.١ ا) مناطقC190G ، لمجموعة (ب.ص.ح.١ ب) مناطق A1267G و لمجموعة (ب.ص.ح.١ ل) مناطقT2437C .

النتائج: اثبتت الدراسة زيادة ذات دلالة احصائية في مستوى (ب.ص.ح.٧٠) في المصل في مريضات تسمم الحمل مقارنة مع الحمل الطبيعي. اثبتت الدراسة زيادة معدل حدوث مجموعة (ب.ص.ح.١ ا) مناطقC190G بتحور جيني CG، لمجموعة (ب.ص.ح.١ ب) مناطق A1267G بتحور جيني GG في مريضات تسمم الحمل مقارنة مع الحمل الطبيعي .(P <0.03) كما اوجدت الدراسة اختلافا ذو دلالة احصائية في توزيع النمط الجيني لمجموعة (ب.ص.ح.١ ب) مناطق A1267G في مريضات تسمم الحمل مقارنة مع الحمل الطبيعي. (P <0.004) . كما اوجدت الدراسة عدم اختلاف في توزيع النمط الجيني لمجموعة (ب.ص.ح.١ ل) مناطق T2437Cفي مريضات تسمم الحمل مقارنة مع الحمل الطبيعي. كما اثبتت الدراسة زيادة مستوى ال (ب.ص.ح.٧٠) في المصل في الافراد ذوي التوزيع النمطي الجيني (ب.ص.ح.١ ا) مناطقC190G عن الافراد الذين لا يملكون نفس النمط الجيني بمقدار هو ١.٤ نانوغرام / مل. ولوحظ وجود ارتباط سلبي ذو دلالة احصائية بين سن الأم وتركيز (ب.ص.ح. ٧٠)في المصل لدى النساء على حد سواء الحوامل بتسمم الحمل والصحيحات.

الاستنتاج: ان ارتفاع مستوى (ب.ص.ح.٧٠) في المصل في مريضات تسمم الحمل يعكس عبء الاكسدة التي تحدث اثناء تسمم الحمل. ان تعدد الأشكال لجينات بروتين الصدمة الحرارية (ب.ص.ح.) يؤثر على مستوى المصل كما ثبت من قبل في زيادة مستوى ال (ب.ص.ح.٧٠) في المصل في الافراد ذوي التوزيع النمطي الجيني (ب.ص.ح.١ ا) مناطقC190G عن الافراد الذين لا يملكون نفس النمط الجيني بمقدار هو ١.٤ نانوغرام / مل.