Testicular heat shock protein effects of Methanolic and Phenolic extracts of *N.* sativa seed in chronic heat-stressed male Wistar rats

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التأثير الجيني للبروتين المضاد للاجهاد للمستخلصين الميثانولي والفينولي لبذور الحبة السوداء في ذكور جرذان الوستر المجهدة حراريا

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المستخلص

أجريت الدراسة الحالية لغرض تقييم فعالية المستخلص الميثانولي والفينولي لبذور الحبة السوداء (*Nagilla Sativa*) لغرض تحسين خصوبة اللبائن تحت تأثير الإجهاد الحراري المزمن .

بعد التكيف على ظروف التجربة وتسجيل الأوزان الابتدائية، تم تقسيم (49) جرذا ذكراً بالغاً بعمر 8 أسابيع ومعدل وزن 250±10 غم عشوائياً على سبع مجموعات متساوية العدد عوملت أربع منها تحت ظروف حرارية طبيعية (20 ± 1 درجة سيليزية) تضمنت مجموعات السيطرة السالبة (Nc) بدون تجريع والسيطرة الموجبة (C) بتجريعها بماء الشرب والمعاملة (M) التي جرعت بالمستخلص الميثانولي لبذور الحبة السوداء (1 غم/ كغم من وزن الجسم) والمعاملة (P) التي جرعت بالجزء الفينولي من المستخلص (0.3 غم/ كغم من وزن الجسم). أما المجموعات الثلاث الأخرى فقد عوملت تحت ظروف الإجهاد الحراري (35 ± 1 درجة سيليزية)، إذ جرعت الاولى (Hc) بماء الشرب وجرعت الثانية (Hm) بالمستخلص الميثانولي وجرعت الثالثة (Hp) بالجزء الفينولي من المستخلص. إستمرت عملية التجريع مدة 18.

بعد مرور 24 ساعة على آخر تجريع، تمت التضحية بالحيوانات تم حساب الوزن الرطب لخصي الجرذان . ثم أخذت عينات منها لغرض دراسة التعبير الجيني للبروتين المضاد للإجهاد (Heat Shock Protein 70; HSP70) باستخدام تقانة الاستنساخ الراجع لتفاعل سلسلة إنزيم البلمرة (RT-PCR). ولم تشهد أوزان الخصى أية فروقات معنوية عند المقارنة بين مجموعات التجربة السبع . أما نتائج التعبير الجيني لبروتين الإجهاد HSP70 في عينات الخصى فقد سجلت أعلى مستوى معنوي في مجموعة Hp تلتها مجموعات Hm و P وM التي تقاربت مستوياتها إلا أنها كانت أعلى معنوياً من مستوى مجموعة Hc التي كانت بدورها أعلى معنوياً من مجموعة C التي كانت اوطأ معنويا من مجموعة Nc.

Abstract

In order to evaluate the efficacy of crude methanolic and phenolic extracts of *Nigella sativa* seed in modulating protective role response in mammals under chronic heat stress. Forty nine mature male Wister rats (weighted: 250 ± 10 g, aged: 8 weeks) has been randomly divided into 7 equal groups. Four groups were reared under normal room temperature (20±1ºC) which included negative control (Nc) was reared without any treatment, positive control (C) was drenched with drinking water, treated group (M) was drenched with metanolic extract of *N. sativa* (1 g/ kg bw), and treated group (P) was drenched with phenolic extract of *N. sativa* (0.3 g/ kg bw). Other three groups has been reared under high room temperature (35 ± 1 cº) which included heated positive control (Hc) was drenched drinking water, heated treated group (Hm) was drenched with methanolic extract of *N. sativa* (1 g/ kg bw), and heated treated group (Hp) was drenched with phenolic extract of *N. sativa* (0.3 g/ kg bw). The experiment extended for 18 days.

Twenty four hours after the last day of experiment, male rats has been sacrificed and body organs testis was removed and weighted .Testis samples (100 mg) were quickly obtained and putted in liquid

nitrogen for heat shock protein 70 (HSP70) gene expression analysis using reverse transcriptase polymerase chain reaction (RT-PCR). Revealed insignificant differences in testes weights among the experimental groups.

RT-PCR results showed that level of mRNA expression of HSP70 gene in testis tissues in treatment groups methanolic extract (M, Hm) (1g/ kg, BW, daily) and phenolic compound (P, Hp, 0.3g/ kg, BW, daily) of *Nigella sativa* seed was significantly higher than that of non-treated groups (Nc, C, and Hc). We have suggested that crude methanolic extract and phenolic compounds obtained from *Nigella sativa* seed have an efficient role in modulating protective role response of mammals under chronic heat stress in mature male Wistar rats.

Introduction

Psychological stress is a common event in daily life Human being may benefit from a mild stress, because it promotes or improves physiological functions in the body such as a mild increase in noradrenalin in the blood improves circulation; a little more secretions of thyrotrophic releasing hormone help basic metabolism in the body. However, severe stress or sustained stress may cause detrimental effect in some organs or tissues in the body (1). There is evidence that hypertension associates with long-term stress (2). Hyperthyroidism may relate to sustained stress (3). The mechanism of stress inducing pathology and phathophysiology in the body remains unclear. The growing evidence implicates that over-secretion of some hormones such as corticotrophin releasing hormone from the hypothalamus may be involved in stress-induced disorders in the body(4). Fertility losses do not immediately follow heat exposure, as heat stress is not detrimental to mature spermatozoa (5, 6), heat exposure is damaging to developing spermatozoa as evidenced by reduced fertility coinciding with maturation of these spermatozoa. In mice, this period of subfertility occurred approximately (18-28) days post stress, as spermatozoa in the ejaculate would have been at spermatid or spermatocyte developmental stages at the time of heat exposure (5). Differences in expression of genes implicated in the response to heat exposure may contribute to the observed variation in fertility (5,7).

Differential expression of DNA repair proteolysis, spermatogenesis, and stress response genes was identified in knock-out male mice exhibiting infertility. Since the observed variation in fertility following heat stress was heritable (6). Annual economic losses could be partially alleviated by improving fertility after heat stress through genetic selection of highly fertile sires (8). Jaquire-sarlin et al (9) suggest that Hsp70 can prevent DNA strand breaks, protect mitochondrial structure and function and thus inhibit apoptosis. Dix (10) showed in mouse model that the disruption of the Hsp70 gene by gene targeting results in failed meiosis, germ cells apoptosis and male infertility. Spermatocyte of mice in which the Hsp70 gene had been knocked out became arrested during meiosis, this failure of meiosis was associated with an increase in spermatocte apoptosis **(**11). Hsp70 participation during spermatogenesis is required for successful completion of meiosis in mouse spermatocyte (12). The mRNA for one of the Hsp70 has also been shown to be induced by cell-free seminal fluid as well as by isolated motile spermatozoa, and exposure to semen resulted in transcription of the Hsp70 gene (13). The mechanism of semen induced HSP70 gene activation and biological consequences of this activity remain a matter of speculation, human semen is a rich source of prostaglandins proteases, polyamines and other products which conceivably could induce a stress response in cell after physical contact. This response and Hsp70 expression may activate lymphocytes that were previously sensitized to cross-reaction regions common to microbial Hsp70, by this mechanism the immune system might initiate a

rapid response to micro-organisms in semen, even to those organisms never previously encountered .Hsp70 gene activation by promoting suppression of pro-inflammatory immune response (14,15).

N. sativa belongs to the Ranunculaceae family and is found wild in southern Europe, northern Africa, and Asia Minor. Common name of the plant is "black seed" (16). Among several compounds, thymoquinone (TQ), dithymquinone (DTQ), thymohydroquinone (THQ), and thymol (THY) are the main active ingredients of the black seed. *Nigella sativa* possesses several pharmacological properties including hypotensive, anti-nociceptive, choleretic, anti-diabetic, anti-oxidant, anti-inflammatory, anti-microbial, anti-tumor, immunomodulation and immunological effect under chronic heat stress (15, 17, 18)

Materials and methods

Experimental animals

Mature male Wister rats were used in the experiment. Male rats were allowed one week to acclimatize to the animal house environment before beginning of experiment. Rats were fed on the standard chow and drinking water throughout the experiment. Room temperature was maintained at (20-22) $\overset{o}{C}$, the light-dark cycle was on a 12:12 h with light and off at 08:00 p.m throughout the experimental period.

Preparation of Black Seeds Extract

Methanolic extract of *Nigella sativa* has been obtained according to Harborne **(**19**)** using Soxhlet apparatus. using 1kg of *Nigella sativa seed*s was put in a cellulose bag of Soxhlet container, adding liter of 99.9% methanol in the cellulose bag, adding 3 liter of 99.9% methanol in the round bottom flask of the apparatus and adjustment of heater temperature (56 C°), then adding the total extraction content in the big round container of the rotavaporator (40 C° and 50-60 rpm), the device turned on and allowed to continue evaporation for at least 2 hrs, and lyophilized by dry freezer, extract was weighted and stored in deep freeze.

Phenolic extraction**:** The extract (P.E.) was prepared by put the seeds in reflex condenser in water bath at 70Cº for 8 hour. Filtration after cooling in separating funnel, added equal volume of n-propanol with sodium chloride was added to the solution till saturation, after separation of two layers, we selected upper layer which includes the phenolic extract. Then evaporated in the rotary evaporator and lyophilized for 24 hours till dryness and then kept at 4 Cº (20).

Heat stress protocol

Rats were kept in a room, in which the temperature was at (20±1Cº). For aday, then room temperature was gradually increased 1-2 °C per day until it reaches (35±1 °C). Then, room temperature was kept constant at (35±1 °C) till the end of experiment for (18 days) (21).

Experimental design

Forty nine mature male Wistar rats were randomly divided into seven equal groups: non-stressed groups (Nc,C,M,P) have been kept at room temperature (20 ±1Cº). Non-treated control (Nc), control (C) daily drenched with vehicle daily for 18 days, *N. sativa* methanolic extract (M) rats have been fed with methanolic extract of *N. sativa* seed at the dose of 1.0 g/ kg b.w. for 18 days. Phenolic extract *(P)* rats have been fed with phenolic extract of *N. sativa* seed at the dose of 0.3 g/ kg b.w. for 18 days.

Stressed groups (Hc,Hm,Hp), rats have been kept at high room temperature (35±1Cº) heat stress control (Hc) drenched with vehicle daily for 18 days, *N. sativa* treated with heat stress (Hm) rats have been fed with methanolic extract of *N. sativa* seed at the dose of 1.0 g/ kg b.w. daily for 18 days. *N. sativa* treated with heat stress (Hp) rats have been fed with phenolic extract of *N. sativa* seed at the dose of 0.3 g/ kg b.w. daily for 18 days. Twenty four hours after late treatment, rats were anaesthetized with thiopental (100 mg/ kg, i.p.), sacrificed and organ testis were removed. Samples from testis of rats in all groups have been quickly removed, dipped in DEPC solution, and frozen in liquid nitrogen for determination of HSP (HSP70) gene expression by semiquantitative RT-PCR analyses.

RNA Isolation from testis tissues

The isolation of RNA from the testis was carried out according to the following protocol (22). Testis was taken from each animal and kept directly in liquid nitrogen for 1 hour, and then stored at (-82) C.Samples were thawed on ice and 50 g. were obtained from each sample.Trizol (0.5 ml.) for each sample, was prepared. The sample put in the grinder and liquid nitrogen was added for three times. After completion of homogenization, sample was transferred to Trizol by small spoon and mixed by insulin syringe and needle should be changed after mixing from 22-25 G for 10 times. After that, samples were kept at room temperature for 10 min. Samples were spined at 11,000 rpm , 4 C° for 10 min.Supernatant was transfered into new eppendorf.100 µl of chloroform was added.Vortexed for 15 sec.Kept at room temperature for 10 min.Spined at 11,000 rpm , 4 C° for 10 min.Supernatant was transferred to a new eppendorf and continued with Isopropanol (250 µl).Vortexed for 15 sec. and kept at room temperature for 10 min.Spined at 11,000 rpm , 4 C° for 10 min.Supernatant was discarded.Adding 75 % EtOH (0.5 ml).Vortexed again.Spined at 8,000 rpm , 4 C° for 5 min.Supernatant was discarded.Adding 75 % EtOH (0.5 ml).Vortexed again.Spined at 8,000 rpm , 4 C° for 5 min.Supernatant dry pellet was discarded.RNase free water was added to the sample with vortexing until is dissolved.RNA samples that show acceptable integrity were diluted with Tris HCl buffer (1:50 v/v) and then the optical density was estimated with spectrophotometer at wavelength 260 nm. The concentration of the RNA in µg/ ml of sample was calculated using the following formula: [OD at 260 nm x dilution (≈50) x 40]/1000

Designing of primers: The primers used in the present study were designed depending on the web site of *NCPI* http://www.ncbi.nlm.nih.gov. method as Select search dialog box for (gene) and type the name of interested gene in the right dialog box i.e. Hsp70 rat**,** The result contains many of genes we should choose specific type of species like (*Rattus novegitus*). web site to decide the primer http://www.eu.idna.com and select (Real time PCR).

Polymerase Chain Reaction (PCR): Master Mix for each sample was prepared according to the recommendations of the manufacturers, and the same procedure followed in the determination of endogenous gene and target genes.

Gel Electrophoresis: PCR products have been transferred into gel electrophoresis apparatus for obtaining and determining the studied genes bands. This step has been done by mixing 2 µl of loading dye with 10 µl of each PCR product (each sample) and 2 µl of ladder. The mixture was loaded into gel lanes. Finally, gel electrophoresis apparatus was turned on at 90 mV for 40 minutes (or adequate time).

Gel Documentation: Gel documentation step has been carried out according to the following protocol (22).

Statistical analysis

Results were presented as mean ± standard deviation of the mean (SD). Comparisons were performed using one way analysis of variance (ANOVA1) and newman- keuls to test all groups' unpaired values. Differences were considered to be significant at the level of $P<0.05$. All statistical analysis was carried out using the GraphPad Prism (SAS Institute, Inc., USA, 2011).

Results

Concentration of RNA in testis

Result of RNA concentration in testis tissues clarified in table (1) and figure(1) showed that mature male Wistar rats administered with crude metanolic extract (1g/ kg, BW, daily) and phenolic compound (0.3g/ kg, BW, daily) of *Nigella sativa* seed revealed significant decrease ($P\leq 0.05$) of mRNA concentration in heat stressed methanolic group (Hm) and increased heat stressed phenolic compounds treated group (Hp) when compared with heat stressed control(Hc) other experimental groups, which showed insignificant differences between methanolic (M) and phenolic (P) groups when compared with control (C) .

RNA normalization

Table (1) illustrated the optical density of each sample at 260nm and 280nm as well as the dilution folds needed to prepare the final concentration (100ng/µl) necessary to complete the following steps of semi quantitative reverse transcriptase polymerase chain reaction (RT-PCR). It has been shown that ratio between optical densities at 260nm and 280 nm within normal range (more than 1.8 and less than 2.1).

mRNA Expression Level of HSP70 in testis tissues

Results of mRNA expression level of heat shock protein70 gene clarified in figures (4 and 5) revealed that testis tissues of male rats treated with crude methanolic extract (1g/ kg, BW, daily) and phenolic compound (0.3g/ kg, BW, daily) of *Nigella sativa* seed showed highly significant level of HSP70 gene expression in in (Hp,Hm) and increased (P,M) all of treated groups compared with that of non-treated groups (Nc, C, and Hc). On the other hand, Hp group registered gene expression level significantly ($P\leq 0.05$) higher than that of other treated groups which showed insignificancy ($P\geq 0.05$) when compared with each other.

mRNA Expression Level of beta actin in testis tissues

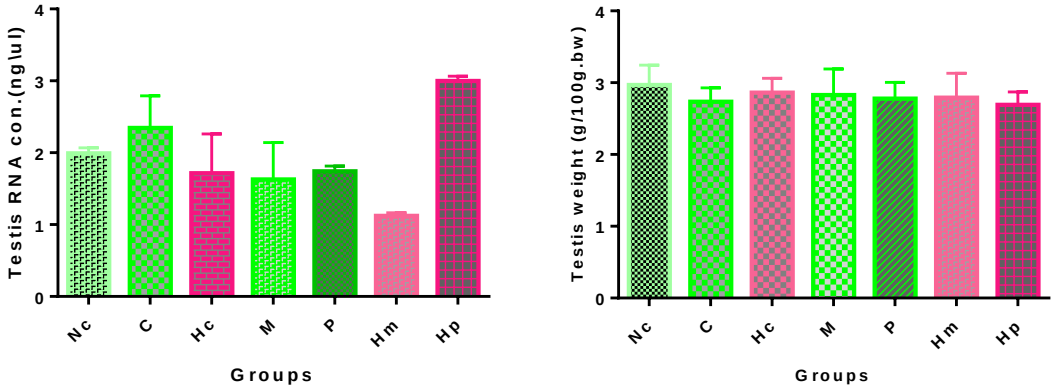
Figure (3 and 6) demonstrates results of mRNA expression level of beta actin concentrations throughout the experimental period. Interestingly, levels of beta actin in (Hp and Hm) groups were highly increased ($P\leq 0.05$) in Hp group, compared with stressed control Hc , Hc group was sharply decreased ($P\leq 0.05$). While in non-stressed groups (Nc, C, M, and P) showed highly increased ($P\leq 0.05$) in methanlic group(M) and increased in phenolic group (P) when compared with control (C). This gene bands were used as corresponding bands for normalization and quantification of heat shock protein70 gene.

Nc = Control (-1) kept at 20º C without drenching. C = Control (+1) kept at 20^{o}C and drenched vehicle without treatment for 18 days. Hc= Control (+2)(heat stressed control) kept at 35^{o}C and

drenched vehicle without treatment for 18 days. M= Treated 1, kept at 20°C and drenched with crude *N. s* S.E. (1g/kg bw) for 18 days. P= Treated 2, kept at 20°C and drenched with phenolic compound of *N. s* S.E. (0.3g/kg bw) for 18 days. Hm= Treated 3, kept at 35°C and drenched with crude *N. s* S.E. (1g/kg bw) for 18 days. Hp= Treated 4, kept at 35°C and drenched with phenolic compound of *N. s* S.E. (0.3g/kg bw) for 18 days.

***** Deferent letters represent significant difference (P<0.05) between groups.

**** No. 7 per each group.**



Figure(1-2) : Effect of methanolic and phenolic extract of *N. sativa* seed on RNA concentration of testis tissues and testis weight in chronic heat- stressed adult male Wistar rats.

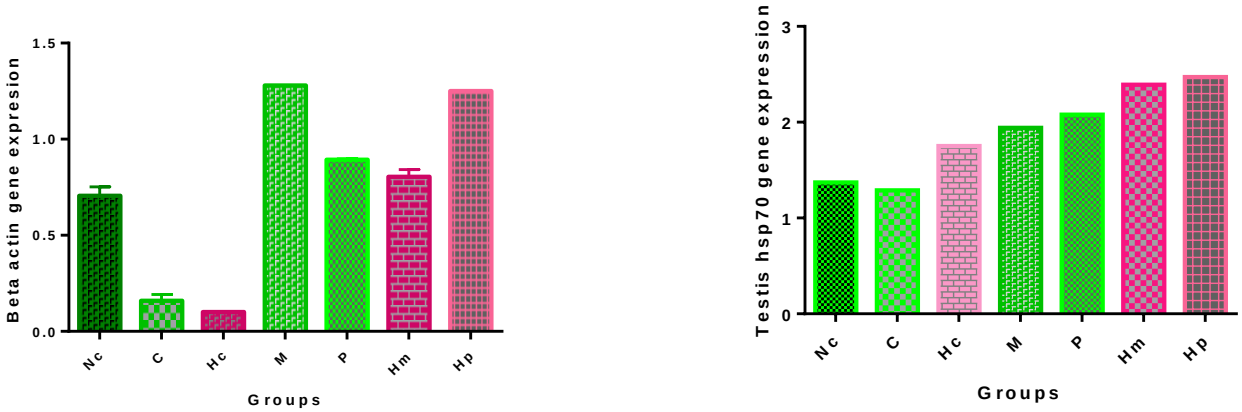


Figure (3-4): Effect of methanolic and phenolic extract of *N. sativa* seed on mRNA expression level of Hsp70 in testis tissue and mRNA expression level of Beta actin in chronic heat-stressed adult male rats.

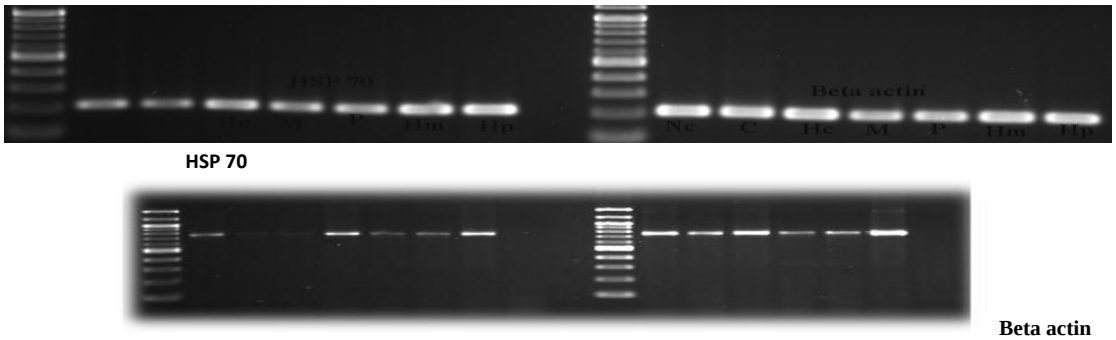


Figure (5-6): Effect of methanolic and phenolic extract of *N. sativa* seed on cDNA bands of beta actin and Hsp70 in testis tissues. in chronic heat-stressed adult male Wister rats.

Table (1): Effect of methanolic and phenolic extract of *N. sativa* seed on testis RNA conc. in chronic heat-stressed adult male Wistar rats

Total RNA Conc.					
Group	260nm	280nm	260/280R	Conc.ng/ul	M ± SD
Nc1	50.124	25.714	1.949	2.005	1.98 ± 0.063
Nc2	48.540	24.383	1.991	1.942	
Nc3	52.060	27.651	1.883	2.082	
Nc4	48.025	23.699	2.026	1.921	
C1	70.675	36.515	1.936	2.827	2.62 ± 0.4
C2	67.680	33.520	2.019	2.707	
C3	73.665	39.510	1.864	2.947	
C4	50.500	26.343	1.917	2.020	
Hc1	32.208	15.976	2.016	1.288	1.68 ± 0.58
Hc2	56.232	28.873	1.948	2.249	
Hc3	27.008	13.776	1.961	1.080	
Hc4	53.201	25.843	2.059	2.128	
M1	53.860	27.520	1.957	2.154	1.63 ± 0.54
M2	51.661	24.320	2.124	2.066	
M3	28.632	14.218	2.014	1.145	
M4	29.662	15.248	1.945	1.186	
P1	43.155	21.553	2.002	1.726	1.73 ± 0.08
P2	45.265	23.655	1.914	1.811	
P3	44.105	22.502	1.960	1.764	
P4	40.520	20.060	2.020	1.621	
Hm1	28.25	14.24	1.983	1.13	1.11 ± 0.041
Hm2	29.32	15.31	1.915	1.172	
Hm3	27.301	13.291	2.054	1.092	
Hm4	26.979	13.914	1.938	1.079	
Hp1	74.574	38.723	1.925	2.982	3.019 ± 0.0049
Hp2	76.682	40.826	1.878	3.067	
Hp3	73.475	35.515	2.068	2.939	
Hp4	77.265	41.42	1.865	3.090	

The optical density of pure RNA is (1.8 -2.1) at ratio of absorbance (260/280nm)

Discussion

The present experiment aimed to evaluate the protective activity of methanolic and phenolic extract of *Nagila sativa* seed in heat stressed mature male rats. Since the finding that heat stress induced highly increased in Hsp70 in heart and liver tissue treated with methanolic and phenolic extract of *Nagila sativa* seed (15). Under normal and high ambient temperature, male rats treated with methanolic and phenolic extracts of *N. sativa* seed revealed significant increase in mRNA expression level of HSP70 in testis tissue when compared with the male rats in the corresponding groups. These findings are indicative to the protective role of *N. sativa* seed components under normal temperature and chronic heat stress. It has been shown that HSP70 expression levels are rapidly increased, and several-folds, in response to cellular stress (23), which provides protection to the cell. While the function of HSP70 as a molecular chaperone becomes especially important during cellular stress, the mechanisms by which increased levels of HSP70 provide cytoprotection are increasingly being linked to the direct regulation of specific pathways by HSP70 (24). It is therefore, speculated that a decrease in HSP70 may contribute to the pathogenesity that occurs in response to stressful stimuli. Heat shock proteins critical for survival of cells under adverse condition, including heat stress (25). In that regard, HSP is one of many well characterized molecular chaperones, known for their ability to prevent aggregation of unfolded or improperly folded protein. In particular, members of the Hsp90 class, including Hspcb, tend to associate with cellular substrate proteins that are either inactive or unstable. This association prevents the aggregation of such proteins and can also inhibit their rapid activation (26). Furthermore, Hsp90is hypothesized to have role in spermatogenesis, as it was expressed in the germ cells, and variation in Hsp90 may be associated with infertility (27). Additionally, Hsp90 may be involved in the apoptotic path way (28). Fertility losses do not immediately follow heat exposure , as heat stresses not detrimental to mature spermatozoa (5,6,31), rather heat exposure is damaging to developing spermatozoa as evidenced reduced fertility coinciding with maturation of the spermatozoa , in mice this period of sub fertility occurred approximately (18-28) days post stress, as spermatozoa in the ejaculated would have been at spermatid or spermatocyte development stage at the time of heat exposure (5,6,31).

The potent role of *N.sativa* seed extracts in the activation of the Hsp70 expression level that lead to protective role of *N.sativa* seed extracts anti-heat stress after chronic heat stress. The expression of Hsp70 inhibited chronic stress induced intestinal barrier dysfunction may be via preventing stress induced intestinal epithelial cell apoptosis (8). There are also large number of studies demonstrating neuroprotection by the chaperone Hsp70 (32). The released extracellular HSP70 may play key role in initiating immunoregulatory functions. Therefore, it can be speculated that elevating in extracellular HSP70 levels after *N. sativa* seed extracts administration, during normal temperature and heat stress, might act as beneficial danger signals protecting against stressful condition via cytokines secretion (33;15). On the other hand it is not clear as to whether HSP70 might be exposed on the cell surface of cardiac muscle cells or hepatocytes following heat stress and treatment with *N. sativa* seed extracts and thus might provide a protective role from the effect of chronic heat stress (15).
This hypothesis might be supported by the finding that treatment with *N. sativa* seed extract, under normal and high ambient temperature, results in an increase of pro-fertility by increasing HsP70 in testis tissue thus high role in spermatogenesis, meiosis and maturation.

References

1. Gardener R J R., (2001). Evolutionary perspectives on stress and affective disorder semin. Clin. Neuropsychiatry;6:32-42

2. Pickering TG;(2001). Mental stress as a causal factor in the development of hypertension and cardiovascular disease. Curr,Hypertens,Rep;3:249-54

3. Matos-Santos A, Nobre EL, Costa JG, Nogueira PJ Macedo A, Galvao-Teles A, and de Castro JJ (2001). Relationship between the number and impact of stressful life events and the onset of Graves' disease and toxic nodular goitre. Clin Endocrinol (Oxf);55(15-9)

4. Sfikakis A, Papadopoulou-Daifotis Z, and Bikas N., (2002). Inverse relationship of hippocampal serotonin to avoidance behavior serotonergic activation by emotional stress differentiated by estrous cycle and surgical stress. Behav Brain Res.;41:128-52.

5. **Rockett JC, Mapp FL, Garges JB, Luft JC, Mori C, and Dix DJ, (2001)**. Effects of hyperthermia on spermatogenesis, apoptosis, gene expression, and fertility in adult male mice. Biol Reprod; 65:229–39

6. **Cammack KM, Mesa H, and Lamberson WR, (2006)**. Genetic variation in fertility of heat-stressed male mice.Theriogenology;66: 2195-2201.

7. Rockett J.C.,Patrizio P., Schmid J.E., Hecht N.B., and Dix D.J.;(2004). Gene expression patterns associated with infertility in humans and rodent models. Mut. Res. 549:225-240

8. Yang P. C. , Tu Y. H., M. H. Perdue, C. Oluwole, and S. Struiksma, (2009). Regulatory effect of Hsp70 in stress induced rat intestinal epithelial barrier dysfunction. North American J. Med. Sci. 20091(1):9-15.

9. Jaquire-sarlin,M.R.; Fuller, k.; Dinb-Xuan, A.T.et al.,(1994). Protective effect of Hsp70 in inflammation Experiential.50:1031-1038.

10. DIX, D.J. ; Allen , J. W. ; Collins, b. w. et al., (1996).targeted gene disruption of Hsp70 results in failed meiosis , germ cell apoptosis and male infertility .proc.Nati.Acad.Sci. U.S.A.93,3264-3268

11. Mori, c., Nakamura, N.; Dix, D.J. et al., (1997). Morphological analysis germ cell apoptosis during postnatal testis development in normal and Hsp70-2 Knockout mice . Dev.Dyn.208;(125-136).

12. Mori, C.; Allen, J. W.; Dix, D.J. et al ., (1999). Completion of meiosis not always required for a crosome formation in Hsp70-2 null mice .Biol.Reprod. 61,(813-822).

13. Jeremias, J.; Bongiovanni, A. M. and Witkin, S.S.,(1999). Induction of heat shock protein expression in cervical epithelial cells by human semen.Infect.Dis.obstet.Gynecol.,7, 17-22

14. Cahill, M.C.;Waterman, W.R.; Xie, Y. etal ., (1996). Transcriptional repression of the pro-interleukin 1 beta gene by heat shock factor 1.J.Bio.Chem.,271, (24874-24879).

15. Hamady,J.J. (2011). Immunological effects of methanolic and phenolic extracts of *N. sativa* seed in chronic heat-stressed male wister rats. MSc thesis, College of Veterinary Medicine, Al-Qadisiya University, Iraq.

16. Salem, M.L., (2005). Immunomodulatory and therapeutic properties of the *Nigella sativa* L. seed.; *Internat Immunopharmacol* 5: 1749-1770

17. Ali BH, and Blunden G (2003). Pharmacological and toxicological properties of *Nigella sativa*. Phytother Res 17; 299- 305

18. El-Dakhakhny M, Madi NJ, Lembert N, and Ammon HP.(2002). Nigella sativa oil, nigellone and derived thymoquinone inhibit synthesis of 5-lipoxygenase products in polymorphonuclear leukocytes from rats. J.Ethnopharmacol, 81; 161-16 4.

19. Harborne J B., (1984). Phytochemical Methods: A Guide to Modern Techniques of plant Analysis. Chapman and Hall, London, UK., pp: 1-34

20. Ribereau-Gayon, P**.,** (1972).Plant phenolics (University reviews in botany, 3

21. Sutherland, M. A.; Niekamp, S. R.; Rodriguez-Zas, S. L., and Salak-Johnson, J. L., (2006). Impacts of chronic stress and social status on various physiological and performance measures in pigs of different breeds. J. Anim. Sci. 84:588-596.

22.Surzcki, S. (2000): Basic Techniques in Molecular biology. Springer Lab. Manual.

23. Liu, Y.; Gampert, L.; Nething, K., and Steinacker, J. M. (2006). Response and function of skeletal muscle heat shock protein 70. Front. Biosci. 11: 2802–2827

24. Mayer, M.P., and Bukau, B., (2005). Hsp70 chaperones: cellular functions and molecular mechanism. Cell Mol Life Sci 62: 670–684

25. Neuer A, Spandorfer SD, Giraldo P, Dieterle S, Rosenwaks Z, and Witkin SS. (2000). The role of heat shock protein sinre production .Hum.ReprodUpdate;6:149-159.

26.Burston SG,and Clarke AR;(1995). Molecular chaper ones:physical and mechanistic properties.EssaysBiochem;29:125-136.

27. Hassun-Filho PA, Cedenho AP, Lima SB, Ortiz V, Srougi M; (2005). Single nucleotide polymorphisms of the heat shock protein 90 gene in varicocele-associated infertility. Int. Braz. J. Urol. 31:236-242.

28. Stangl K, Gunther C, Frank T, Lorenz M, Meiners S, Ropke T, et al.; (2002). Inhibition of the ubiquitin-proteasome pathway induces differential heat shock protein response in cardiomyocytes and renders early cardiac protection. Biochem Biophys. Res. Comm. 291:542-559.

29.Cammack K.M., E. Antoniou, L. Hearne,and W.R. Lamberson; (**2009).** Testicular gene expression in male mice divergent for fertility after heat stress. Theriogenology 71, 651–661**.**

30.Yenari MA, Giffard RG, Sapolsky RM,and Steinberg GK, (1999). The neuroprotective potential of heat shock protein 70 HSP70). Mol. Med. Today; 5:525-31.

31. Campisi, J.L.; Leem, T.H., and Fleshner, M., (2003). Stress-induced extracellular Hsp72 is a functionally significant danger signal to the immune system. Cell Stress Chaperones,8: 272-286.