Testicular Expression Levels of inhibin-α, βA, and βB Genes in Chronic Heat-Stressed Male Wistar Rats Treated with Phenolic Extracts of *Nigella sativa* Seeds

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Abstract

The present study aimed to examine the role of phenolic compounds obtained from *Nigella sativa* seeds in modulating testicular endocrine response of mammals under chronic heat stress in mature male Wistar rats. Thirty mature males were randomly assigned to three equal groups, one non stressed group (C) served as a non-treated control and two stressed groups (HC; as heat stressed control, andHp; as heat stressed and treated with phenolic extract of *N. sativa* seed at the dose of 0.3 g/ kg b. w. daily for 18 days). At the end of treatment period, rats have been anesthetized with thiopental (100 mg/ kg, i.p.), sacrificed and testes were quickly removed, dipped in DEPC solution, and frozen at -80 ºC for determination of inhibin alpha and beta (βa and βb) isotypes gene expression by semi-quantitative RT-PCR analyses. Stressed rats (HP) showed marked increased of inhibin α, βa, and βb genes in Hp group compared with Hc group. In conclusion phenolic extract of *N. sativa* seed have potent role as a pro-fertility by increased hormonal activities response under chronic heat stress condition.

KEYWORD:inhibin, *Nigella sativa*, fertility, testis, heat stress.

Introduction

Stress is defined as a real or interpreted threat to the physiological or psychological integrity of an individual, which results in a physiological and/or behavioral response. With regards to its duration, heat stress can be classified to acute, when it lasts for a period of a few minutes to a few hours and chronic, when it persists for several hours per day for several days (McEwen, 2005).The cellular stress response can be defined as a reaction to the threat of macromolecular damage, the cellular stress response is associated with essential aspects of protein and DNA processing and stability (Khan et al., 2003). Stress hormones released in response to hypothalamic-pituitary-adrenal (HPA) activation, such as corticotropic releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and cortisol, has all been shown to have an effect on hormonal function (McEwen, 2005). The damaging effects of heat stress on spermatogenesis and sperm quality have been well documented in several species including cattle (Casady et al., 1953; Rhynes and Ewing, 1973), sheep (Howarth, 1969), swine (Cameron and Blackshaw, 1980), rats (Shiino and Rennels, 1971) and mice (Rockett et al., 2001; Cammack et al., 2006; 2009).

Inhibin, as a member of transforming growth factors (TGFβ) superfamily, is a disulphide-linked heterodimeric glycoprotein consisting alpha(α) and beta(β) subunits. Inhibin α and β subunits are synthesized as pro-proteins (pro-αN-αC and pro Bβ). There are two forms of β subunits (βA and βB). Inhibin A is a complex of α and βA subunits whereas inhibin B is a complex of α and βB subunits. The free subunit do not suppress FSH, therefore, the bioactivity of the inhibin depends on formation of a dimeric α-β strictures (Illingworth et al., 1996; Anderson et al., 1997). Researches generally agree that Sertoli cells is the

predominate site of inhibin B production in the testis (Anderson et al., 1998; Young et al., 2000). The Sertoli cells contain only the α-subunit whereas the βB subunits are localized in the pachytene spermatocytes and in the round spermatids (Anderson et al., 1998). It has been mentioned that inhibin B is produced by the Sertoli cells but the process depend on the presence of specific germ cells (Anderson et al., 1998; Anderson, 2001).

Nigella sativa Linn, commonly known as black seed or black cumin, is an important medicinal herb in many Arabian, Asian, and African countries (Meral et al., 2001; Kalus et al., 2003). The seeds have anti-inflammatory, analgesic, anti-pyretic, anti-microbial and anti-neoplastic activities. The anti-inflammatory effect of *N. sativa* is associated with inhibition of cyclooxygenase and lipoxygenase enzymes leading to decreased prostaglandins and leukotriens, which are mediators of inflammation (Rice-Evans et al., 1996; Middleton et al., 2000 Ali and Blunden, 2003; Scalbert et al., 2005). To examine the ameliorating role of methanolic and phenolic extracts of *Nigella sativa* seed in testicular functions, in the mean of inhibin alpha and beta subunits, of mammals under chronic heat stress, the present study was conducted in mature male Wistar rats.

Materials and Methods

Experimental Animals: Adult male Wister rats (average weight was 250 ± 10g.), were obtained from the National Laboratory Animal Center and reared under controlled conditions (12L:12D cycles at 20-22 C°) and fed standard laboratory food (19% protein ratio and 3000 kilocalories energy) and drinking water.

Preparation of *N. sativa*extract:*N. sativa* was purchased from the local market and classified by State Board for Seed Testing and Classification, Ministry of Agriculture, Iraq (SBSTC). Phenolic extract has been prepared according toRibereau-Gayon(1972).

Heat stress protocol: At first, the rats have been kept at a room temperature (20±1Cº). In the following days, the room temperature has been gradually increased (1-2 °C per day) until it reaches (35±1 °C) in the first week and kept constant until the end of experiment (18 days) (Sutherland et al., 2006).

Experimental Design: Thirty male rats were randomly assigned to three equal groups, treated for 18 days as follow: non-stressed group (C) has been kept at normal room temperature (20 ±1Cº) and heat-stressed groups (HC andHp)have been kept at high room temperature (35±1Cº). Non-treated control (C) rats were daily drenched with 1 ml of drinking water, heat-stressed control (Hc) rats were daily drenched with vehicle, and heat stressed Hp rats have been daily drenched with phenolic extract of *N. sativa* seed at the dose of 0.3 g/ kg b.w. Twenty four hours after the last treatment, male rats were anaesthetized with thiopental (100 mg/ kg, i.p.), sacrificed and testis were removed. Samples from testis of rats in all groups have been quickly dipped in DEPC solution, and frozen at -80 ºC for determination of inhibin alpha and beta gene expression levels by semi-quantitative RT-PCR analyses.

RNA extraction: RNA has been extracted from the testis tissues according to the protocol mentioned by Surzcki (2000).

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.

Gel Documentation: Gel documentation step has been carried out according to the protocol mentioned by Surzcki (2000).

Statistical Analysis: the results were expressed as mean ± standard deviation of the mean (SDM). 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 $P<0.05$. The statistical analysis was carried out using the GraphPad Prism (SAS Institute, Inc., USA, 2011).

Results

Concentration of RNA in the testis: the result of RNA concentration in testis tissues clarified in figure (1) showed that heat-stressed male rats that administered with phenolic extract of Ns (0.3g/ kg, b.w.) recorded significant increase ($P\leq 0.05$) compared with heat stressed control (Hc).

RNA normalization:the present findings illustrates that the ratio between the optical densities at 260 nm and 280 nm for each sample as well as the dilution folds needed to prepare the final concentration (100 ng/µl) necessary to complete the following steps of semi-quantitative RT-PCR, was within normal range (more than 1.8 and less than 2.1).

mRNA expression level of beta actin: the results illustrated in figure (2) shows the endogenous (beta actin) gene bands. This gene bands has been used as a corresponding bands for normalization and quantification of inhibin alpha and beta (βa and βb).

mRNA Expression Level of inhibin alpha in testis tissues: the results of mRNA expression level of inhibin alpha gene illustrated in figures (3) revealed that testis tissues obtained from male rats treated with phenolic compound (0.3g/ kg, BW, daily) of *Nigella sativa* seed (Hp group) registered gene expression level significantly ($P\leq 0.05$) higher than that of non-treated groups (C and Hc) throughout the experimental period.

mRNA Expression Level of inhibin beta a and b in testis tissues: the results of mRNA expression level of inhibin βa and βb genes illustrated in figures (4 and 5) recordedsignificant ($P\leq 0.05$) higher level in Hp group than that of non-treated groups (C and Hc) throughout the experimental period.

Discussion

The protective activity of methanolic and phenolic extract of *Nigella sativa* seed in heat stressed mature male rats has been demonstrated by Hamady (2011), as polyphenols are chemopreventers and therefore act to protecting the body tissues against oxidative stress, modulating gene expression, and inhibiting carcinogen-induced tumorigenesis (Scalbert et al., 2005). In particular, they have found*Nigella sativa* seed to be strong antioxidants with free radical scavenging, inhibiting enzymatic systems responsible for free radical generation, metal chelation, and reducing properties (Rice-Evans et al., 1996).

It has been postulated that fertility losses do not immediately follow heat exposure, where heat stresses not detrimental to mature spermatozoa, rather heat exposure is damaging to developing spermatozoa as evidenced reduced fertility coinciding with maturation of the spermatozoa (Rockett et al., 2001; Cammack et al., 2006; 2009).

The present findings reported that phenolic extract of *Nigella sativa* seed possess pro-fertility role in heat-stressed mature male rats, as it performed a potent role in the activation of reproductive hormonal expression. Follicle stimulating hormone is one of these hormones that will be increased after treatment with *Nigella sativa* seed extract (Al-

Sa'aidi et al., 2009), which in turn will upregultes the expression level of inhibin subunits in Sertoli cells inside the seminiferous tubules of the testis, where inhibin immunoreactivity was detected mainly in Sertoli cells from puberty to adulthood, as previously indicated for rat testis (Noguchi et al., 1997). Regarding to spermatogenesis, inhibin B concentration was the sensitive marker for the assessment of sperm production (Brugo-Olmedo et al., 2001; Kumanov, 2005), therefor, the increment of inhibin alpha and beta B subunits, registered in the present study, could be a result of the high secretion levels of FSH from adenohypophesis. The increment of inhibin alpha and beta B subunits in testicular tissues confirm the potent role of inhibin B instead of inhibin A in testicular functions and/or the reproductive efficiency, whereas the increment of beta A subunit indicate the potent role of activin A in testicular functions. The mRNA expression levels of inhibin alpha and inhibin beta β isotypes (βa, βb) were closely associated with testicular maturation (Schmitt et al., 2002; Seok et al., 2004). Inhibin alpha was reported to have significant association with acrosome integrity and inhibin beta with semen volume per ejaculate and motility (Sang et al., 2013), accordingly inhibin B and activin A act as markers of persistent spermatogenesis (Toulis et al., 2010), and the secretion of FSH in conjunction with estrogen and testosterone (Bhardwaj et al., 2012). In contrast, after chronic heat-stress, all subunits showed insignificant decrease in their expression levels when compared with control, which could be attributed to the effect of heat stress on testicular structure and activity. In spite of the role of inhibin as a negative feedback regulator of FSH secretion (O'Connor and de Kretser, 2004), the role of phenolic extract of *Nigella sativa* seed increase the maturation of Sertoli cells by FSH stimulation, which further promotes the expression of the inhibin alpha, beta A, beta B isotypes (Kim et al., 2008).

In conclusion, the present findings, including the increase expression of inhibin α and β(βa and βb) activities level, might be considered as indicative to the potent role of *N. sativa* seed extract in the activation of the hormonal response and activation of the spermatogenesis in heat-stressed mature male rats, when used at the given doses for 18 days.

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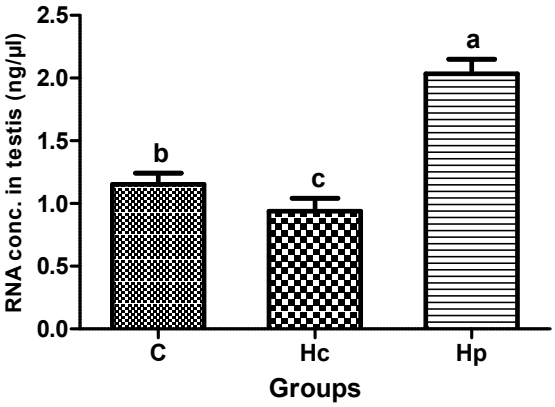

Figure (1): effect of phenolic extract of *N. sativa* seed on RNA concentration (ng/µl) in testis tissues of chronic heat- stressed adult male Wistar rats.

The results represented as mean ± SE.

Different small letters denotes the present of significant differences (P<0.05) between groups.

C: control male rats kept at normal temperature (20 ±1Cº).

Hc: heat-stressed male rats kept at high temperature (35 ±1Cº).

Hp: heat-stressed male rats kept at high temperature (35 ±1Cº) and treated with phenolic extract of *N. sativa* seeds.

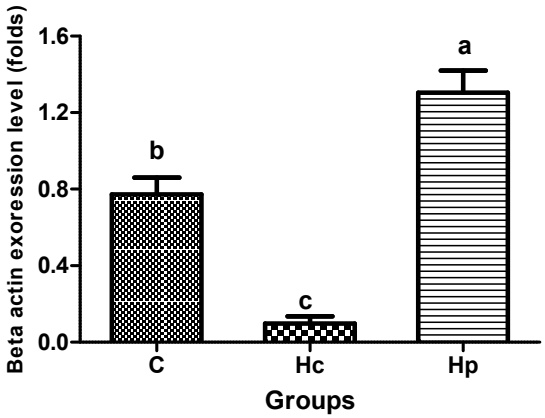

Figure (2): effect of phenolic extract of *N. sativa* seed on mRNA expression level of Beta actin (folds) in testis tissues of chronic heat- stressed adult male Wistar rats.

The results represented as mean ± SE.

Different small letters denotes the present of significant differences (P<0.05) between groups.

C: control male rats kept at normal temperature (20 ±1Cº).

Hc: heat-stressed male rats kept at high temperature (35 ±1Cº).

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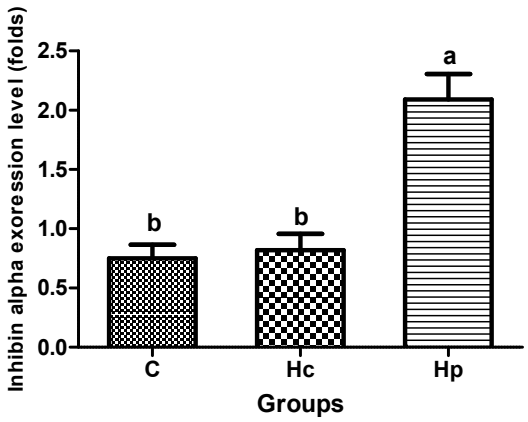

Figure (3): effect of phenolic extract of *N. sativa* seed on mRNA expression level of inhibin alpha subunit (folds) in testis tissues of chronic heat- stressed adult male Wistar rats.

The results represented as mean ± SE.

Different small letters denotes the present of significant differences (P<0.05) between groups.

C: control male rats kept at normal temperature (20 ±1Cº).

Hc: heat-stressed male rats kept at high temperature (35 ±1Cº).

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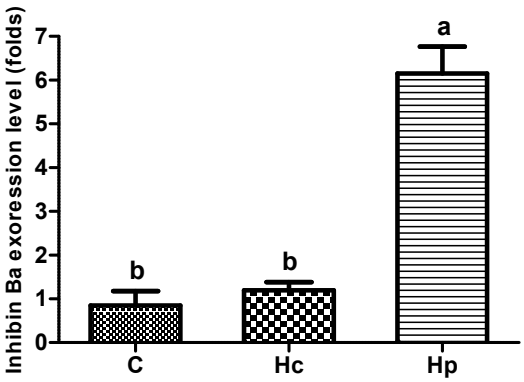

Figure (4): effect of phenolic extract of *N. sativa* seed on mRNA expression level of inhibin Beta-A subunit (folds) in testis tissues of chronic heat- stressed adult male Wistar rats.

The results represented as mean ± SE.

Different small letters denotes the present of significant differences (P<0.05) between groups.

C: control male rats kept at normal temperature (20 ±1Cº).

Hc: heat-stressed male rats kept at high temperature (35 ±1Cº).

Hp: heat-stressed male rats kept at high temperature (35 ±1Cº) and treated with phenolic extract of *N. sativa* seeds.

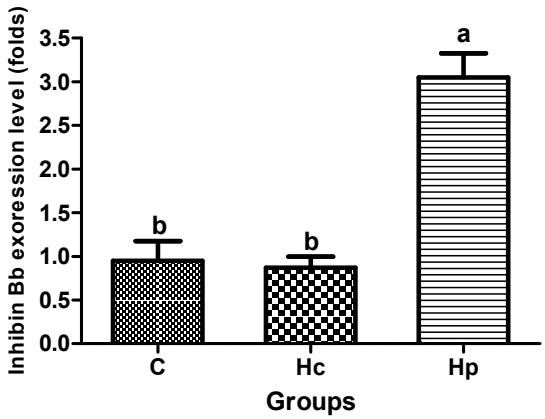

Figure (5): effect of phenolic extract of *N. sativa* seed on mRNA expression level of inhibin Beta-B subunit (folds) in testis tissues of chronic heat- stressed adult male Wistar rats.

The results represented as mean ± SE.

Different small letters denotes the present of significant differences ($P<0.05$) between groups.

C: control male rats kept at normal temperature (20 ±1Cº).

Hc: heat-stressed male rats kept at high temperature (35 ±1Cº).

Hp: heat-stressed male rats kept at high temperature (35 ±1Cº) and treated with phenolic extract of *N. sativa* seeds.