Histological and Immunohistochemical expression of testicular testosterone in male rats treated with thymoquinone .

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Abstract :

The objective of this study was to examine the effects of thymoquinone (TQ), which has antioxidant properties in the expression level of testes testosterone hormone in pubertal male rats , a total of 48 male rats aged 60 days were assigned to two equal groups. First group was drenched distilled water and served as control while second group was drenched with thymoquinone suspension (50 mg/kg) daily for six weeks.Two weeks internal eight males were sacrificed and testes tissues were fixed in formalin for histopathological and immunohistochemical study. Histopathological findings revealed moderate improvement of testicular tissue with normal cellularity of testes.

Immunohistochemical results revealed that male rats drenched with thymoquinone showed actively stained of Leydig cells with actively stained of Sertoli cells ,this refers to higher expression of testosterone hormone in thymoquinone treated males with gradual increment as treatment priods progressed .

It can be concluded that drenching of 50 mg/ kg, b.w, of thymoquinone from *Nigella sativa* seed has potent role in elevation of testes hormone related to reproduction and fertility .

Introduction :

Thymoquinone (TQ) is the main bioactive constituent of an oil extract of Nigella sativa. The last fifteen years have witnessed hundreds of research reports regarding its therapeutic biological effects as an anti-inflammatory, analgesic, anti-diabetic, antihistaminic and anticancer agent (Woo et al., 2012) .TQ exerts its biological functions by modulating the physiological and biochemical processes involved in reactive oxygen species (ROS) generation both in normal and tumor cells where it acts as antioxidant and pro-oxidant, respectively (Dergarabetianetal.,2013). In the prognosis of cancer, the cells must have some features hallmarks in order to enable their transformation to malignant tumors (Hanahan et al.,2014) .TQ has been proved to affect nine out of the ten known cancer hallmarks, and drugs that affect or modulate even one of them should be considered as good candidates

for clinical trials (Schneider etal.,2014) . However, to date TQ has not been used in clinical trials, mainly due to formulation problems . The already reported formulations of TQ clearly share problems of low drug loading and burst release (Ganea etal.,2010 ; Abdel wahab et al., 2013) .

The testes are encapsulated ovoid organs consisting of seminiferous tubules separated by interstitial tissues containing leydig cells which are responsible for the production of testosterone. The testis has two main functions namely production of sperms and testosterone. Testosterone plays an important role in maintaining spermatogenesis, accessory sex organs and secondary sexual characters. The epididymis is a single highly convoluted duct, closely applied to the surface of the testes extending from the anterior to the posterior pole of the testis (Al-Hassan ,2010). The metabolic procedure required for testosterone production takes place in approximately 500 million Leydig cells which are located in the testes. Testosterone is secreted under the influence of Luteinizing Hormone (LH), which is the most important factor for regulation of Leydig cell number and function The source for synthesis of testosterone is cholesterol , which may be synthesised from acetate but it may also be taken up from plasma lipoproteins (Rommerts, 2004).

Successful male fertility requires an adequate sperm count, adequate sperm motility, the appropriate functioning of accessory sex organs (to produce and concentrate semen and to activate and capacitate sperm), and appropriate sexual behavior (i.e., mounting, intromission, ejaculation). The production of viable sperm (spermatogenesis) in the testes is under genetic control (a male-determining gene on the Y chromosome) and neuroendocrine regulation initiated in the brain by the hypothalamic-pituitary-gonadal (HPG) axis(Lucio et al., 2005; Tyl, 2001) . Regulation of the neuroendocrine system begins with specialized cells in the hypothalamus in the brain that release gonadotropin-releasing hormone (GnRH) in a pulsatile pattern. GnRH travels via the hypothalamic-pituitary portal system directly to the anterior lobe of the pituitary. In a receptor-mediated process, GnRH stimulates cells of the anterior lobe of the pituitary to secrete the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). LH and FSH travel via the systemic blood supply to the testes. LH binds to receptors on the interstitial cells of Leydig to stimulate Steroidogenesis (i.e., synthesis of testosterone) in these cells. FSH binds to receptors on the intra tubular sertoli

cells, which act as nurse cells for the developing germ cells and are necessary for spermatogenesis(O'Donnell et al., 2001). LH and FSH are produced by the anterior pituitary. The production of these two hormones is stimulated by gonadotropin releasing hormone (GnRH) made by the hypothalamus. It is largely known how LH, FSH and testosterone successful completion of the spermatogenesis process Without it, conversion of round spermatids to spermatozoa during spermatogenesis is impaired. (singh etal.,2011).

Material and method:

Experimental animals:

Sexty days old adult male Wistar rats (average weight: 130±1.6 g), were breed at the animal house of the college of Veterinary medicine Al-Qadisiya University. Animals were reared under controlled conditions (12L:12D cycles at 20-22 C°) and fed on standard laboratory food (19% protein ratio and 3000 kilocalories energy) and drinking water *ad libitum*.

Experimental protocol :

Fourty eight adult male rats were randomly assigned to two equal groups (24 per each) ,and was further divided into three sub groups, based on period of treatment *i.e* two, four and six weeks .

Group 1: **Control group** (**C1, C2, C3),** (intact rats): daily administered with drinking water orally for 6 weeks.

Group 2: **Treatment group:** (**T1, T2, T3**) rats will daily receive thymoquinone (50 mg/kg bw,po) for 6 weeks.Twenty four hours after the last administration of thymoquinone. The animals will be processed in the similar manners to those in experiment. All animals were sacrificed after general anesthesia by combination of Xylazine and Ketamine (10mg and 90mg/kg, *ip*, respectively).

After scarification, testes tissue will be removed for histological and immunohistochemical examination.

Preparation of TQ suspension

TQ suspension at a dose of 50 mg/kg bw were prepared by dissolving 5 mg of TQ powder (from Sigma Chemical Co.) in 1 ml of drinking water to be used as 5 mg / 100 g bw, so that each 100 g bw will need drenching 1 ml of TQ suspension to be contain 5 mg (TQ50) (Kanter,2009).

Histological study:

The rat tissues(testes) excised previously saved in formalin 10% used for the preparation of histological slides depending on the method of Humason (1972).

Immunohistochemistry-Paraffin protocol

According to According to the manufacture instructions (Abcam, UK; www.abcam.com/technical), immunohistochemistry (or IHC) is a method for demonstrating the presence and location of proteins in tissue sections.

Statistical Analysis

The data was entered and analyzed on SPSS version 16.0. Mean ± standard deviation of mean were computed for the quantitative variables like weight of animals, weight of pituitary glands and weight testes Two sample t-tests were used to calculate P-values in comparison of population means.

Results:

Body weight gain and relative weight of testes:

The results showed a significant increase ($P<0.05$) in body weight gain (g) inT1,T2, T3 compared with Control groups for(2,4,6)weeks . It also showed a significant increase ($P<0.05$) in relative weight of testes of T1,T2 and T3 compared with control group (table–1).

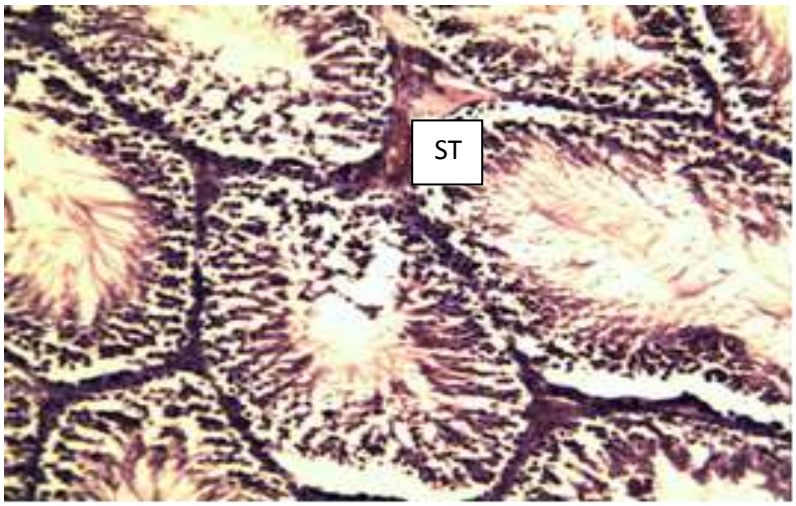
Table (1) : Effect of thymoquinone on the body weight gain, relative weight testes.

Group	Control			Treatment		
Measurement	2wnd	4wth	6wth	2wnd	4wth	6wth
Body weight gain g/100bw	17.37± 0.73 Aa	34± 1.0 Ab	49± 0.08 Ac	44.89± 1.39 Ba	62.25± 1.08 Bb	78.25± 3.55 Bc
Testes weight g/100g bw	0.638± 0.007 Aa	0.641± 0.002 Ab	0.699± 0.014 Ac	0.705± 0.01 Ba	0.859± 0.014 Bb	0.868± 0.004 Bc

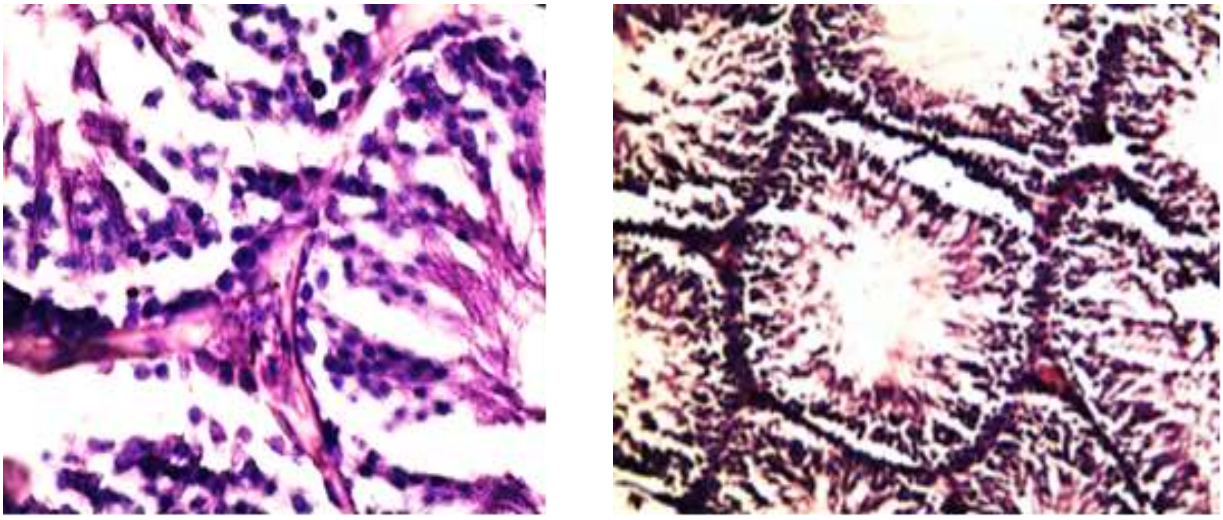
*Different letters indicate significant differences($P<0.05$).

Results of histopathological study:

The histological study of the testicular of experimental animals significant increase (P<0.01) in number of sertoli cells and Leydig cells in T group compared with C. (Fig 1 a,b)



(Fig 1,a) : Section of testicular tissue of a rat belongs to the control group. The seminiferous tubules (ST) have ordinary shape; normal number of sertoli cells and Leydig cells; their epithelium is structurally intact and shows normal association of germ cells. H&E.(10X).



(Fig 1,b) : Treated group show normal size of cells; normal seminiferous tubules ; significant increase (P<0.01) in number of sertoli cells and Leydig cells in testes cells. H&E 40X.

Results of immunohistochemical study:

Immunohistochemical results revealed that male rats drenched with thymoquinone showed actively stained of Leydig cells with actively stained of Sertoli cells in T group than in C group. Figure(2).

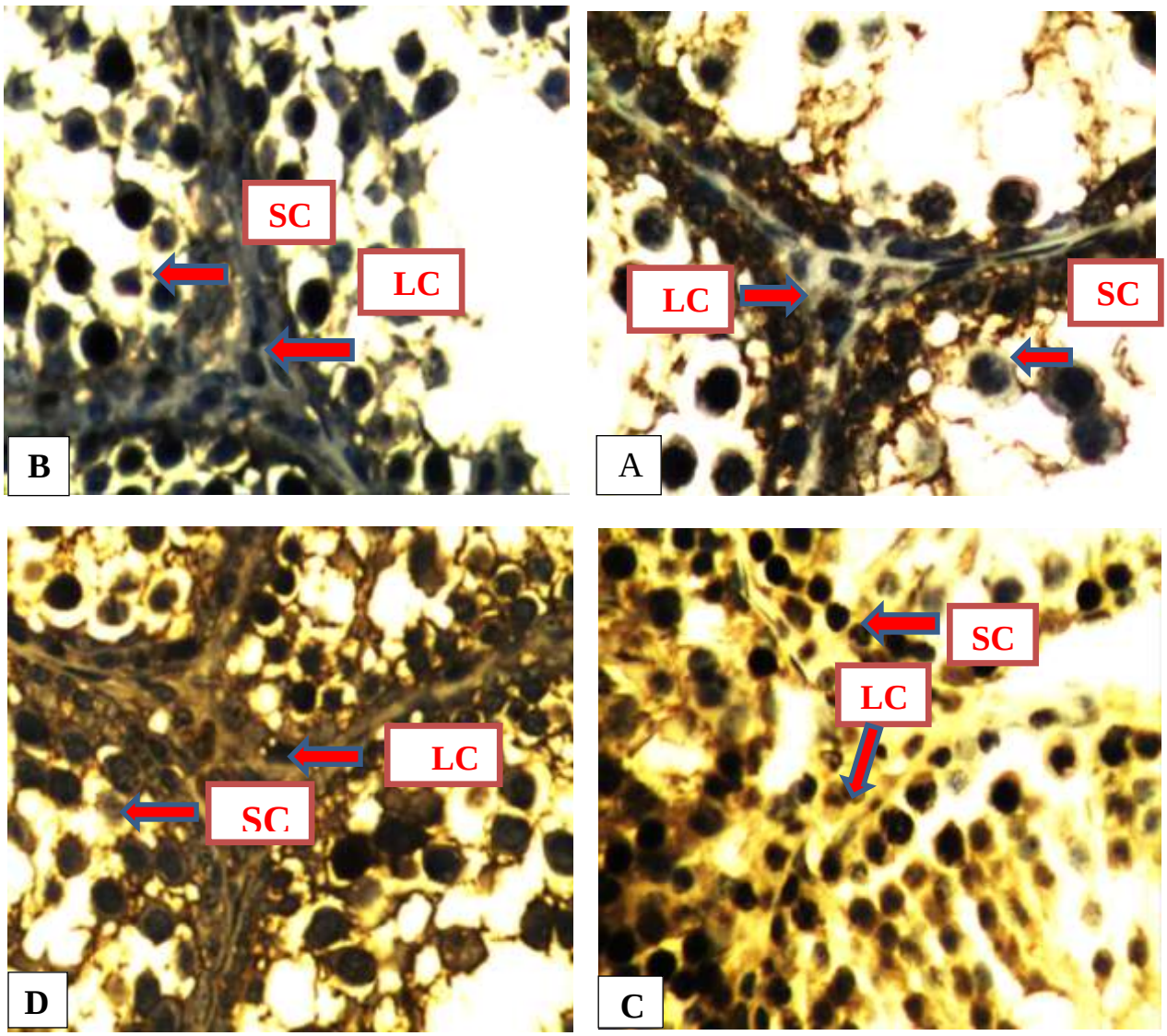


Figure (2): Cellular expression of testosterone in testis during treatment with TQ , shows positive immune staining in Sertoli cells(S.C) and Leydig cells (L.C) in (T1,T2,T3) figure (B,C,D) respectively compared with control (A).H.E.400X.

Discussion:

Administration of male rats with thymoquinone caused insignificant changes in body weight gains in comparison with intact control male rats. The result at end of 2weeks and continue to end of experimental showed significantin increase of body weight gains this shows a clear in group 3 this related to effect of Thymoquinone on increases the catabolism of glucose and energy production which ultimately lead to improve the growth (Passos and Von Zglinicki, 2006) . previous studies have been shown that there were no toxic effects or inflammatory reaction (18, 42-43). This suggests that this extract at the applied doses has no general toxic effect on body weight. The present study showed that testes weight at dose 50 mg/kg significantly increased and this effect was seen in previous studies (29-31).

The testes are structurally and physiologically dependent upon the testosterone and other androgens. Testosterone stimulates growth and secretary activity of the reproductive organs (44-46) so a significant increase of these hormones in our study could increase the number and function of somatic and germinal cells of testis and in results increase the testis weight.

Histological and immunohistochemical study:

Results for histological study showed a significant increase in producing testosterone to lyedic cells in animals of treated groups compared with the control group. This increase was attributable to the positive impact of thymoquinone due to its antioxidant properties and this result conform with Mahdavi *et al*. (2005) as it pointed out that the black seed have positive impact on reproductive system, and they said that this effect may be due to the essential ingredient of the black seed which thymoquinone which has a role in improving the fertility standards by strengthening antioxidants defenses, where they works on the equation of free radical in the body which

works to sperm harm. The results of the study are consistent with what they referred Menzo *et al*. (2014) that the components of anti-effect in improving the spermatogenesis and steroidogenesis including testosterone the increase of this hormone results in an increase in the number of producing lyedic cells under the control of LH hormone from the pituitary this leads to the development of spermatogenesis and fertility improve .(Spaliviero *et al*., 2004)**.** That the increase in Srtoli cells for male treatment group compared with the control group may be due to the reaction of follicle stimulating hormone as increased rates by TQ treatment. Studies indicated that the number of these cells depends primarily on the basis of the concentration of the pituitary hormones that Sertoli cells do not generated in to in seminiferous tubules and increased their numbers depends on the growing number of cells in seminiferous tubules.(Sharpe *et al*., 2004). The results of the study are consistent with findings of Al- Sa'aidi et al. (2009) on the role of the alcoholic extract of black seeds in improving the standards of male reproductive tract, including the numbers of Sertoli and leydig's cells and all levels of testosterone and follicle stimulating hormone. TQ has demonstrated some protective roles in relation to oxidative status, such as superoxide anion scavenger, direct cytoprotective effects and indirect antioxidant and androgen activities (Hala 2011). Therefore, it may protect cells and testes against a testicular toxin .

The present immunohistochemical study has showed gradual increase in positive immune staining for testosterone product of the leydig cells in male rats treated with TQ ,the increase started from second week and continued to end of experiment . The immune interaction density increased in the sixth week as the amount of testosterone produced is greater than the quantity in the rest of the totals and the strength of this interaction back to the antibody linked to hormone with antigen (testosterone) located in leydig cells

compared with the control group, which appear immune reaction but in less extent .Those findings were in agreement with that reported by Majdic et al.(1997).The elevation in positive immunostanining in result especially at T3 may be reason of hypothalamo- pituitary- testicular axis activation in TQ treated, being correlated with rising levels of the gonadotropins and testosterone. Sasso-cerri et al.(2005) they confirmed that testosterone secreted by interstitial cells in testis in all animals observed when using immune reaction of leydig cells . They explained that the interaction increases by seasons of the year where they are in severity in the summer season, which is the breeding season compared to the winter season, which will be the strength of the interaction is weak for lack of the amount of the hormone as well as in the spring and fall season. As to these results agree with the results of Haseena *et al*.(2015) Who confirmed the role of Thymoquinone in improving and prevention fertility parameters and raise the level of testosterone in rats. Thymoquinone is the major active component derived from Nigella sativa and many of the pharmacodynamic effects reported above for N. sativa are due to Thymoquinone (54). Gokce *et al* (55) has been confirmed that Thymoquinone treatment has protective effects on testicular parameters

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