Available online on www.ijpqa.com
International Journal of Pharmaceutical Quality Assurance 2018; 9(3); 340-347
doi: 10.25258/ijpqa.v9i3.13670
ISSN 0975 9506

Research Article

Effect of Allicin in Reducing the Cytotoxicity of Cyclophosphamide on Reproductive System of Wister Male Rats

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Received: 23rd Jun, 18; Revised: 13th Jul, 18, Accepted: 29th Aug, 18; Available Online: 25th Sep, 2018

ABSTRACT

The aim of the present study is to investigate the positive role of Allicin in reducing the side effects induced by cyclophosphamide drug in reproductive system of male rats. In this experiment, fifty adult male Wister rats were used, about four month old, with average weight about (162.5±13gm). They were divided randomly into five equal groups (10 animals for each group) and drenched for 60 consecutive days as follows: The results of this study revealed significant differences ($P \leq 0.05$) represented by increasing in concentration of testosterone, SSH and ICSH hormones in T1 group as compared with other groups, while there was a significant decrease in T2 group as compared with other groups. Also, there was a significant difference represented by increasing levels of these hormones in T3 and T4 groups as compared with T2 group ,and there was a significant difference in concentration of testosterone, SSH and ICSH between T3 and T4 groups represented by increasing the concentration of these hormones in T4 group as compared with T3 group. It is concluded that allicin has in dose of 50 mg/kg/B. W. both a preventive and a therapeutic role in ameliorating cyclophosphamide toxicity in adult Wister male rats. The use of allicin as a therapeutic agent showed more amelioration in cyclophosphamide toxicity than a preventive agent.

Keywords: Allicin, Cyclophosphamide, Male reproductive hormones, Gene expression.

INTRODUCTION

An antioxidant is any substance that delays, prevents or removes oxidative damage to a target molecule. It is stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage. These antioxidants inhibit cellular damage mainly through their free radical scavenging property[1].

Allicin (diallyl-thiosulfinate) is one of the main biologically active compounds derived from garlic, it has been shown to exert various pharmacological activities and considered to has therapeutic potential effect for many pathologic conditions[2]. It certainly acts as a "physiological antioxidant and its structure and biological properties were first described by Cavallito and Bailey in 1944[3]. It has been used as a medicine to therapy a wide range of diseases and conditions related the heart and blood system including high blood pressure, high cholesterol coronary, heart disease, heart attack, and hardening of the arteries (atherosclerosis)[4]. A recent study has confirmed that in vitro the vasoactive ability of garlic sulfur compounds whereby red blood cells convert garlic organic polysulfides into hydrogen sulfide, a known endogenous cardio-protective vascular cell signaling molecule[5]. Allicin is not present in raw garlic, but it is rapidly produced by the action of allinas (CS-lyase) on alliin . Allinase is activated by crushing or cutting the garlic cloves[6]. Intact garlic typically contains ~1% alliin, together with (+)-S-methylL cysteine sulphoxide (methiin) and (+)-S-(trans-1-propenyl)-L-cysteine sulphoxide. S-(2-carboxypropyl) glutathione, γ-glutamyl-S-allyl-L-cysteine, glutamyl-S-(trans-1-propenyl)-L-cysteine and γ -glutamyl-S-allyl-mercapto-L-cysteine are also reported to be present in garlic cloves[7].

MATERIALS AND METHODS

Experimental Animals

In this experiment fifty adult male Wister rats were used, about four month old, with average weight about (162.5±13gm) obtained from animal house in college of veterinary Medicine at university of AL-Qadisiyah. The animals housed in well ventilated wire-plastic cages with dimensions 40×60 cm and reared under controlled conditions about 12 hour light and 12 hour dark at 22C°) .The animals were allowed to acclimatize for 14 days before experimentation.

Experimental Design

Fifty adult male Wister rats were divided randomly into five equal groups (10 animals for each group) and treated for 60 consecutive days as following:

Control group (C), was given 1 ml distilled water orally.

The first treated group (T1) was given allicin orally in a dose of 50 mg\kg B .W once daily dissolved in 1 ml distilled water for 60 day (19).

The second treated group (T2) was given cyclophosphamide orally in dose of 10 mg /kg /B .W

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once daily dissolved in 1 ml distilled water for 60 day (20).

4-The third treated group (T3) was given allicin orally (50mg/kg/B. W)for 30 days then given orally cyclophosphamide 10 mg /kg B. W/day for 30 days.

5-The fourth treated group (T4)was given cyclophosphamide orally

(10 mg/kg B.W/day) for 30 days then given orally allicin (50 mg/kg B.W/day) for 30 day.

Animals Sacrificing

Body weight has been recorded Weekly because of administration of doses of allicin and cyclophosphamide depending on body weight. Twenty four hours after last administration all animals were anaesthised by mixing of Ketamine and xylazin (9mg/kg/B.W, 10mg/kg/B.W) respectively intraperitonial, to sacrified then blood samples were collected from the heart to obtain the serum of animals for assessment of testosterone hormone, ICSH and SSH. Testis samples from all animals were removed for study gene expression.

Collection of Blood Samples

Blood was collected from each animal directly from the heart by using 5 ml disposable syringe, then putting in gel and clot activator tube and left at room temperature until clotted, then it were centrifuged at 5000 rpm for 15 minutes, the serum was aspirated from the tube and stored at -20C^{o} until used for analysis (8).

Laboratry Measurements

The animals were investigated for the following parameters:-

Hormonal Assays in Blood Serum by Using ELISA Technique.

Interstitial stimulating hormone (ICSH) by using ELISA ICSH Kit (Monobind, USA)

Spermatogenic stimulating hormone (SSH) by using ELISA SSH Kit(Monobind, USA)

Testosterone hormone by using ELISA Testosterone Kit(Monobind, USA)

Quantitative Reverse Transcription Real-Time PCR.

Quantitative Reveres Transcription Real-Time PCR technique was performed for the quantification of relative gene expression analysis for ICSH receptor, and CYP11A1 (Testosterone) gene. These genes were normalized by using housekeeping gene (GAPDH). This technique was done according to method described by Livak and Schmittgen, (2001) (11).

Statistical Analysis

A computerized program, the statistical package for social sciences (SPSS), was used to analyze data. The data were expressed as means ± standard errors (SE). Differences between group means were estimated using a one-way analysis of variance (ANOVA) with least significant difference LSD was detected to compare between groups, and results were considered statistically significant at P <0.05 (13).

RESULTS

The Effect of Allicin on Reproductive Hormones Levels in Wister Male Rats Treated with Cyclophosphamide.

Concentration of Testosterone Hormone (ng/ml).

Table (1) revealed there was a significant increase ($P \leq o.o5$) in testosterone concentration in T1 group (1.81 ± 0.04) as compared with other groups, while there was a significant decrease in testosterone concentration in T2 group (0.29 ± 0.006) when compared with other groups. And there was a significant difference represented by the increase in testosterone concentration in T3 and T4 groups (0.92 ± 0.003), (1.06 ± 0.013) respectively as compared with T2 group, and there was a significant increase in testosterone concentration inT4 group as compared with T3group.

Concentration of ICSH (mIU/ml)

Table (4-2) showed there was a significant difference ($P \leq 0.05$) represented by increasing of ICSH concentration in T1 group (1.94 ± 0.01) as compared with other groups, while there was a significant decrease in ICSH concentration in T2 group (0.65 ± 0.02) as compared with other groups. There was a significant difference represented by increasing of ICSH concentration in T3 and T4 groups (1.43 ± 0.01), (1.52 ± 0.004) respectively as compared with T2 group, and there was a significant increase in ICSH concentration in T4 group as compared with T3 group.

Concentration of SSH (mIU/ml)

Table (1) showed that T1 group appeared a significant increase ($P \leq 0.05$) in concentration of SSH (2.84 ± 0.01) when compared with other groups. While there was a significant decrease in SSH concentration in T2 group (0.81 ± 0.01) as compared with other groups. Also, there was a significant increase in concentration of SSH in T3 group and T4 group (1.87 ± 0.02), (2.03 ± 0.02) respectively as compared with T2 group, and there was a significant increase in ICSH concentration in T4 group as compared with T3 group.

Molecular Analysis

Total RNA Concentration and Purity

Total RNA concentrations and purity that extracted from rat testis tissue samples of experimental were measured by Nanodrop spectrophotometer. Results (Mean ± SD) concentrations exhibited as ng/µl. Testis RNA concentration in C, T1, T2, T3, T4 (571.94±295.42, 522.44±120.36, 652.35±156.92, 678.32±176.17, 456.266±232.55) respectively.

The purity of the extracted RNA was estimated by measuring the ratio of A_{260}/A_{280}.Testis total RNA purity in C, T1, T2, T3, T4 (1.81±0.12, 1.854± 0.16,1.69±0.21,1.73±0.25, 1.81±0.18) respectively.

Gene Expression of Testosterone (CYP11A1 gene).

Results in table (2) indicated there was a significant difference ($P \leq 0.05$) represented by increasing the fold change of testosterone gene expression levels in T 1 group (12.63 ± 0.48) when compared with other groups, while there was a significant difference represented by decreasing the fold change in T 2 group (0.31 ± 0.03) as compared with other groups. And there was a significant difference represented by increasing the fold change of testosterone gene expression levels in T3 and T4 groups (5.26 ± 0.35), (7.08± 0.47) respectively as compared with T2 group, and there was a significant difference in fold change of testosterone gene expression levels

Table 1: The Effect of Allicin on Reproductive Hormones Levels in Wister Male Rats Treated with Cyclophosphamide.

Parameters Groups	Testosterone hormone (ng/ml)	ICSH (mIU/ml)	SSH (mIU/ml)
C group	A 1.18 ± 0.007	A 1.63 ± 0.01	A 2.17 ± 0.02
T1 group	B 1.81 ± 0.04	B 1.94 ± 0.01	B 2.84 ± 0.01
T2 group	C 0.29 ± 0.006	C 0.65 ± 0.02	C 0.81 ± 0.01
T3 group	D 0.92 ± 0.003	D 1.43 ± 0.01	D 1.87 ± 0.02
T4 group	E 1.06 ± 0.013	E 1.52 ± 0.004	E 2.03 ± 0.02
LSD 0.05	0.0014	0.046	0.065

Numbers = mean ± Standard Error (S.E).
Different litters = Significant Differences ($p<0.05$).
C= Control group, drenched with distilled water orally for (60) days.
T1= Drenched with allicin orally (50 mg /kg B.W/day) for (60) days.
T2= Drenched with cyclophosphamide orally (10 mg/kg B.W/day).
T3= Drenched with allicin orally (50 mg/kg/B.W/day)for (30) days then with cyclophosphamide (10 mg/kg/B.W/day) for (30) days.
T4= Drenched with cyclophosphamide orally (10 mg/kg/B.W/day) for (30) days then with allicin (50 mg/kg/B.W/day) for (30) days.

Table 2: The Effect of Allicin on Gene Expression in Wister Male Rats Treated with Cyclophosphamide.

Groups	Genes	
	CYP11A1 gene for Testosterone(fold change)	ICSH receptors gene expression(fold change)
C group	A 1.31 ± 0.52	A 1.11 ± 0.27
T1 group	B 12.63 ± 0.48	B 8.46 ± 0.44
T2 group	A 0.31 ± 0.03	A 0.21 ± 0.04
T3 group	C 5.26 ± 0.35	C 3.89 ± 0.37
T4 group	D 7.08 ± 0.47	D 5.17 ± 0.52
LSD 0.05	1.35	1.207

Numbers = mean ± Standard Error (S.E).
Different litters = Significant Differences ($p<0.05$).
C= Control group, drenched with distilled water orally for (60) days.
T1= Drenched with allicin orally (50 mg /kg B.W/day) for (60) days.
T2= Drenched with cyclophosphamide orally (10 mg/kg B.W/day).
T3= Drenched with allicin orally (50 mg/kg/B.W/day)for (30) days then with cyclophosphamide (10 mg/kg/B.W/day) for (30) days.
T4= Drenched with cyclophosphamide orally (10 mg/kg/B.W/day) for (30) days then with allicin (50 mg/kg/B.W/day) for (30) days.

between T3 and T4 groups represented by the increase levels of gene expression for testosterone hormone in T4 group as compared with T3 group.

Gene Expression of ICSH Receptors.

Results in table (2) indicated there were significant differences ($P \leq 0.05$) represented by increasing the fold change of ICSHr gene expression levels in T1 group (8.46 ± 0.44) when compared with other groups, while there was a significant difference represented by decreasing the fold change in T 2 group (0.21 ± 0.04) as compared with other groups. And there was a significant difference represented by increasing the fold change of ICSHr gene expression levels in T3 and T4 groups (3.89 ± 0.37), (5.17 ± 0.52) respectively as compared with T2 group, and there was a significant difference in fold change of ICSHr gene expression levels between T3 and T4 groups represented by increasing levels of gene expression for LH receptors in T4 group as compared with T3 group.

DISCUSSION

The Effect of Allicin on Reproductive Hormones Levels in Wister Male Rats Treated with Cyclophosphamide.

The toxic effects of chemotherapy medications such as cyclophosphamide can be directly or indirectly imposed on the interstitial cells through seminiferous epithelium cell damage[14]. And thus lead to reduced synthesis and testosterone secretion[15]. It was established earlier that cyclophosphamide causes testicular toxicity by germ cell degeneration and inhibits androgen production in adult male rats, probably by affecting pituitary ICSH hormones and thereby inhibiting Leydig cell testosterone production. The decrease in the serum level of testosterone in rats exposed to cyclophosphamide may be due to both direct effect on testis and suppression of ICSH secretion. The abnormal testosterone levels following chemotherapy are due to the leydig's cells damages which is a side effect outcome of the chemotherapy[16]. The results of this study showed that serum levels of ICSH, SSH and testosterone hormone in a group that had received cyclophosphamide decreased compared to the other group. The harmful effects of cyclophosphamide and its active metabolites by affecting DNA molecule and breaking it, affecting RNA molecules, and synthesizing proteins[17,18]. It may affect the ICSH generating cells located in the anterior pituitary and cause serum level of ICSH and SSH to lower. Or it was affect hypothalamus and GnRH-generating cells, number of GnRH produces hormone decreases considerably and

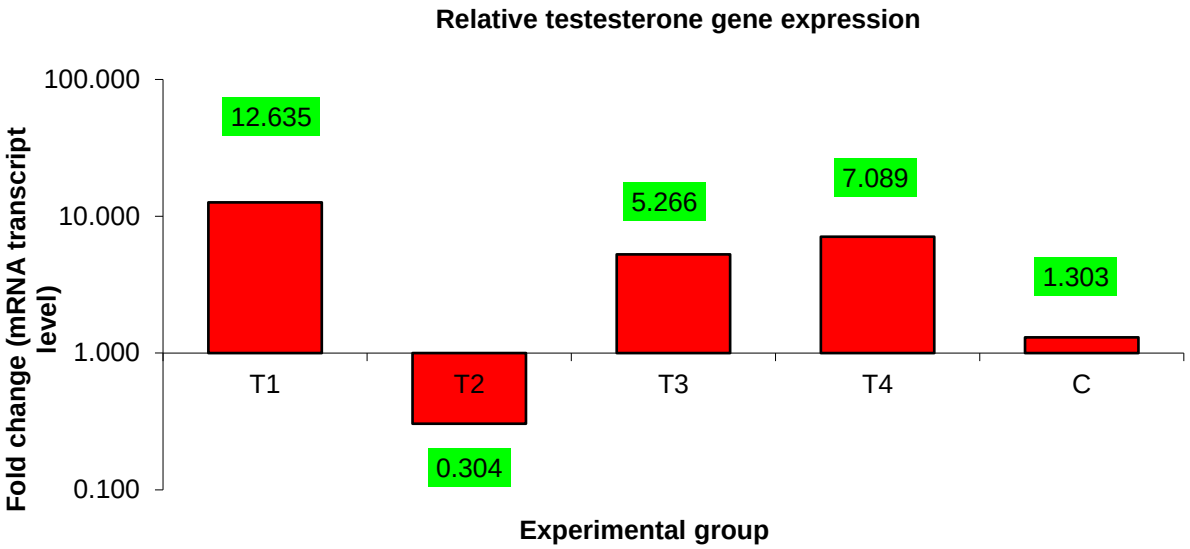

Fig 1: Relative Testosterone Gene Expression
C= Control group, drenched with distilled water orally for (60) days.
T1= Drenched with allicin orally (50 mg /kg B.W/day) for (60) days.
T2= Drenched with cyclophosphamide orally (10 mg/kg B.W/day).
T3= Drenched with allicin orally (50 mg/kg/B.W/day)for (30) days then with cyclophosphamide (10 mg/kg/B.W/day) for (30) days.
T4= Drenched with cyclophosphamide orally (10 mg/kg/B.W/day) for (30) days then with allicin (50 mg/kg/B.W/day) for (30) days .

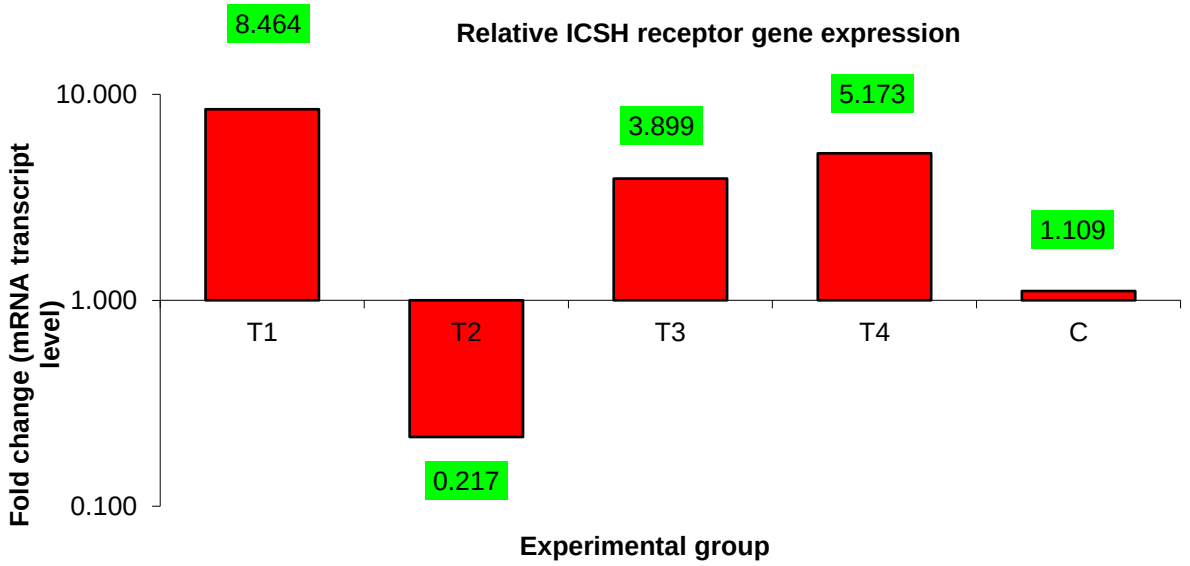

Fig 2: Relative ICSH Receptor Gene Expression
C= Control group, drenched with distilled water orally for (60) days.
T1= Drenched with allicin orally (50 mg /kg B.W/day) for (60) days.
T2= Drenched with cyclophosphamide orally (10 mg/kg B.W/day).
T3= Drenched with allicin orally (50 mg/kg/B.W/day)for (30) days then with cyclophosphamide (10 mg/kg/B.W/day) for (30) days.
T4= Drenched with cyclophosphamide orally (10 mg/kg/B.W/day) for (30) days then with allicin (50 mg/kg/B.W/day) for (30) days .

GnRH reduction, in turn, leads to lowering ICSH and SSH. After DNA molecule breakage, replication does not occur, which is followed by protein synthesis reduction. As ICSH, SSH and GnRH have apolypeptide structure, therefore,ICSH ,SSH and GnRH levels decrease with respect to their chemical structure, which is protein (polypeptide)[19]. We can conclude that cyclophosphamide reduces ICSH and SSH hormones concentration. ICSH

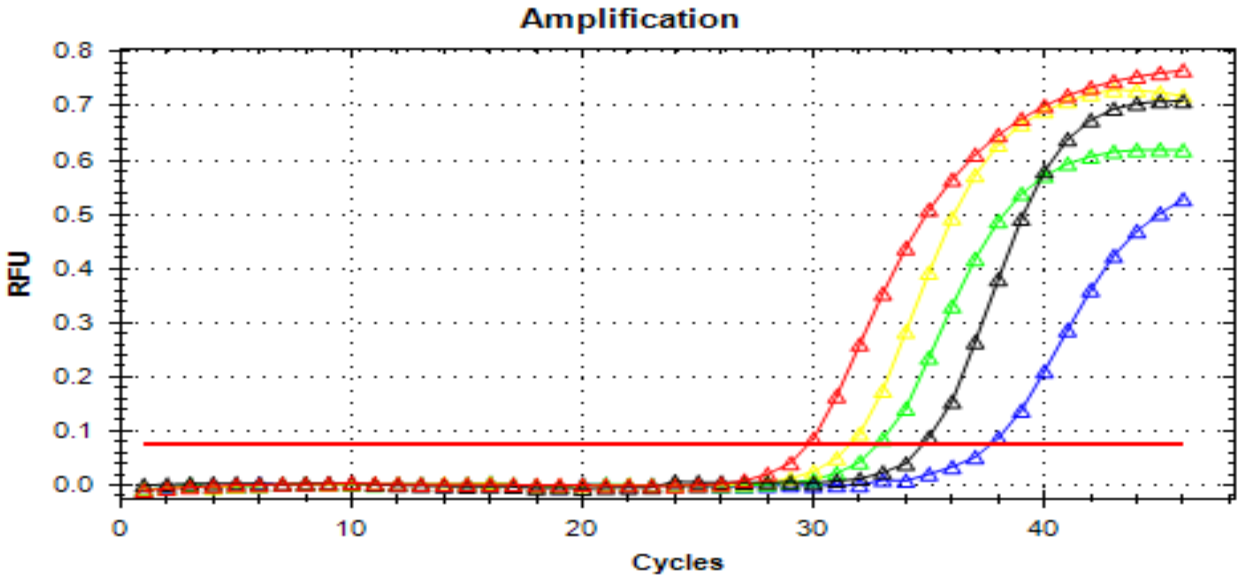

Fig 3: Real time PCR amplification plot for CYP11A1 gene for Testosterone in testis that showed difference in threshold cycle numbers (Ct value) between treatments and control groups.
Black plot: Control group, drenched with distilled water orally for (60) days
Red plot: T1 group, drenched with allicin orally (50 mg /kg B.W/day) for (60) days.
Blue plot: T2 group, drenched with cyclophosphamide orally (10 mg/kg B.W/day) for (60) days.
Green plot: T3 group, drenched with allicin orally (50 mg/kg/B.W/day) for (30) days then with cyclophosphamide (10 mg/kg/B.W/day) for (30) days.
Yellow plot: T4 group, drenched with cyclophosphamide orally (10 mg/kg/B.W/day) for (30) days then with allicin (50 mg/kg/B.W/day) for (30) days.

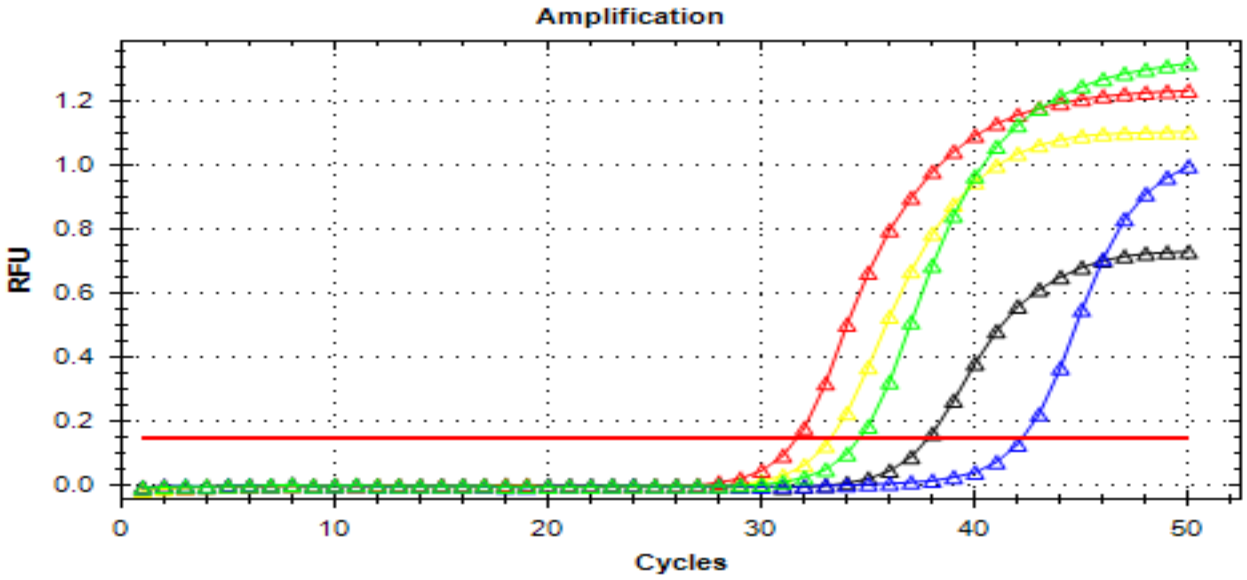

Fig 4: Real time PCR amplification plot for ICSH receptor gene for in testis that showed difference in threshold cycle numbers (Ct value) between treatment and control groups.
Black plot: Control group, drenched with distilled water orally for (60) days
Red plot: T1 group, drenched with allicin orally (50 mg /kg B.W/day) for (60) days.
Blue plot: T2 group, drenched with cyclophosphamide orally (10 mg/kg B.W/day) for (60) days.
Green plot: T3 group, drenched with allicin orally (50 mg/kg/B.W/day) for (30) days then with cyclophosphamide (10 mg/kg/B.W/day) for (30) days.
Yellow plot: T4 group, drenched with cyclophosphamide orally (10 mg/kg/B.W/day) for (30) days then with allicin (50 mg/kg/B.W/day) for(30)days .

stimulates Leydig cells in the testis to produce testosterone hormone[20]. Decrease of ICSH concentration leads to lowering concentrations of testosterone hormone. This results agree with Rajabi and Karimijashni, (2014)[21]. Our results demonstrated that animals which were treated with allicin (T1) showed increasing in concentration of ICSH, SSH and testosterone hormone. Garlic extract compounds are responsible for the significance increase by affecting the Performance of steroid-generating enzymes, the effective compounds of garlic extract increase secretion of testosterone hormone and its metabolites[22]. Aqueous extract of *Allium sativum* treatment ameliorated the pituitary-testicular injury and dysfunction in Wister rats Induced reproductive disturbances[23]. This result leads to conclude that allicin has improver effect on hypothalamic-pituitary-testis axis causes increasing in Testosterone hormone ICSH and SSH by stimulating the hypothalamic-pituitary-testis axis. It has been reported that these results suggest that garlic administration increases testosterone production in the testis due to the enhancement of ICSH secretion from the pituitary gland.

Also, the results demonstrated that animals were received allicin and then cyclophosphomide (T3), and animals were received cyclophosphamide and then allicin (T4) a significant increase in ICSH, SSH and testosterone hormone levels as compared with T2 group. Several studies showed that garlic (*Allium sativum)* inhibits lipid peroxide formation[24]. Antioxidant ability of garlic depended on the presence of organic sulfur compounds especially allicin compound that regulate glutathione level and GST activity[25]. Therefore, they are able to remove the harmful effects of cyclophosphamide and it's active metabolite. The antioxidant compounds in garlic (Sulfur-containing compounds that are abundant in garlic) by removing active and negative metabolites from body, reduce the effect of such metabolites on the anterior pituitary secreting SSH and ICSH hormone[26]. These reasons may explain how the reproductive hormones are increased by treating with allicin. Also, our results indicated there was a significant difference between T3 and T4 groups represented by increasing in level of reproductive hormones in T4 group when compared with T3 group due to increase gene expression for these hormones in T4 group more than T3 group.

Molecular Analysis

Our study intended to evaluate the role of allicin in improving male reproductive system by using the Wister male rat as a model of mammalian through the assessment level of mRNA of CYP11A1 gene expression which is responsible for production of testosterone hormone and ICSH receptors gene that is responsible for producing ICSH receptors .The results of this study indicated that allicin treatment induces up regulation in gene expression of CYP11A1 gene and ICSH receptors gene in T1 group when compared with other groups.

Allicin presumably strengthens its antioxidant action through increasing removal of free radicals, activity of superoxide dismutase, glutathione peroxide, and increasing glutathione level[27] this leads to the increase of the gene expression of ICSH receptors and CYP11A1 genes through the increase of signaling, transport , metapolism, and control of transcription and oxidative phosphorylation.

The allicin improves of lydig's cells act as antioxidant action lead to Cholesterol transport within the Leydig cells. The steroidogenic enzymes are critical for the production of androgens; up-regulation of the mRNA expression of genes coding for these enzymes can increase the efficiency of testosterone and estradiol synthesis[28]. Steroidogenesis requires the transport of free cholesterol from the outer to the inner mitochondrial membrane enzyme reaction. These enzymes reaction are catalyzed by the CYP11A1 gene lead to increase production of testosterone[29]. In T2 group which treated with cyclophosphamide there was a significant decrease in gene expression of CYP11A1 gene and ICSH receptors gene when compared with other groups.

Free radicals attack important macromolecules leading to cell damage and homeostatic disruption. Targets of free radicals include all kinds of molecules in the body. Among them, lipids, nucleic acids, and proteins are the major targets[30]. There is evidence that biological responses triggered by oxidative products are associated with lipid peroxidation derivatives, which are able to induce various pathogenic intracellular signals involving of inflammatory genes, calcium, G-proteins, cAMP, cGMP, phospholipase C and D, protein kinase C, IL-1, IL-6, TNF-α, ceramide, and MAP kinase cascade leading to cellular dysfunction[31].

Cyclophosphamide caused increased the lipid peroxidation, induced of ROS production and down regulation of antioxidant genes, glutathione peroxidase, catalase, and superoxide dismutase[32].

Cyclophosphamide produces two active metabolites such as phosphoramide mustard and acrolein. Lipid peroxidation is one of the principal causes of CP-induced toxicity and is mediated by the production of acrolein, a metabolite for much of its toxicity[33]. The increasing of ROS causes changes in all bases, deletion and uncoupling of complement bases, morphological and cross junction changes of DNA and changes in reorganization of chromosomes[34] cyclophosphamide develops many complications by impacting the DNA molecule and breaking it and also by impacting the RNA molecule and protein synthesis[35]. The ICSH receptors are a protein in structure, thus the cyclophosphamide may cause change in gene that is responsible for the production of enzymes that stimulate formation of ICSH receptors in leydig's cells .

Steroidogenic enzymes are sensitive indicators of testicular testosterone synthesis, the alterations of enzymes, which might result in change of testosterone synthesis. The changes in gene expression were correlated with corresponding levels oftestis testosterone (intratesticular testosterone) concentration and finally the increased secretion of sex hormones. (28). Cyclophosphamide causes reduction of 17β-Hydroxysteroid dehydrogenases (17β-HSD) which is an enzyme that catalyzes the interconversion of

androstenedione to teoststerone in tissues testes[36] cyclophosphamide may cause down regulation of mRNA expression of gene coding for this enzyme.

Inaddation, our results demonstrated that in T3 group which was treated with allicin then with cyclophosphamide, and T4 group which was treated with cyclophosphamide then with allicin, there was a significant inecrease in gene expression of CYP11A1 gene and ICSH receptors gene when compared with T2 group due to the allicin suppresses ROS overproduction, reduces lipid peroxidation and increases the endogenous antioxidant enzyme activities[37].

A study by Horev-Azaria *et al*., (2009)[38] suggested a putative role for allicin and it's derivatives in preventing reactive oxygen species damage by up-regulating the phase II detoxifying enzymes and increasing the cellular glutathione level in vascular endothelial cells. Allicin can be able to remove the harmful effects of cyclophosphamide and its active metabolite and protects DNA from the damages caused by free radicals and cyclophosphamide metabolites. In case the DNA of the testes cells is damaged due to cyclophosphamide metabolites, the allicin recovers them[25].

Allicin is responsible for the significance increase in testosterone hormone by affecting the performance of steroid-generating enzymes, these include the ability of allicin to act as key enhancer of the endogenous levels of GSH and glutathione peroxidases which are protect against oxidative damage to DNA, proteins, and membrane lipids. Furthermore, it can prevent and/or detoxify intermediate metabolites of chemical carcinogens and stimulate the immune responses. Allicin is reported to be metabolized to intermediate metabolites that modify cell cycle and apoptotic factors and modulate thio redoxin reductase, protein disulfide isomerase, quinine reductase, glutathione reductase, and intracellular redox status, which in turn, regulate cell-signal transduction, transcription factor activation and DNA repair[39].

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