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STUDY THE EFFECT OF QUERCETIN ON FSH AND ESTROGEN GENE EXPRESSION IN FEMALE WISTER RATS EXPOSED TO OXIDATIVE STRESS BY LEAD ACETATE

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ABSTRACT

This study was aimed to evaluate the effects of Quercetin to improve efficiency of the female reproductive system of wester rats which exposed to oxidative stress by lead acetate. 40 mature female rats were used with weights at (180± 20 gm) and randomly divided into equal 4 groups (10 animals in each group). group 1(control group) received drinking water freely, group 2: administered Querctin (300mg/kg) orally once daily for 60 days, group 3: administered daily dose of lead acetate (10mg /kg) orally, once daily for 60 days and group 4: administered daily dose of lead acetate (10mg/kg) orally for 30 days and then administered Quercetin (300mg/kg) orally once daily for another 30 days. after 60 days the animals were sacrificed and samples of ovary and pituitary glands was taken for gene expression study for Aromatase cytochrome P450 gene, FSH beta subunit gene and FSH receptor gene The results were as follows: gene expression of estrogen showed a significant increase ($p<0.05$) in (T1) compared with control groups and T2, while results of T2 showed significant decrease ($p>0.05$) in compared with other groups, also there was a significant increase($p<0.05$) in (T3) compared with control group and T2.while the results gene expression of FSH receptor gene showed significant increase ($p<0.05$) in T1 in compare with T2 and control group, also significant increase ($p<0.05$) in T3 compared with control groups and T2, while T2 showed significant decrease ($p> 0.05$) compared with other groups. In the same time the results of gene expression of FSH beta subunit gene showed significant increase ($p < 0.05$) in T1 compared with control

groups and T2, also showed significant decrease (p> 0.05) inT3 compared with T1 andT3, and T3 showed significant increase (p<0.05) compared with T2 and control group.

KEYWORDS: Quercetin, lead acetate, gene expression, antioxidants, Oxidative stress.

INTRODUCTION

Oxidative stress(OS) defined as an imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage[1]. Heavy metals, including lead (Pb), are known to cause over production of reactive oxygen species (ROS) and consequently enhance lipid peroxidation, increase the unsaturated fatty acid and decrease the saturated fatty acids contents of membranes[2]**.** Lead is reported to cause oxidative stress by generating the release of ROS such as hydrogen peroxide, superoxide radicals, lipid peroxides and hydroxyl radicals[3]**.** A variety of ROS are produced during the body which are presence to be the by-products of aerobic metabolism of the cell, and exposure to UV light or X-rays and ongoing stress[4]. Humans are exposed to lead through food, water and air. Also it is well known that is red blood cells the main target for lead toxicity, and exert lead toxicity on reproductive organs in females and males[5]. The human body has several mechanisms to counteract oxidative stress by formation antioxidants, either naturally produced in situ (endogenous) or externally supplied through foods and supplements (exogenous). So, endogenous and exogenous antioxidants act as "free radical scavengers" by preventing and correcting damages caused by reactive oxygen species and therefore can enhance the immune defence and decrease the risk of disease and cancer[6]. An antioxidant is a molecule able of inhibiting the oxidation of other molecules[7]. Antioxidants scavenge excess reactive oxygen species(ROS) to counteract potential for significant damage of the cell by excess reactive oxygen species. Antioxidants also help create a balance between useful oxidant generation (commonly act as cell signaling molecules) also damaging oxidative stress[8]. Example on antioxidant Flavonoids which are a group of naturally occurring compounds extensively distributed as secondary metabolites in the plants[9]**.** One of these flavonoids, quercetin QE (3,5,7,3',4'-pentahydroxyflavone), prevents oxidant injury and cell death by a number of mechanisms, such as scavenging oxygen radicals protecting against lipid peroxidation and chelating metal ions[10]**.** The quercetin a glycone is a flavonol that occurs naturally in fruits and vegetables, including onions, apples, grapes and grapes nuts. Therefore, it is commonly included in human diets[11]**.** It is known to use a strong antioxidant therefore and already used in treating human disease (phytotherapy), as it is present in the seeds, barks, stems, roots

and/or flowers of several medicinal plants[12]. It is present in humens daily food and is recognized for biological activities such aantioxidant, antiviral, anticancer, antimicrobial, anti-inflammatory and many more[13].

MATERIALS AND METHODS

Laboratory animals: used in our experiment forty adult females Wister rats with about 6 months in old, with average weight about (175±10 gm.) obtained from animal house of veterinary medicine college of Al-Qadisiyah university .The animals housed in well ventilated wire-plastic cages and reared under controlled conditions about 12 hour light and 12 hour dark at about 25̊. And given drinking water freely. The animals were allowed to acclimatize for 7 days before experimentation.

biological material: quercetin 95% from onion provided by brightol company/ China.

Experiment design: forty adult female Wister rats divided randomly to the equal four groups and treated for 60 days as following-:control group given drinking water only .second group (T1): given quercetin orally in dose (300mg/kg/b.w) [14]. The third group (T2):given lead acetate orally in dose (10mg/kg/b.w) [15]. The four group (T3): given lead acetate orally in dose(10mg/kg/b.w) for 30 days then treated by quercetin orally in dose (300mg/kg/b.w) for 30 day.

Samples Collection: made flashing to the samples of ovary and pituitary gland by put it in liquid nitrogen (-196) and Store in -20.

Primers: The real time PCR primers that used in this study were designed by NCBI gene Bank data base and Primer designed online, these primers were supported from (Bioneer, Korea) company**.**

Primer	***Sequence***		***Amplicon***
LH subunit	F	AGTTCTGCCCAGTCTGCATC	79bp
	R	GCTGGCAGTACTCGAACCAT	
LHr	F	ATTTCCTTCTGCTGCTGAGC	110bp
	R	TCCTGGGAAGCCATTTTTGC	
CYP11A1	F	GACGCATCAAGCAGCAAAAC	79bp
	R	ATGGACTCAAAGGCAAAGCG	
GAPDH		ATGCCCCCATGTTTGTGATG	136bp
		TCCACGATGCCAAAGTTGTC	

Total RNA extraction: total RNA were extracted from ovary and pituitary tissue of rat by using (TRIzol® reagent kit) and done according to Bioneer company instructions/korea.

Estimation of extracted total RNA yield: The extracted total RNA was assessed and measurement by Nanodrop spectrophotometer (THERMO. USA), there are two quality controls were performed on extracted RNA. firstly is to determine the concentration of RNA (ng/µL), secondly is the purity of RNA by reading the absorbance at 260 nm and 280 nm in same Nanodrop machine.

DNase I Treatment: The extracted total RNA were treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using (DNase I enzyme kit) and done according to method described by Promega company, USA.

cDNA synthesis: DNase-I treatment total RNA samples were used in cDNA synthesis stage by using (AccuPower RocktScript RT PreMix kit) that provided from Bioneer company, Korea and prepared according to instructions of company.

Quantitative Real-Time PCR (qPCR) master mix preparation: qPCR master mix was prepared by using AccuPower^{TM} Green Star Real-Time PCR kit based SYBER green dye which detection gene amplification in Real-Time PCR system and done according to Bioneer company instructions/korea. After that, these qPCR master mix above Accopwer Green star qPCR premix standard plate tubes that contain the syber green dye and other PCR amplification components, then the plate mixed by Exispin vortex centrifuge for 3 minutes, than placed in Miniopticon Real-Time PCR system. After that the qPCR plate was loaded and the following thermocycler protocol in the following table:

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	5min	1
Denaturation	95 °C	15 sec	45
Annealing\Extension Detection(scan)	60 °C	30 sec	45
Melting	60-95°C	0.5 sec	1

Data analysis of qRT-PCR: The results of qRT-PCR for housekeeping and target genes were analyzed via the relative quantification gene expression level (fold change) ΔCT according to the Livak method [**15**] Relative quantification method quantity obtained from qRT-PCR experiment must be normalized in such method that the data become biologically significant. in this method one of experimental samples is the calibrator as control sample

each of the normalized CT values (target values) is divided by the calibrator normalized target value to produce the relative expression levels. after that, the ΔCT method with a reference gene was used as following equations:

Gene	Test (Treatment group)	Cal. (Control group)
Target gene	CT (target, test)	CT (target, cal)
Reference gene	CT (ref, test)	CT (ref, cal)

First, normalize the (CT) of the reference gene to the target gene, for calibrator sample:

ΔCT (control) = CT (ref, control) – CT (target, control)

Second, normalize the CT of the reference gene to the target gene, for the test sample:

ΔCT (Test) = CT (ref, test) – CT (target, test)

ΔΔCT= ΔCT (test) – ΔCT (control)

Fold change = 2-ΔΔCT

Ratio (reference/target) = 2CT (reference) – CT (target)

So, the relative expression was divided by the expression value of chosen calibrator for all expression ratio of test sample.

7 statistical analysis: For analysis the results of study used Anova 1 (one way analysis of variance) with least significant differences LSD was detected to compare between groups by using (SPSS) program software.[16]

RESULTS AND DISCUSSION

Total RNA concentration and purity

Total RNA concentrations and purity that extracted from experimental ovary and pituitary tissue samples were measured by using Nanodrop spectrophotometer. The concentrations results (Mean ± SE) exhibited as (ng/µl) whereas, the purity was estimated by measuring the absorbance ratio of A_{260}/A_{280}. as following tables (Table 4.1 and Table 4.2).

Table 4.1: ovary total RNA level and purity

Total RNA experimental group	Concentration (ng/µl) Mean ± SE	Purity A_{260}/A_{280} Mean ± SE
C	249.04 ± 26.25	1.724 ± 0.013
T1	376.24 ± 59.51	1.742 ± 0.016
T2	355.50 ± 50.76	1.588 ± 0.081
T3	423.74 ± 97.73	1.682 ± 0.082

Table 4.2: Pituitary total RNA level and purity

Total RNA experimental group	Concentration (ng/µl) Mean ± SE	Purity A_{260}/A_{280} Mean ± SE
C	226.32 ± 96.43	1.692 ± 0.081
T1	213.18 ± 47.10	1.684 ± 0.027
T2	261.54 ± 50.00	1.656 ± 0.026
T3	384.02 ± 86.46	1.522 ± 0.62

(C)Control group: animal that received standard food and drinking water.(T1) group: animal that received (300mg/kg/b.w dissolved in 1ml tap water) Quercetin for 60 days. (T2) group: animal that received (10mg/kg/b.w dissolved in 1ml tap water) lead acetate for 60 days.(T3) group: animal that received (10mg/kg/b.w dissolved in 1ml tap water) lead acetate for 30 days. then received (300mg/kg/b.w dissolved in 1ml tap water) Quercetin for another 30 days.

Quantitative Reverse Transcriptase Real-time PCR

Was performed for measurement of relative quantification "gene expression analysis" for ovarian estrogen hormone (Aromatase cytochrome P450 gene), FSH subunit gene, FSH receptor genes expression levels normalized by housekeeping gene expression (GAPDH). Rt –qPCR quantification method in real-time PCR system was dependent on the values threshold cycle numbers (Ct)of amplification plot of target genes and housekeeping gene as show in the following figures:

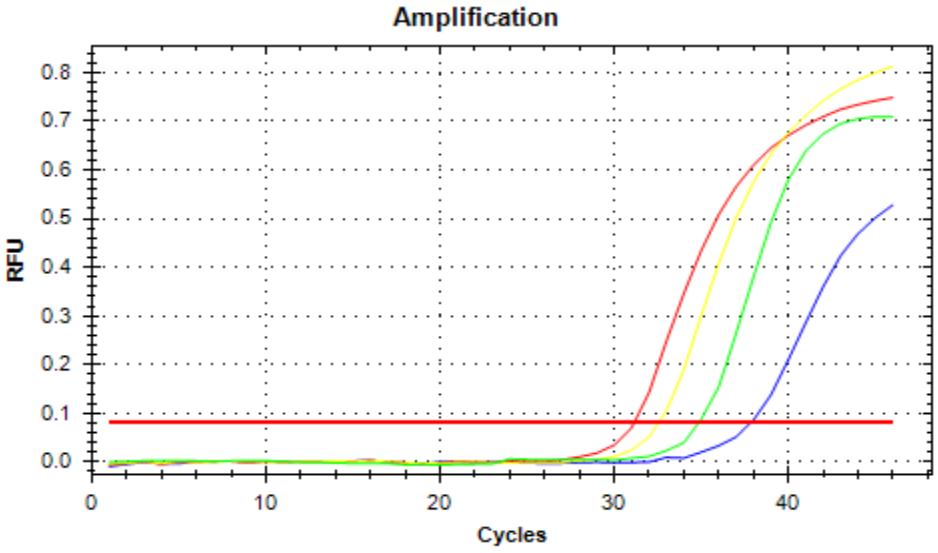

Figure (4-1): Real time PCR amplification plot for aromatase cytochrome P450 gene for (Estrogen) in ovary tissue that show difference in threshold cycle numbers (Ct value) between treatment and control groups. Green plot: C(Control group). Red plot: T1 group animal that received (300mg/kg/b.w dissolved in 1ml tap water) Quercetin for 60

days. Blue plot: T2 group animal that received (10mg/kg/b.w dissolved in 1ml tap water) lead acetate for 60 days. Yellow plot: T3 group animal that received (10mg/kg/b.w dissolved in 1ml tap water) lead acetate for 30 days. then received (300mg/kg/b.w dissolved in 1ml tap water) Quercetin for another 30 days.

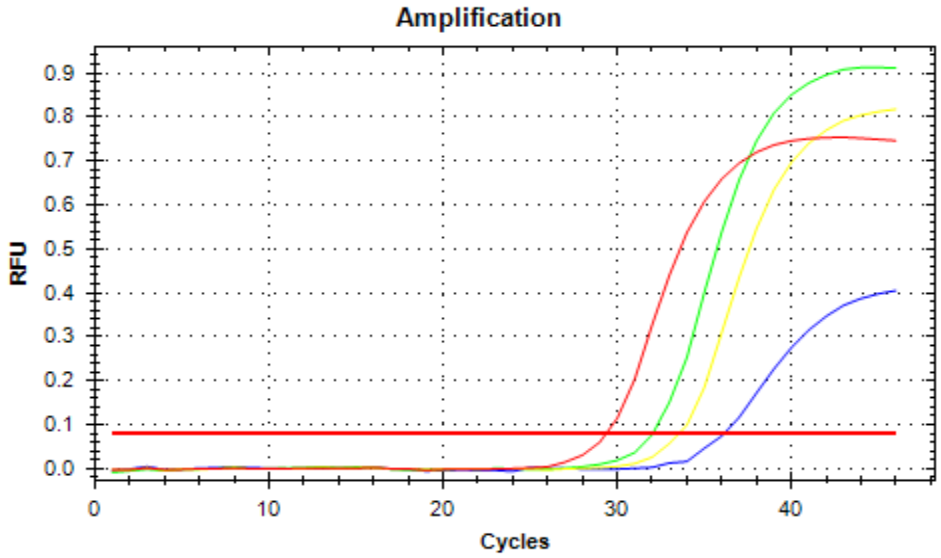

Figure (4-2): Real time PCR amplification plot for FSH receptor gene for in Ovary tissue that show difference in threshold cycle numbers (Ct value) between treatment and control groups. Green plot:C (Control group). Red plot: T1 group animal that received (300mg/kg/b.w dissolved in 1ml tap water) Quercetin for 60 days. Blue plot: T2 group animal that received (10mg/kg/b.w dissolved in 1ml tap water) lead acetate for 60 days. Yellow plot: T3 group animal that received (10mg/kg/b.w dissolved in 1ml tap water) lead acetate for 30 days. then received (300mg/kg/b.w dissolved in 1ml tap water) Quercetin for another 30 days.

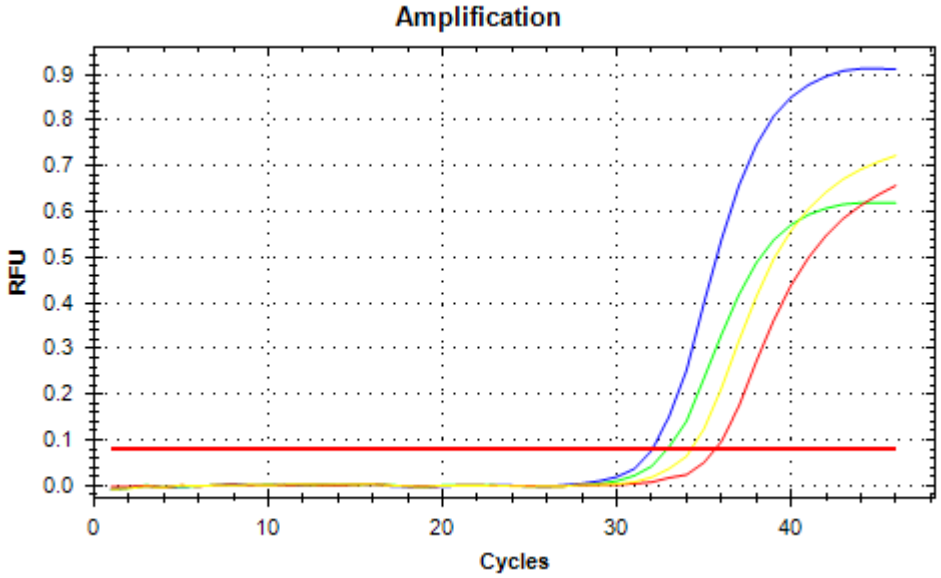

Figure (4-3): Real time PCR amplification plot for FSH beta subunit gene for in pituitary that show difference in threshold cycle numbers (Ct value) between treatment and control groups. Green plot:C(Control group). Red plot: T1 group animal that

received (300mg/kg/b.w dissolved in 1ml tap water) Quercetin for 60 days. Blue plot: T2 group animal that received (10mg/kg/b.w dissolved in 1ml tap water) lead acetate for 60 days. Yellow plot:T3 group animal that received (10mg/kg/b.w dissolved in 1ml tap water) lead acetate for 30 days. then received (300mg/kg/b.w dissolved in 1ml tap water) Quercetin for another 30 days.

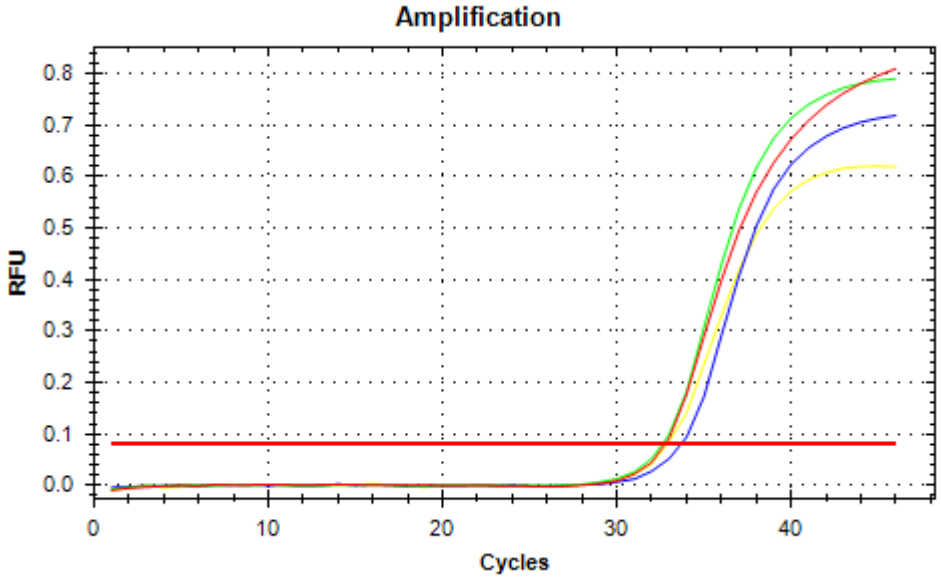

Figure (4-4): Real time PCR amplification plot for GAPDH housekeeping gene that show no difference in threshold cycle numbers (Ct value) between treatment and control groups. Green plot:C(Control group). Red plot: T1 group animal that received (300mg/kg/b.w dissolved in 1ml tap water) Quercetin for 60 days. Blue plot: T2 group animal that received (10mg/kg/b.w dissolved in 1ml tap water) lead acetate for 60 days. Yellow plot:T3 group animal that received (10mg/kg/b.w dissolved in 1ml tap water) lead acetate for 30 days. then received (300mg/kg/b.w dissolved in 1ml tap water) Quercetin for another 30 days.

Relative gene expression results

Aromatase cytochrome P450 gene expression: The results of aromatase cytochrome P450 gene expression for synthesis of estrogen hormone were showed the mRNA expression level change in (T1,T2 and T3)groups according to C(control group) . Highly significant increase of gene expression (up to7.016±0.29468) in T1 group compared with T2 and C . Also there were significant decrease (0.018±9.01228) in T2 group compared with C, T1 and T3 groups. while in (T3) group there were significant increase(3.726±0.97687) compared with C and T2 groups as show in figure (4-5).

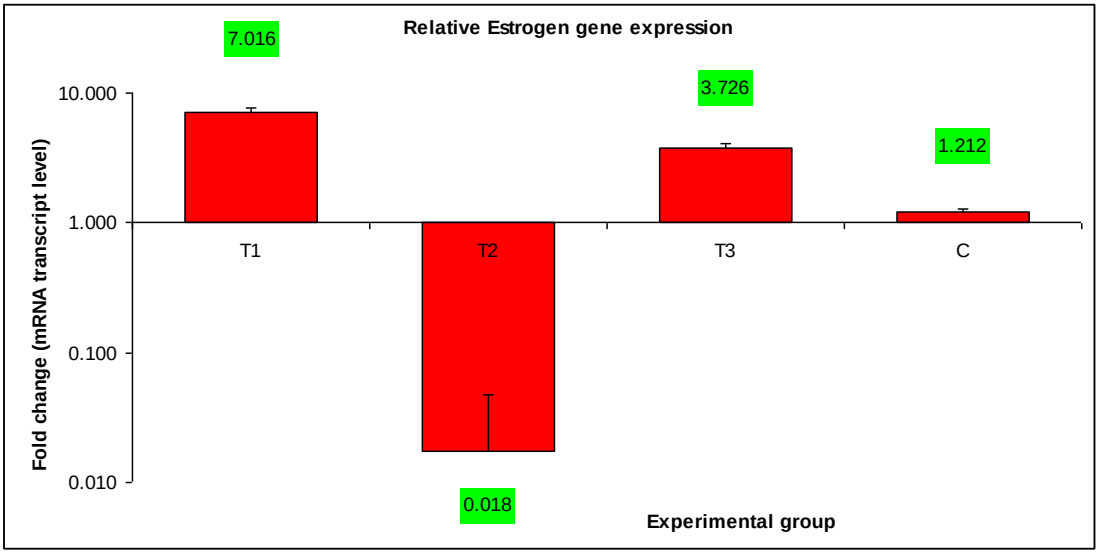

Figure (4-5) Histogram that show relative gene expression of aromatase cytochrome P450 gene, where C=control group, T1=orally gavage Quercetin (300mg/kg/b.w dissolved in 1ml tap water) for 60 days, T2=orally gavage lead acetate (10mg/kg/b.w dissolved in 1ml tap water) for 60 days, T3=orally gavage lead acetate (10mg/kg/b.w dissolved in 1ml tap water) for 30 days then given Quercetin (300mg/kg/b.w dissolved in 1ml tap water) for 30 days.

FSH receptor gene expression: The results of FSH receptor gene expression for synthesis were showed the mRNA expression level change in (T1,T2 and T3)groups according to C(control group) . Highly significant increase of gene expression (up to 10.110±2.57847) in T1 group compared with T2 and C. Also there were significant decrease (0.139±0.08611) in T2 group compared with C, T1 and T3 groups. while in (T3) group there were significant increase(8.231±2.19234) compared with C and T2groups as show in figure (4-6).

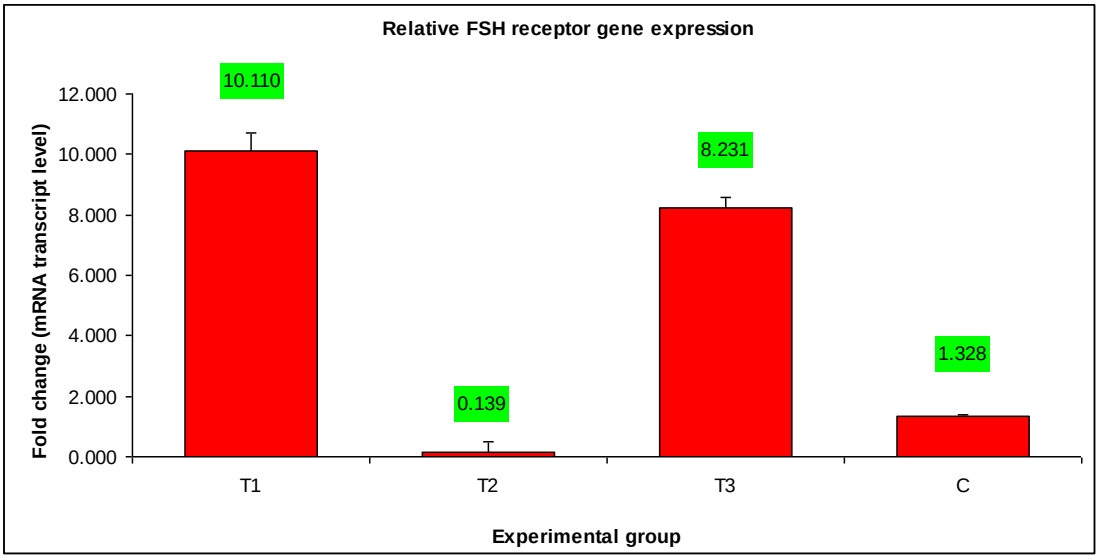

Figure (4-6) Histogram that show relative gene expression of FSH receptor gene, where C=control group, T1=orally gavage Quercetin (300mg/kg/b.w dissolved in 1ml tap

water) for 60 days, T2=orally gavage lead acetate (10mg/kg/b.w dissolved in 1ml tap water) for 60 days, T3=orally gavage lead acetate (10mg/kg/b.w dissolved in 1ml tap water) for 30 days then given Quercetin (300mg/kg/b.w dissolved in 1ml tap water) for 30 days.

FSH beta subunit gene expression: The results of FSH beta subunit gene expression for synthesis were showed the mRNA expression level change in (T1,T2 and T3)groups according to(control group). Highly significant increase of gene expression (up to12.073 ± 1.01736) in T1 group compared with T2 and C. Also there were significant decrease (0.889±0.24370) in T2 group compared with T1 and T3 groups. while in (T3) group there were significant increase(9.188±1.84393) compared with C and T2 groups as show in figure (4-7).

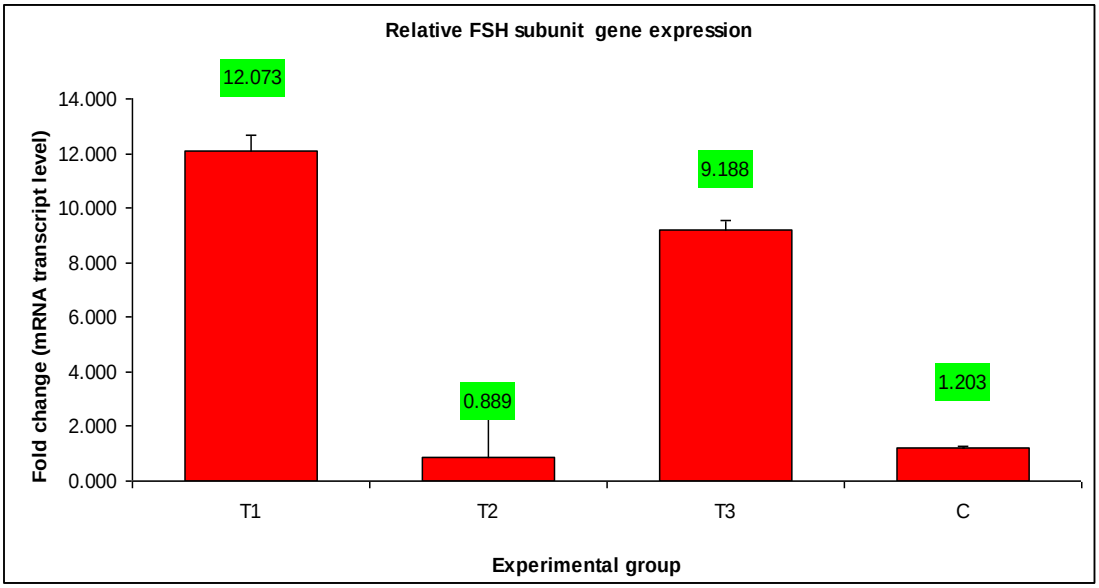

Figure (4-7) Histogram that show relative gene expression of FSH beta subunit gene, where C=control group, T1=orally gavage Quercetin (300mg/kg/b.w dissolved in 1ml tap water) for 60 days, T2=orally gavage lead acetate (10mg/kg/b.w dissolved in 1ml tap water) for 60 days, T3 =orally gavage lead acetate (10mg/kg/b.w dissolved in 1ml tap water) for 30 days then given Quercetin (300mg/kg/b.w dissolved in 1ml tap water) for 30 days.From the present results, the activity of Quercetin as antioxidant activity of free radical scavenging[17]**.** Therefore, we tried to reach to the purpose of our results to study the role of Quercetin by using a mammalian model (female rats). In the present finding we have investigated expression of mRNA levels of such genes in pituitary and ovary tissues, related to pituitary and ovary hormones secretion, as an another way to study the powerful of Querctin in effect of FSH beta subunit gene, FSH receptor gene and aromatase genes which responsible for of

pituitary and ovary hormones biosynthesis.[18] showed that the P450aromatase is a key enzyme for biosynthesis of estrogen by granulosa cells of the ovary, it is essential for oogenesis, follicular maturation, normal luteal functions and ovulation in females.Statistical analysis of present result found up regulation of aromates genes, these results showed significant increase ($p< 0.05$) in T1 in compare with control group and T2.

We think that the Quercetin in T1 effect to up regulation of P450 aromatase gene by acting as strong antioxidant to reduction the ROS and activated the most important factors which responsible for release P450 aromatase. Also for the same reason we showed significant increase ($p< 0.05$) in T3 in compare with control group and T2.[19] showed that the super family descended FSH receptor (FSHr) from it, characterized by their relations with the intracellular G proteins. It is extent the cell membrane 7 times, with an intracellular C-terminus and an extracellular N-terminus. Recently, The FSHr gene has been sequenced in some species, including human[20]**.** FSHrs are found in granulosa cells of growing follicles at Graafian stages, and FSH stimulated P450 aromatase[21]**.** The results of present study found significant increase ($p< 0.05$) of FSH receptor gene in T1 in compare with control group and T2. Quercetin may increase the number and development of growing follicles and also may influence gene expression as antioxidant effects. It is scavenges oxygen radicals**[22].[23]** showed that antioxidants substance trigger reactive oxygen species-sensitive intracellular pathways that regulate the induction of specific gene. Free radicals are showed as dangerous by-products of cell metabolism, and it is well known that the accumulation of ROS in cells will provoke the oxidation of DNA, proteins, and lipids, which results in cell damage and causes genomic instability. However, a number of studies have identified a critical physiological role of ROS in intracellular signaling[24]**.** Also results showed significant increase ($p< 0.05$) of FSH receptor gene in T3 in compare with T2 and control group because protective effects of Quercetin to the ovary after oxidative stress.[25]**,** reported that quercetin significantly inhibited the stimulation of NADPH oxidase expression with increased the expression of the antioxidant enzymes catalase, glutathione peroxidase and superoxide dismutase . Results showed up regulation of FSH beta subunit(FSH beta s) gene and significant increase ($p< 0.05$) in T1 in compare with control group and T2, the antioxidant properties of Quercetin may explain this results by the effect of quercetin on both hypothalamus to stimulate secretion of GnRH and pituitary to FSH released, and there was significant increasing ($p < 0.05$) in group 3 compare withT2 and control group ,this may be discussed as the effect of quercetin as antioxidant by treated the harmful effect of lead acetate

on female reproductive system. Results showed down regulation of P450 aromatase gene, and significant decrease ($p<0.05$) in FSHr and FSH beta subunit genes in lead acetate administration in group 2 compare with T1 and T3, this result indicated of harmful effect of lead acetate on these genes, because it is a cause of oxidative stress. Inhibition of P450 aromatase activity may involve several mechanisms. One of these mechanism is the direct lead inhibitory effect. It may be mediated by the creation of a complex with the cysteine residues, due to a strong affinity of lead for sulfhydryl groups[26]**.** On lead treatment, decrease in binding of FSH to ovarian homogenate may be due to co-precipitation of metal ions and receptor, binding to amino acids like cysteine residues of the receptor or interference of metals with stability of hormone-receptor complex[27].[28] reported that is Lead cause oxidative stress by generating (ROS) such as hydroxyl radicals, lipid peroxides, superoxide radicals and hydrogen peroxide.An accumulation of excess ROS has the capability to damage DNA, RNA, proteins, lipid and nucleic acids[29]**.** Oxidative stress cause toxic effects by changing cellular molecules such as nucleic acids, proteins and lipids. This can lead to an increase in enzyme inactivation, loss of membrane integrity, structural damage to DNA, mitochondrial alterations and membrane permeability[30]**.** In one study used mice exposed to an oxidative stress inducer and showed that inducer could cause DNA damage in endometrial cells and then embryo loss[31]**.** Lead may cause change in sperm quality in men, and irregular menstruation in fmales induce preterm delivery, stillbirth, and spontaneous abortion[32]**.** Our finding showed up regulation of P450 aromatase, FSHr and FSH beta subunit gene and significant increase ($p< 0.05$) inT3(lead acetate then Quercetin) compare with control and T2, we suggested that Quercetin as an antioxidant, protects tissues and cells and prevents oxidative damages caused by ROS. An increase of total RNA concentration in ovary tissues obtained from rats in T1,Quercetin act as antioxidant substance. Was a result of increase of ovary restoration cellular function in protein synthesis as the results exposed ovary to high level of events which can be established by high expression level of genes in end of experiment[33]**.** In Quercetin treated female rats, demonstrated high level of expression while decrease in group 2.Our data investigated a significant increase in serum estrogen and level in group 1 and 3 after two months oral administration of Quercetin. This increase in the ovary hormone level resulted from activation of granulose and theca cells of the ovary from the Quercetin treated female rats as demonstrated by mRNA expression level of P450aromatase genes.

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