Effect of quercetin on gene expression of male hormone in adult Wistar rats exposure to the oxidative stress by lead acetate

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Abstract

The study was aimed to investigate the beneficial effect of plant source quercetin extracted in improvement of reproductive system efficiency of adult males rats exposed to the oxidative stress by lead acetate. Forty males Wistar rats (6 months old, and 175±10gm weight) were divided randomly into four equal groups and treated for 60 days as follows: The first group (C) was given drinking water as a control group. Second group (T1) was given quercetin (300 mg/kg/ B.W) orally. Third group (T2) given lead acetate (10 mg/kg/ B.W) orally, and the fourth group (T3) was given lead acetate (10mg/kg/B.W) orally for 30 days then given quercetin (300mg/kg/B.W) for 30 days. All animals were sacrificed at the end of experiment and samples of testis were taken to study gene expression for CYP11A1 which responsible for testosterone hormone production, LH receptor gene which responsible for production ICSH receptors and LH beta sub unit gene which responsible for ICSH production. Results showed there was significant increase ($p \leq 0.05$) in fold change of gene expression levels of (CYP11A1,LH beta sub unit gene and LHr gene) in T1 group compared with control. Also there was significant decrease ($p \leq 0.05$) in fold change of gene expression levels of (CYP11A1) in T2 in compared with control. While the fold changes of gene expression levels of LH beta sub unit gene and LHr gene not show significant differences between C and T2 groups. Also there was significant increase ($p \leq 0.05$) in fold change of gene expression levels of (CYP11A1, LH beta sub unit gene and LHr gene) in T3 compared with T2. It could be concluded that quercetin causes up regulation to gene expression of (CYP11A1) gene, LH beta subunit gene, and LH receptor gene (LHr) in adult males Wistar rats exposed to the oxidative stress by lead acetate.
Key words: quercetin, gene expression, oxidative stress, lead acetate, male hormones.

دراسة تأثير مادة الكيرستين على التعبير الجيني للهرمون الذكري في الجرذان المعرضة للإجهاد التأكسدي بواسطة خلات الرصاص

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الخلاصة

هدفت الدراسة الحالية لأثبات دور مادة الكيرستين المستخلصة من مصدر نباتي في تحسين الاداء التناسلي لذكور الجرذان المعرضة للإجهاد التأكسدي بواسطة خلات الرصاص. استخدم في التجربة اربعون جرذا بالغ جنسياً بعمر حوالي 6 اشهر ووزن حوالي 175±10 غم وزعت عشوائياً الى أربعة مجاميع متساوية وجرعت لمدة 60 يوم وكالاتي: المجموعة الاولى C اعطيت ماء الشرب باعتبارها مجموعة سيطرة اما المجموعة الثانية T1جرعت مادة الكيرستين (300 ملغم /كغم) اما المجموعة الثالثة T2جرعت مادة خلات الرصاص (10 ملغم /كغم) اما المجموعة الرابعة T3جرعت مادة خلات الرصاص (10 ملغم /كغم) لمدة 30 يوم بعدها أعطيت مادة الكيرستين (300 ملغ/كغم) لمدة 30 يوم. بعد انتهاء التجربة تمت التضحية بجميع الحيوانات وأخذت نماذج من الخصية لغرض قياس التعبير الجيني لجين (CYP11A1)

المسؤول عن انتاج الهرمون الذكري وجين LHr المسؤول عن انتاج مستقبلات الهرمون المحفز للخلايا البينية. واخذت نماذج من الغدة النخامية لقياس التعبير الجيني للجين LH beta sub unit المسؤول عن انتاج الهرمون المحفز للخلايا البينية. بينت الدراسة ارتفاعا معنويا ($p\leq0.05$) في مستوى التعبير الجيني لجينات (CYP11A1 و sub unit gene LH beta و LHr gene) في المجموعة الثانية T1 مقارنة مع مجموعة السيطرة ايضا لوحظ انخفاضاً معنويا ($p\leq0.05$) في التعبير الجيني للجين (CYP11A1) في المجموعة الثالثة T2 مقارنة مع مجموعة السيطرة في حين لم يتأثر التعبير الجيني لجينات (beta sub unit و LHr gene) في المجموعة الثالثة مقارنة بمجموعة السيطرة ايضا لوحظ ارتفاعا معنويا ($p\leq0.05$) في التعبير الجيني للجينات (CYP11A1, LH beta sub unit gene, LHr gene) في المجموعة الرابعة T3 مقارنة مع المجموعة الثالثة T2. اوضحت النتائج ان مادة الكيرستين حسنت من التعبير الجيني للجينات المذكورة أعلاه .

الكلمات المفتاحية: الكيرستين ، التعبير الجيني ، الاجهاد التأكسدي ، خلات الرصاص ، الهرمون الذكري.

Introduction

Oxidation is a chemical reaction that removals electrons from a substance to the oxidizing agent, and cause cells damage by produce free radicals (1). The important mechanisms of free radicals by restrict the cellular functions by lipid peroxidation which leading to the cell death (2). Heavy metals like lead is important oxidative agents. Lead is an abundant element in the environment, it is use in many industrial actions including mining, refining and making lead acid batteries (3). Lead interferes with some of body functions and generating many sicknesses (4). Lead cause oxidative stress by generation the reactive oxygen species (ROS) like hydrogen peroxide, superoxide radicals, lipid peroxides and hydroxyl radicals (5).
The exposure to the lead acetate in male rat causes decrease in spermatids number, epididymis sperms count, testosterone serum level and effect on prostate function (6). The harmful effect of oxidative agents by remove antioxidants which are substances have ability to protect cells from the damage which cause by unstable molecules famous as free radicals, antioxidants reaction with free radicals and prevent some of the damage causes by free radicals (1). Antioxidants found in vegetables and fruits and divided into two groups: synthetic antioxidants like butylated hydroxyl anisole, and natural antioxidants like minerals, vitamins and phytochemicals like flavonoids (7). Flavonoids have the ability to inhibit some enzymes like cyclooxygenases and protein kinases which involved in cell proliferation and cell apoptosis (8). Quercetin is type of flavonoids it's a plant‘s pigment found in many plants especially onion, apples, tea, broccoli, and berries (9). Quercetin has importance in pharmacology as antioxidant (10). Quercetin improves the antioxidative defense system by up regulating of antioxidant enzymes (11). Also quercetin enhances sperms feature by preventing lipid peroxidetion (12). So the present study was aimed to investigate the beneficial effect of quercetin in improving the male reproductive system that exposure to oxidative stress by lead acetate by measurement the gene expression of CYP11A1 gene, LH beta subunit gene and LH receptor gene (LHr).

Materials and methods

Forty adult male Wistar rats (6 months old, 175±10 gm weight) obtained from animal house of Veterinary Medicine College of Al-Qadisiyah University were used in this experiment. Animals were housed in well ventilated wire-plastic cages, reared under controlled conditions (12 hours light and 12 hours dark at 25Cº), given drinking water freely, and were allowed to acclimatize for 7 days before experimentation. Animals divided randomly into four equal 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) (13). The third group (T2) given lead acetate orally in dose (10mg/kg/B.W) (14). The fourth group (T3) given lead acetate orally in dose (10mg/kg/B.W) for 30 days then treated by quercetin orally (Onion quercetin (95%) provided by Brightol Company-China) in a dose of (300mg/kg/B.W) for 30 days. All animals were sacrificed at the end of experiment and samples of testis and pituitary glands were taken, made flashing by put them in liquid nitrogen (-196 Cº) and

store in -20Cº, to study gene expression for CYP11A1 which responsible for testosterone hormone production, LH receptor gene which responsible for production ICSH receptors and LH beta sub unit gene which responsible for ICSH production. The used real time PCR primers 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	AGTTCTGCCCAGT CTGCATC	79bp
	R	GCTGGCAGTACTC GAACCAT	
LHr	F	ATTTCCTTCTGCT GCTGAGC	110bp
	R	TCCTGGGAAGCCA TTTTTGC	
CYP11A1	F	GACGCATCAAGCA GCAAAAC	79bp
	R	ATGGACTCAAAG GCAAAGCG	
GAPDH		ATGCCCCCATG TTTGTGATG	136bp
		TCCACGATGCCAA AGTTGTC	

Total RNA were extracted from testis and pituitary tissue of rat by using (TRIzol® reagent kit) and done according to Bioneer company instructions/Korea. The extracted total RNA was assessed and measurement by Nano drop spectrophotometer (THERMO. USA). Two quality controls were performed on extracted RNA. Firstly to determine the concentration of RNA (ng/µL), and secondly the purity of RNA; by reading the absorbance at 260 nm and 280 nm in same Nano drop machine. 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. 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. qPCR master mix was prepared by using AccuPower™ 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, then 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	Temp. Cº	Time	Repeat cycle
Initial Denaturation	95	5min	1
Denaturation	95	15 sec	45
Annealing\Extension Detection(scan)	60	30 sec	45
Melting	60-95	0.5 sec	1

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.

Statistical analyses:
For analysis the results of study used ANOVA test (one way analysis of variance) with least significant differences LSD was detected to compare between groups by using (SPSS) program software (16).

Results

Relative expression of CYP11A1 gene: There was significant increase ($p \le 0.05$) in fold change of gene expression levels in T1 group (4.69±0.46) compared with other groups and there was significant decrease ($p \le 0.05$) in T2 group (0.062±0.014) compared with other groups. While there was no significant different between C and T3 groups.

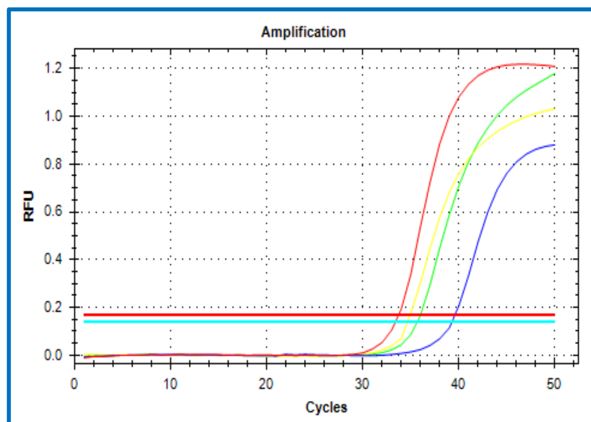
Relative expression of LH beta subunit gene: There was significant increase ($p \le 0.05$) in fold change of gene expression levels in T1 group (11.97± 0.95) compared with other groups and there was significant increase ($p \le 0.05$) in fold change of gene expression levels in T3 group (4.39±1.16) compared with C and T2 groups (1.29±0.38, 0.136±0.048) respectively. While there was no significant different between C and T2 groups.

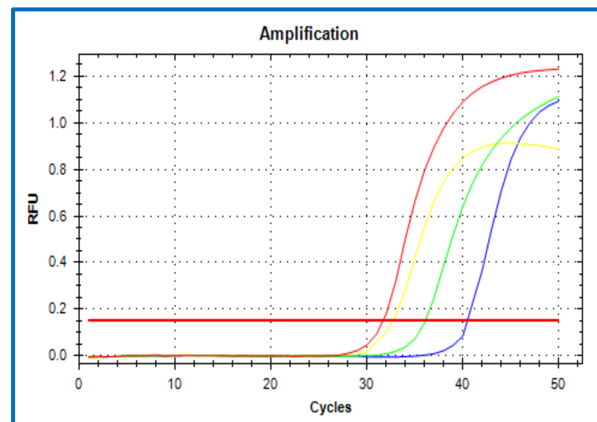
Relative expression of LH receptor gene: There was significant increase ($p \le 0.05$) in fold change of gene expression levels in T3 group (35.44 ±3.39) compared with other group and there was significant increase ($p \le 0.05$) in fold change of gene expression levels in T1 group (21.29±2.3) compared with C and T2 groups (1.19±0.34, 0.33±0.22) respectively. While there was no significant differences between C group and T2 group.**(Table 1, Fig 1,2,3,4)**

Table (1): The effect of quercetin on gene expression of CYP11A1 gene, LH beta subunit gene and LHr gene in males Wistar rats treated with lead acetate (Mean ± SE).

Groups / Genes	C	T1	T2	T3
CYP11 A1 (fold change)	b 1.3± 0.44	a 4.69±0. 46	c 0.062±0. 014	b 2.19±0. 36
LH beta subunit (fold change)	c 1.29±0. 38	a 11.97± 0.95	c 0.136±0. 048	b 4.39±1. 16
LH recepto r (fold change)	c 1.19 ±0.34	b 21.29± 2.3	c 0.33±0.2 2	a 35.44 ±3.39

Different litters =significant differences ($p<0.05$).

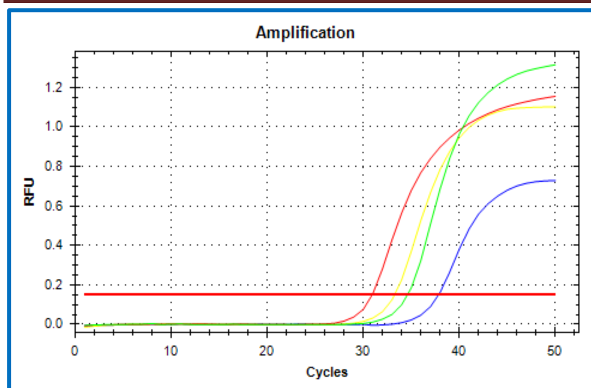


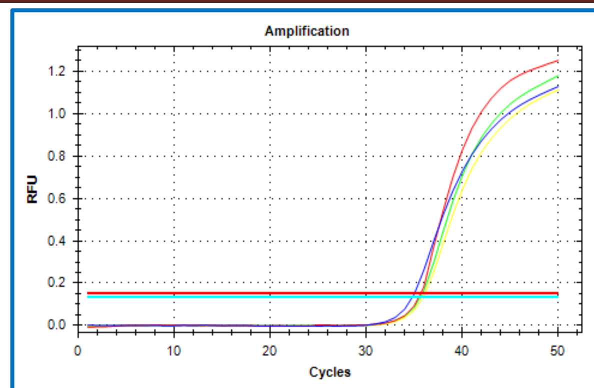


Green plot: Control group, Red plot: T1 group, Blue plot: T2 group, Yellow plot:T3 group

Fig. (1): Real time PCR amplification plot for CYP11A1 gene for (Testosterone) in testis shows difference in threshold cycle numbers (Ct value) between treatment and control groups.

Fig. (2): Real time PCR amplification plot for LH receptor gene in testis shows difference in threshold cycle numbers (Ct value) between treatment and control groups.





Green plot: Control group, Red plot: T1 group, Blue plot: T2 group, Yellow plot:T3 group.

Fig. (3): Real time PCR amplification plot for LH subunit gene in pituitary that shows difference in threshold cycle numbers (Ct value) between treatment and control groups.

Fig. (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.

Discussion

The study was aimed to investigate the role of quercetin to improvement testis functions by using males rats as a model of mammalian through study mRNA expression level of LH beta subunit gene in pituitary gland which responsible for production ICSH, and CYP11A1 gene in testis tissue which responsible for production steroid-genic enzymes, and LHr gene in the testis which responsible for production LH receptors. Antioxidant substances like quercetin trigger reactive oxygen species-sensitive intracellular pathways that regulate the induction of specific gene. Free radicals are harmful products of cell metabolism, and it is well known that the accumulation of ROS in cells will induce the oxidation of DNA, lipids, and proteins, which results in cell damage and causes genomic instability (16). Quercetin act as strong antioxidant hunt ROS and restore antioxidant enzymes this lead to increase the gene expression of LH beta sub unit, CYP11A and LHr genes through increase of cell signaling, transport, metabolism, and control of transcription, and oxidative phosphorylation, these process necessary for hormones synthesis. Testosterone biosynthesis requires steroid-genic proteins. Steroidogenic act as regulat-ory protein, CYP11A gene produce cholesterol side-chain cleavage enzyme (17). The steroidogenic enzymes are essential for the production of androgens (up regulation of the mRNA expression of genes coding for these enzymes) (18). Quercetin act as strong antioxidant which improve of leydig's cells and lead to cholesterol transport within the Leydig's cells membrane, steroidogenesic necessary for transport of free cholesterol from the outer to the inner leydig's cells mitochondrial membrane, these enzymes reaction catalyze by the enzyme coded in CYP11A1 gene (19). In T2 group received lead acetate, the gene expression for LH beta sub unit gene showed there is no significant differences with control group. This may be the lead acetate cannot cross blood brain barrier, but the gene expression of CYP11A1 decrease due to the Lead acetate cause oxidative stress by generating of ROS such as superoxide radicals, hydrogen peroxide and hydroxyl radicals and lipid peroxides (20). Oxidative stress reduce number of leydig's cells which responsible for testosterone production (21). Lead could accumulate in cell nuclei associated with nuclear proteins and chromatin and change their structure (22). Also excess of ROS has the ability to damage lipid, proteins, and nucleic acids, DNA and RNA (23). In T3group received lead acetate then treated by quercetin, there is significant increase in gene expression of LH beta sub unit, CYP11A and LHr genes due to the quercetin decrease the harmful effect of lead acetate by the ability of quercetin to scavenge ROS, and restore the antioxidant systems (24).

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