Effect of Aqueous Extract of Green Tea on Gene Expression of *CYP17, CYP11A, LH Beta subunit* and *LHr* Genes in Males Wistar Rats Exposed to Oxidative Stress by Streptozotocin

Muntasser Alawi Awad Firas Hasan Hadi

Madenat Alelem University College /Anesthesia and Intensive Care Department

muntasser.alawady@yahoo.com

Abstract

The present study aims to investigate the beneficial effect of green tea to improve the reproductive system efficiency in adult males rats exposed to the oxidative stress by Streptozotocin.

Forty adult male Wistar rats divided randomly to four equal groups , and gavage as following : control group (C) was given drinking water only, second group (T1) : given green tea extract in a dose 100 mg/kg for 60 days. The third group (T2): injected streptozotocine (STZ) by single dose of 60 mg/kg by intraperitoneal (IP) injection. The fourth group (T3) : injected STZ in single dose 60 mg/kg by IP injection then after 30 days were given green tea extract in a dose of 100 mg/kg for 30 consecutive days.

In the end of the experiment all animals were sacrificed, samples of testis were taken for study gene expression of *CYP11A1,CYP17* and *LHr* gene ,also samples of pituitary gland were taken for study gene expression of *LH beta subunit* gene**.**

The results showed there was significant increase ($p \leq 0.05$)in fold change of gene expression levels of (*CYP11A1,CYP17,* and *LHr* gene) in T1 group compared with control group. Also there was significant decrease ($p \leq 0.05$) in fold change of gene expression levels of (*CYP11A1*and *CYP17*) in T2 compared with control group. And there was significant increase in fold change of gene expression levels of *LH beta subunit* gene in T2 compared with other groups. While *LHr* gene not show significant differences in the C and T2 groups . Also there was significant increase ($p \leq 0.05$)in fold change of gene expression levels of (*CYP11A1,CYP17* and *LHr* gene) in T3 compared with T2.

It could be concluded that green tea causes up regulation to gene expression of *CYP11A1* ,*CYP17*, *LH beta subunit*, and *LHr* in adult males Wister rats exposed to the oxidative stress by STZ.

KEYWORDS: Green Tea, gene expression, Oxidative stress, Streptozotocin *CYP17, CYP11A, LH beta subunit* and *LHr genes*

تأثير المستخلص المائي للشاي الأخضر على التعبير الجيني للجينات *CYP17,CYP11A,LH beta subunit* and *LHr* في ذكور جرذان الوستر المعرضة لإجهاد تأكسدي بمادة الستربتوزوتوسين

منتصر علاوي عواد فراس حسن هادي

كلية مدينة العلم الجامعة / قسم التخدير والعناية المركزة

الخلاصة

تهدف الدراسة الحالية لأثبات دور المستخلص المائي للشاي الاخضر في تحسين الاداء التناسلي لذكور الجرذان المعرضة للإجهاد التأكسدي بواسطة الستربتوزوتوسين. استخدم في التجربة اربعون جرذا بالغاً جنسياً بعمر حوالي 6 اشهر ووزن حوالي 180±10 غم وزعت عشوائياً الى أربع مجاميع متساوية وجرعت لمدة 60 يوم وكالاتي: المجموعة الاولىC جرعت بماء الشرب باعتبارها مجموعة سيطرة اما المجموعة الثانيةT1جرعت بمادة الشاي الاخضر بتركيز)

100 ملغم /كغم) لمدة 60 يوم اما المجموعة الثالثة T2 احقنت بمادة الستريتوزوتوسين داخل البريتون وبتركيز (60 ملغم /كغم) اما المجموعة الرابعة T3احقنت بمادة الستريتوزوتوسين داخل البريتون وبتركيز (60 ملغم /كغم) وبعد مرور 30 يوم جرعت بمستخلص الشاي الاخضر بتركيز (100 ملغ/كغم) لمدة 30 يوم. بعد انتهاء التجربة تمت التضحية بجميع الحيوانات وأخذت نماذج من الخصية لغرض قياس التعبير الجيني للجينات *LHr, CYP17, CYP11A1* . تم اخذ نماذج من الغدة النخامية لقياس التعبير الجيني للجين *LH beta subunit*.

بينت الدراسة ارتفاعا معنويا ($p\leq0.05$) في مستوى التعبير الجيني لجينات (*CYP17, CYP11A1* و *LHr* gene) في مجموعة الثانية T1مقارنة مع مجموعة السيطرة ايضا لوحظ انخفاضاً معنويا ($p\leq0.05$) في التعبير الجيني *CYP17, CYP11A1* في مجموعة الثالثة T2مقارنة مع مجموعة السيطرة. وزيادة معنويه في التعبير الجيني *LH beta subunit* في المجموعة الثالثة T2 مقارنة مع بقيه المجاميع في حين لم يتأثر التعبير الجيني *LHr* gene في المجموعة الثالثة مقارنة بمجموعة السيطرة ايضا لوحظ ارتفاعا معنويا ($p\leq0.05$) في التعبير الجيني للجينات (*CYP11A1, CYP17* و *LHr*) في المجموعة الرابعة T3مقارنة مع المجموعة الثالثة T2. اوضحت النتائج ان مادة مستخلص الشاي الاخضر حسنت من التعبير الجيني للجينات المذكورة أعلاه .

الكلمات المفتاحية : الشاي الاخضر، التعبير الجيني، الإجهاد التأكسدي ،الستربتوزوتوسين

Introduction

Green tea from the (Camellia *sinensis*) of the Theaceae family is one of the most ancient and the second most widely consumed beverage in the world. Green tea is derived directly from drying and steaming fresh tea leaves [1].Regular drinking of green tea has been associated with many health benefits [2]. Polyphenols components of green tea have been described as having antioxidant activity [3]. Oxidative stress, which is the imbalance between oxidant agents and antioxidant defense, can originate increase in free radicals production either by exogenous processes, such as pollution and cigarette smoking, or by endogenous processes, such as inflammation and respiratory burst [4]."Seminal oxidative stress occurs when there is an increased production of reactive oxygen species (ROS)" or a decrease of antioxidant activity, promoting impaired sperms function [5]. There are many substances cause seminal oxidative stress like streptozotocin (STZ) which is considered cytotoxic to the pancreatic beta cells [6].
Cytotoxic effects of STZ are dependent upon DNA alkylation by site specific action with DNA bases [7]. Sex steroids have crucial roles in reproductive phenomena, the biosynthesis of sex steroids and their enzymes have been well studied in vertebrates [8]. There are three cytochrome P450 enzymes (CYP) such as P450 side chain cleavage (CYP11A), (CYP17), and (CYP19). CYP17 and CYP11A are an enzyme that regulates the conversion from cholesterol to pregnenolone (P5) by its side chain cleavage activity, and it is believed to be a rate-limiting enzyme of steroidogenesis [9]. Luteinizing hormones (LH) are member of glycoprotein hormone family, which regulate gonadal function, LH are α: β heterodimers in which the β-subunit is unique and the α-subunit is common to both [10]. The effect LH is applied by their receptors. Recently, genetic variability of these receptors genes and their receptors has been considered as an important predictive marker in response to testicular stimulation [11].

So the present study aimed to investigate the beneficial effect of Green Tea in improving the male reproductive system that exposure to oxidative stress by STZ through measurement the gene expression of *CYP17*, *CYP11A1*, *LH beta subunit* and *LHr* genes.

Materials and Methods:

Laboratory animals: Forty adult males wistar rats were used in our experiment, with age about 6 months, and weight about (180±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 ,and given drinking water freely. The animals were allowed to acclimatize for 7 days before experimentation.

Experiment design: the animals were divided randomly to four equal groups. After that all treated as following: control group was given drinking water only, second group (T1): given green tea extract in a dose of 100 mg/kg for 60 days [12]. The third group (T2): injected STZ single 60mg/kg by IP injection [13].The fourth group (T3): injected STZ in single dose 60 mg/kg by IP injection then after 30 days given green tea extract in dose of 100 mg/kg for 30 days.

Material: STZ provided from fluka company / England

Preparation Aqueous Extract of Green Tea:

Aqueous extract of green tea was prepared by soxhlet apparatus according to Harbone (1984).

Preparation of Citrate Buffer:

One molar citrate buffer (2.1014g) was dissolved in 50 ml distal water and Adjust solution to final desired pH using NaOH , then is completed the volume to the100ml (according to manufacture).

Preparation of STZ for intraperitoneal injection:

1- Suitable amount of STZ was dissolved in citrate buffer and leave it 20 minutes before injection.

2-The container was covered with an aluminum foil to protect the solution from direct exposure to light [14].

Samples Collection: Made flashing to the parts of testis and whole pituitary glands by putting them it in liquid nitrogen -196 C̊ for gene expression .

Primers: The real time PCR primers that used in this study were designed on line by NCBI gene Bank data base; these primers were supported from (Bioneer, Korea) company shown in table (1).

Table (1) Real Time PCR primers that used in this study

Primer	*Sequence*		*Amplicon*
LH subunit	*F*	5'*AGTTCTGCCCAGTCTGCATC* 3'	79bp
	R	3'*GCTGGCAGTACTCGAACCAT*5'	
LHr	*F*	5'*ATTTCCTTCTGCTGCTGAGC*3'	110bp
	R	3'*TCCTGGGAAGCCATTTTTGC*5'	
CYP11A1	*F*	5'*GACGCATCAAGCAGCAAAAC*3'	79bp
	R	3'*ATGGACTCAAAGGCAAAGCG*5'	
CYP17	*F*	5'*ACAACAACAGCTGTGAAGGC*3'	143bp
	R	3'*AGGATTGTGCACCAGGAAAG*5'	
GAPDH	*F*	5'*ATGCCCCCATGTTTGTGATG*3'	136bp
	R	3'*TCCACGATGCCAAAGTTGTC*5'	

Total RNA extraction:
Total RNA were extracted from testis 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™ 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 table (2):

Table (2) qPCR thermocycler protocol

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 in table (3).

Table (3) Equations that use in data analysis of qPCR

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 analysis:

A computerized program ,the statistical package for social sciences (SPSS) were used to calculated the statistics analysis .The statistical analysis of data had done by

1. Descriptive statistics :mean± stander error
2. Statistical analysis of data was performed on the basis of ANOVA (one way analysis of variance) with least significant difference LSD was detected to compare between groups
3. The confidence limit was accepted at 95% ($p>0.05$) [16].

Results

Relative expression of *CYP11A1* gene:

Table (4): showed significantly increased ($p\leq0.05$) in gene expression levels in T1 group(**4.7±0.45**) compared with other group and there was a significantly decreased in T2 group (**0.069±0.03**) compared with other groups .While there were no significant difference between C (**1.4± 0.43**)and T3 group(**2.2±0.36**).

Relative expression of CYP17 gene:

Table (4) showed significantly increase ($p\leq0.05$) in gene expression levels in T1 group (**4.1±0.33**) compared with other group and

there was a significantly decreased in T2 group (**0.55±0.23**) compared with other groups .While there were no significant difference between C group (**1.30± 0.44**)and T3 group (**2.2±0.44**).

Relative expression of LH beta subunit gene:

Table (4) showed significantly increased($p\leq0.05$) in gene expression levels in T2 group(**11.9± 0.97**) compared with other group .And there was a significantly increased in gene expression levels in T3 group(**4.4±1.15**) compared with C and T1 groups (**1.3±0.4**) (**0.14±0.05**) respectively, while there were no significant difference between C group and T1 group.

Relative expression of LHr gene:

Table(1) showed significantly increased ($p\leq0.05$) in gene expression levels in T3 group(**35.5 ±3.4**) compared with other groups and there was a significantly increased in gene expression levels in T1 group(**21.3±2.3**) compared with C and T2 group(**1.2 ±0.35**),(**0.33±0.3**) respectively, while there were no significant difference between C group and T2 group.

Table (4) Shows Effect of Green Tea on Gene Expression Of *CYP17, CYP11A1*, *LH Beta Subunit* And *LHr* Genes In Males Wistar Rats Exposed To Oxidative Stress By STZ .

Groups / genes	C[1]	T1[2]	T2[3]	T3[4]
CYP11A1(fold change)	b 1.4± 0.4	a 4.7±0.5	c 0.07±0.3	b 2.2±0.4
CYP17(fold change)	b 1.2±0.3	a 4.1±0.3	C 0.55±0.2	b 2.2±0.4
LH beta subunit (fold change)	c 1.3±0.4	c 0.1±0.1	a 11.9± 0.9	b 4.4±1.2
LHr (fold change)	c 1.2 ±0.4	b 21.3±2.3	c 0.33±0.3	a 35.5 ±3.4

Numbers =mean ± S.E.
Different litters =significant Differences ($p<0.05$) .
C 1=control group.
T1 2= Orally gavage Aqueous extracted of green tea 100 mg/Kg for 60 day.
T2 3= given STZ 60 mg/Kg single IP.).
T3 4= given STZ 60 mg/Kg single IP .then orally gavage Aqueous extracted of green tea 100mg/Kg for 30 day.

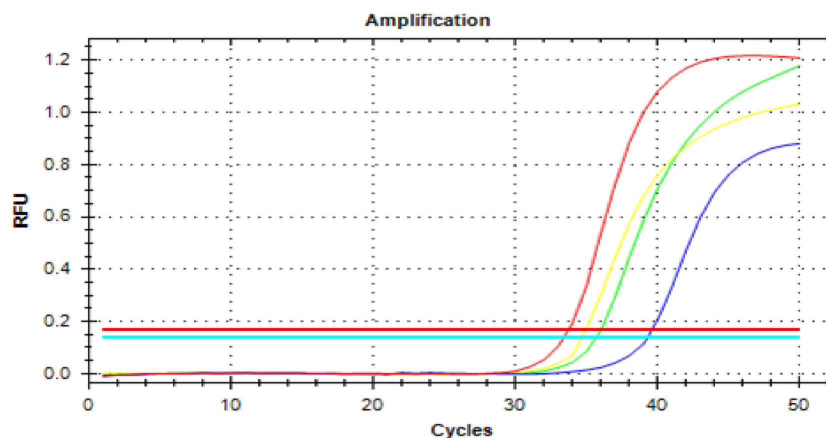

Figure (1): Real time PCR amplification plot for CYP11A1 gene in testis show a difference in cycle threshold numbers (CT value) between treatment and control groups.

Green plot: Control group.
Red plot: Orally gavage Aqueous extracted of green tea 100mg/Kg for 60 day.
Blue plot: given STZ 60 mg/Kg single IP.
Yellow plot: given STZ 60 mg/Kg. single IP. Then orally gavage Aqueous extracted of green tea 100mg/Kg for 30 day.

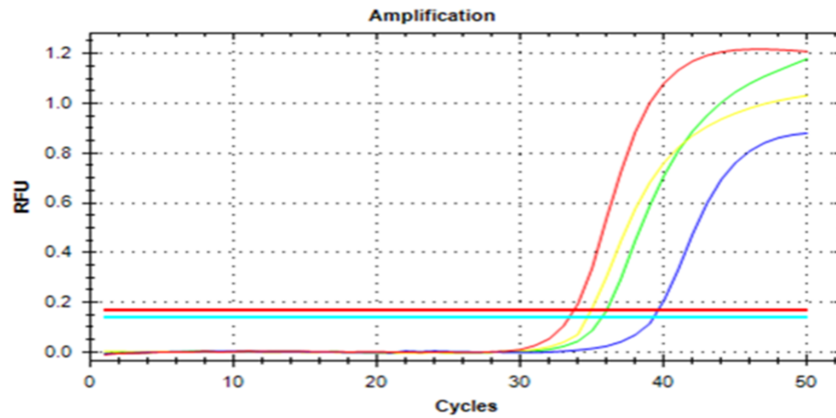

Figure (2): Real time PCR amplification plot for CYP17 gene in testis show a difference in cycle threshold numbers (CT value) between treatment and control group

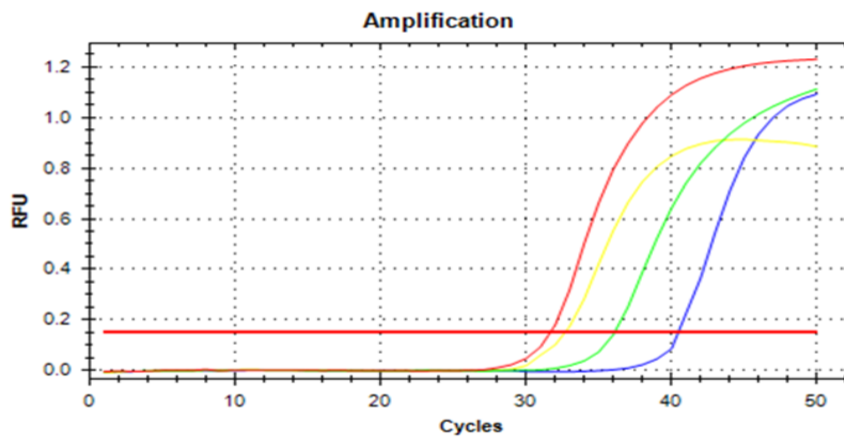

Figure (3): Real time PCR amplification plot for LHr gene in testis show a difference in cycle threshold numbers (CT value) between treatment and control groups**.**

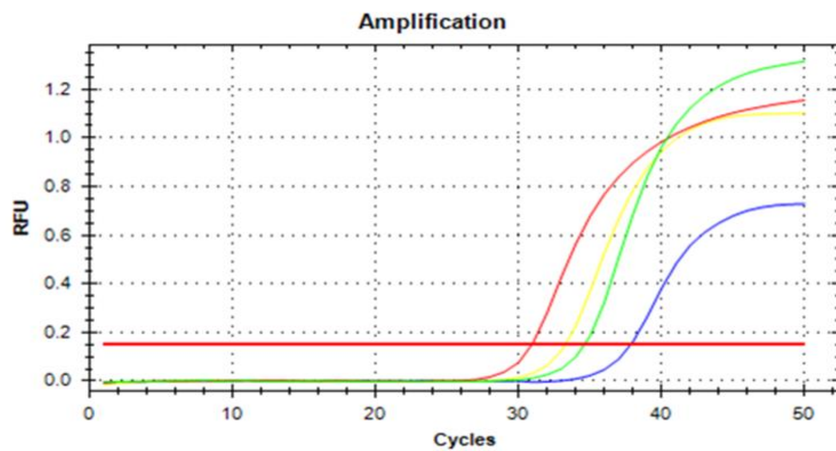

Figure (4): Real time PCR amplification plot for LH beta subunit gene in pituitary show a difference in cycle threshold numbers (CT value) between treatment and control groups

Discussion

Green tea (*Camellia sinensis*) is one of the most commonly consumed beverages worldwide. Its active components are reported to have several biological properties, like antiviral, antioxidant and anti-inflammatory activities [17]. Dried leaves of green tea contain polyphenols (30% -36%), mainly flavanols, more commonly known as catechins, the predominant catechins are epigallocatechin-3-gallat, epicatechin-3 gallate, epigallocatechin , and epicatechin [18].

The present study study used molecular methods (qPCR method) to estimation of male reproductive system effeciency through studying mRNA expression level of *CYP17* and *CYP11A genes* which responsible for steroidogenic enzymes and *LH beta subunit* gene in pituitary gland which is responsible for produce LH, and *LHr* gene in the testis which is responsible for produce LH receptors, that agreed with some researchers that use same the technique like[19][20].

This study agrees with [21] that reported green tea extract could be used to treat the oxidative stress in testicular cells and return the level of reproductive hormones near to the normal after oxidative stress.

Green tea therapy in diabetes was effective; these antioxidants may be use as a supporting therapy for reproductive dysfunction. [22] Green tea extracted may be cause impairment in the morphological and normal functions of rodent testis [23]. Author study Showed green tea extracted has strong castrative effect on male reproductive system efficiency in dose dependent manner. [24]

Steroids are synthesized from cholesterol by a series of enzymatic reactions mediated both by cytochrome P450 enzymes. The steroidogenic cytochrome P450 enzymes are found as single genes (*CYP11A1* and *CYP17*) in multiple species [25].

Free radicals are harmful products of cell metabolism, and it is well known that accumulation of ROS in cells will induce the oxidation of DNA, lipids, and proteins, which results in cell damage and causes genomic instability.

Green tea have polyphenols which act as strong antioxidant that hunt ROS and restore antioxidant enzymes, this leads to Improves the gene expression through the increasing of cell signaling, transport ,metabolism, control of transcription, and oxidative phosphorylation. *CYP17* and *CYP11A* genes which are responsible for produce cholesterol side-chain cleavage enzymes that necessary for testosterone biosynthesis [26]. Seroidogeneic are necessary for transport of free cholesterol from the outer to the inner leydig's cells mitochondrial membrane [27].

In T2 group that received STZ , there was significant increasing in gene expression level of *LH beta subunit* gene, this may be due to increasing of LH through the positive feedback mechanism on the level of pituitary gland and GnRH in hypothalamus when testosterone level fall [28]. In the same group the gene expression level of *CYP17* and *CYP11A1* decreased due to the STZ caused oxidative stress by generating of ROS that reduce number of leydig's cells which responsible for testosterone production this results agreed with [29].

Also [30] showed excess of ROS have the ability to damage lipid, proteins ,nucleic acids .

In T3group that received STZ then treated by green tea, there is a significant increase in gene expression of *LH beta subunit* ,*CYP17* ,*CYP11A* and *LHr* genes , due to the polyphenols compound in the green tea are considered chemopreventive agents because they can quench or prevent the formation of ROS [31].

Conclusions

1-The streptozotocin at dose 60 mg/kg IP caused down regulation to the *CYP17, CYP11A1 , LH beta subunit* due to oxidative stress.

2-Green tea therapy caused up regulation to the *CYP17, CYP11A1 , LH beta subunit and LHr gene* .

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