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QUERCETIN ALLEVIATES THE SIDE EFFECTS OF CLOMID IN THIOACETAMIDE INDUCED INFERTILITY IN RATS

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ABSTRACT

The current work was accomplished to assess the effect of clomid or/ and quercetin on thioacetamide induced infertility in rats. Seventy adult male rats were separated into 2 main groups A as control group & group B as infertility group. GroupA (30 rats) are divided into 3 groups: Group 1A: Control (TAA solvent), Group2A: Rats treated with clomid., Group 3A: Rats treated with quercetin. Infertility group B is induced in male rats (40 rats) by i.p. injection of TAA. These animals are injected by TAA (300 mg/kg) 2 times with 24 hour intervals. The considered infertility rats are divided into 4 groups: Group 1B: Maintained untreated, Group 2B: Rats treated with clomid (0.35mg/kg Clomid i.p.), Group 3B: Rats treated with quercetin (the normal group with Que content of 50 mg/kg bw daily by oral gavage), Group 4B: Rats were treated with both clomid accompanied with querectin. The results confirmed that TAA decreased hormones (FSH, LH, Testesterone), glutathione and catalase while, malondialdehyde was increased. The histopathological study showed several damages in testes tissue. The immunohistochemical invagination revealed an increment in caspase 3 and TNF-α expression. clomid or/ and quercetin administration induced improvement of the biochemical studies. The testes tissue was enhanced in histological, and immunohistochemical investigations.

KEYWORDS: Infertility; clomid; querectin; testes.

1. INTRODUCTION

Male infertility can manifest in the form of premature ejaculation, hypoactive sexuality, erectile dysfunction, oligospermia, azoospermia, *etc*., but its most common manifestation form is as oligospermia. In treating this condition, testosterone and other forms of hormone replacement therapy are often clinically employed due to their ability to stimulate/enhance sexual appetite in hypogonadal male patients **(Sargis *et al.*, 2018).** However, despite the proven efficacy of this replacement therapy in the management of hypoactive sexual desire **(Seidman, 2000).** Many patients still prefer to use natural plants because of the attendant undesirable side-effects associated with these hormonal therapies. Two examples of popular male fertility-promoting herbs are *Panax* spp. (ginseng) and *Lepidium meyenii* (Maca) which are reputed for their supposed aphrodisiac- and spermatogenesis-enhancing effects **(Leung and Wong, 2014).**

Testes are rich in microtubule networks, which form the meiotic and mitotic spindles, and the sertoli cell cytoskeleton, and are of major significance in spermatogenesis. The toxic effect of Thioacetamide (TAA) on the testes and spermatogenesis is known, but there are few studies available in the literature about the toxicity to the testis of TAA **(Kang *et al., 2006*).**

Oxidative stress caused by reactive oxygen species (ROS) (**Zhuo *et al.*, 2017)** is one of the main mechanisms of TAA-induced cytotoxicity. ROS toxicity through adaptive evolution **(Satoh *et al.*, 2013).** In these mechanisms, a series of antioxidant and detoxifying enzymes is activated to maintain the redox balance and weaken the oxidative damage in cells. These enzymes include heme oxygenase-1, glutathione reduces, glutathione peroxidase (GPx), and catalase (CAT) **(Wang *et al.*, 2019).**

Clomiphene citrate (Clomid) is an orally active non-steroidal fertility drug **(Patankar *et al.*, 2007).** It is a selective estrogen receptor inhibitor in the hypothalamus. It acts by inhibiting negative feedback of estrogen on gonadotropin release, leading to the up-regulation of the hypothalamic-pituitary-gonadal axis **(Guay *et al.*, 2001).** It ultimately stimulates testosterone and sperm production. **Patankar *et al*. (2007) and Katz *et al.* (2014)** reported increased levels of serum follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone, and spermatogenesis in infertile and hypogonadal men treated with Clomiphene citrate.

On the other hand, uses of clomiphene citrate were accompanied with many adverse effects, such as ovarian enlargement, vasomotor flashes, nausea, vomiting, breastdiscomfort,

headache, abnormal vaginal bleeding, visual symptoms, weight gain and shortness of breath **(Gore *et al.*, 2014). Yasar and Ertugrul (2009)** reported that CCinduced acute pancreatitis. It also caused myocardial infarction**,** hypertriglyceridemia**,** deep vein thrombosis, and pulmonary embolism**.** The association between birth defects, e.g. neural tube defects, hypospadias and uses of CC was reported by some investigators **(Hawazen and Nahid, 2015, and Abu Hashim, 2012).**

Quercetin (Que; 3, 39, 49, 5, 7-pentahydroxyflavone) is one of the most omnipresent flavonoids **(Mao *et al.*, 2018).** Que is highly concentrated in apples, onion, potatoes, peanuts, soybeans, red wine, and other fruits and vegetables. Recently, quercetin has shown to inhibit an enzyme that converts testosterone into a molecule known as testosterone glucuronide, restoring the testosterone level and testicular function **(Bharti *et al.*, 2014)**.

2. MATERIALS AND METHODS

2.1. Experimental animals

Seventy male albino rats, weighing 150-180 g. each, were used in this study. They were obtained from the Central Animal House of the Farm Animal of the Egyptian Organization for Vaccine and Biological Preparation at Helwan, Egypt.

Animals were housed in groups of ten rats in standardized cages. The animals were kept under suitable laboratory condition through the whole experimental period and were acclimatized to the new environment for 1 week before the onset of experiment. Animals were weighed every week.

2.2. Materials

Thioacetamide (TAA) were purchased from Sigma Chemical Company (St. Louis, U.S.A) as white powder. It was dissolved in citrate buffer PH 4.5.

Quercetin (Que) Quercetin (3, 3', 4', 5, 7-pentahydroxy flavones) was purchased from Sigma Chemical Company (St. Louis, U.S.A) as white powder. It was dissolved in citrate buffer PH 4.5.

Clomiphene citrate (Clomid) was purchased from al- ezaby pharmacy as tablet. It was dissolved in saline.

2.3. Experimental design

Seventy young adult male albino rats (60 days- old, weighing 130-150 g) are used. All rats divided into 2 main groups A as control group & group B as infertility group.

GroupA (30 rats) are divided into 3 groups:

Group 1A: Control (TAA solvent).

Group 2A: Rats treated with clomid.

Group 3A: Rats treated with quercetin

Infertility group B is induced in male rats (40 rats) by i.p. injection of TAA. These animals are injected by TAA (300 mg/kg) 2 times with 24 hour intervals (Shapiro et al., 2006).

The considered infertility rats are divided into 4 groups:

Group 1B: Maintained untreated

Group 2B: Rats treated with clomid (0.35mg/kg Clomid i.p.) dosages were based on the reports by Patankar et al. (2007).

Group 3B: Rats treated with quercetin (the normal group with Que content of 50 mg/kg bw daily by oral gavage) (Abdeen et al., 2019; Ahmed et al., 2019 and Badr et al., 2019).

Group 4B: Rats treated with both clomid companied with querectin.

The animals in all groups were sacrificed 24 hours after the end of the 4th week of treatment at the terminus of the experiment.

2.4. Blood and tissue sampling

At the time of killing the rats, blood samples were taken from each group and allowed to coagulate at room temperature, and then centrifuged to separate serum. The serum from the supernatant is used for biochemical analysis. testes were immediately removed and cleaned. One testis was cut into small pieces (0.5 g) and homogenised in 5 ml of 0.9% NaCl for testis biomarkers. The other testes was fixed in 10% neutral buffered formalin after cutting into two halves for 24 hours to carry out the histological and immuno-histochemical examinations.

2.5. Determination of hormonal analysis

The serum from the supernatant is used to measure FSH, LH, and Testesterone were determined by ELISA technique using a commercial kit purchased from LSBio Company. According to the manufacturer's instructions.

2.6. Determination of oxidative stress and antioxidant levels

Lipid peroxide (MDA), reduced glutathione (GSH), and superoxidase (SOD) were measured using a commercial kit purchased from Bio Diagnostic Co. According to these methods **(Ohkawa *et al.*, 1979, Beutler *et al.*, 1963, and Aebi, 1984)**

2.7. Histological study

The fixed specimens of the testes were washed to remove the excess of fixative, dehydrated ascendingly in ethyl alcohol, cleared with xylol, then embedded in paraffin wax, sectioned at 5 µm and stained with hematoxylin and eosin stain **(Harris, 1900).** Photomicrographs were taken using a Toup View 3.7 for digital camera (Cairo, Egypt).

2.8. Statistical analysis

Group's data stated as mean ± standard error (SE). Statistical analysis was performed with using SPSS version 17 software. $P<0.05$ considered statistically significant.

3. RESULTS

3.1. Hormonal studies

The current results recorded that the essential tool of CC and/or Que to the improvement the normal level of hormones (LH, FSH, and tetesterone) in male rats induced infertility by TAA administration.

3.1.1. Serum LH level

Data recorded for the level of serum LH were presented in table (1). The values of LH content showed a significant decrease in the TAA. group (2.50±.071 ng/ml) as compared with those of the control group (6.24±.04 ng/ml). On the other hand, the groups of TAA+ CC, TAA+ Que and TAA+ CC+Que revealed a significant elevate in LH content recorded (4.40±.055 ng/ml), (3.63±0.211 ng/ml) and (5.19±0.025 ng/ml), respectively.

Table 1: The level of LH hormone (ng/ml) of male rats in the different groups.

	Control	CL	QU	TAA	TAA+CL	TAA+QU	TAA+CL+QU
LH (ng/ml)	6.24±.04	7.07±.035	6.13±.066	2.50±.071[a]	4.40±.055[a]	3.63±.211[ab]	5.19±.025[b]

Values are presented as mean ±SE

a: Statistically significant compared to corresponding value in controls (control, clomid and quercetin groups (group A)).

b: Statistically significant compared to corresponding value in TAA group.

3.1.2. Serum FSH level

The values of FSH level showed a significant decrease in the TAA group (1.40±.02 ng/ml) as compared with those of the control group (5.55±.02 ng/ml). On the other hand, the groups of TAA+ CC, TAA+ Que and TAA+ CC+Que recorded increase in FSH level (3.65±.23 ng/ml), (2.35±.26 ng/ml) and (4.81±.11 ng/ml), respectively.

Table 2: The level of FSH hormone (ng/ml) of male rats in the different groups.

	Control	CL	QU	TAA	TAA+CL	TAA+QU	TAA+CL+QU
FSH (ng/ml)	5.55±.02	6.14±.09	5.17±.09	1.40±.02[a]	3.65±.23[a]	2.35±.26[ab]	4.81±.11[b]

Values are presented as mean ±SE

a : Statistically significant compared to corresponding value in controls (control, clomid and quercetin groups (group A)).

b : Statistically significant compared to corresponding value in TAA group.

3.1.3.Serum testesterone level

The TAA untreated group exhibited a significant decrease in serum testosterone concentration (2.15±.01 ng/ml) in relation to the control (7.37±.12 ng/ml). An important observation was that, the testosterone level increased significantly in the serum of all TAA treated rats (TAA+ CC, TAA+ Que and TAA+ CC+Que). Where the testosterone level reached to 4.26±.06 ng/ml, 3.22±.21 ng/ml and 4.27±.15 ng/ml respectively (table 3).

Table 3: The level of testesterone hormone (ng/ml) of male rats in the different groups.

	Control	CL	QU	TAA	TAA+CL	TAA+QU	TAA+CL+QU
Testesterone (ng/ml)	7.37±.12	9.06±.27	7.29±.17	2.15±.01[a]	4.26±.06[a]	3.22±.21[ab]	4.27±.15[b]

Values are presented as mean ±SE

a : Statistically significant compared to corresponding value in controls (control, clomid and quercetin groups (group A)).

b : Statistically significant compared to corresponding value in TAA group.

3.2. Anti-oxidants and oxidative stress markers

3.2.1. Melondialdehyde (MDA) content

The contents of malondialdhyde (MDA) in testes tissue were shown in table (4). Rats in TAA group showed marked elevation in testes MDA content (7.26±.14 n.mol / mg.tissue) when compared to the control group (.55±.02 n.mol / mg.tissue). Moreover, rats in (TAA+ CC, TAA+ Que and Cis.+ CC+Que groups) showed marked depletion in testes MDA content

reach to (3.52±1.74n.mol / mg.tissue), (2.35±.12n.mol / mg.tissue) and (1.59±.19n.mol / mg.tissue), respectively, when compared to TAA group.

Table 4: The effect of TAA, CC and/or Que on the level of MDA (nmol / g.tissue) of male rats.

	Control	CL	QU	TAA	TAA+CL	TAA+QU	TAA+CL+QU
MDA (n. mol/mg/ protein)	.55±.02	.65±.54	.43±.01	7.26±.14[a]	3.52±1.74[a]	2.35±.12[ab]	1.59±.19[b]

Values are presented as mean ±SE

a : Statistically significant compared to corresponding value in controls (control, clomid and quercetin groups (group A)).

b : Statistically significant compared to corresponding value in TAA group.

3..2. 2. Superoxide dismutase (SOD) activity

The present investigation revealed that the content of SOD decreased significantly in TAA group (.89±.02 u/mg/ protein)) as compared to normal control rats (4.45±.14 u/mg/ protein)). Whereas, treatment with CC, Que or CC+Que produced a significant improvement of SOD activity whereas recorded (1.92±.02 u/mg/ protein), (2.47±.08 u/mg/ protein) and (3.39±.31 u/mg/ protein), respectively, in comparison with TAA group (table 5).

Table (5): The effect of TAA, CC and/or Que on the level of SOD (u/mg/ protein) of male rats.

	Control	CL	QU	TAA	TAA+CL	TAA+QU	TAA+CL+QU
SOD (u/mg/ protein)	4.45±.14	4.03±.02	3.39±.12	.89±.02[a]	1.92±.02[a]	2.47±.08[ab]	3.39±.31[b]

Values are presented as mean ±SE

a: Statistically significant compared to corresponding value in controls (control, clomid and quercetin groups (group A)).

b: Statistically significant compared to corresponding value in TAA group.

3.2.3. Glutathione reduced (GSH) content

The results of tissue GSH content in control and treated groups are illustrated in table (6). The recorded value of GSH level in TAA group showed significant decrease (6.49±.04 m. mol/mg/ protein) when compared to that in the control group (3.14± 0.09 m. mol/ mg/ protein). In contrast, TAA grop treated with CC, Que or CC+Que exposed significant

increase GSH content (2.10±.10 m. mol/mg/ protein), (3.25±.108 m. mol/mg/ protein) and 4.51±.101 m. mol/mg/ protein), respectively, as compared to the TAA group.

Table 6: The effect of TAA, CC and/or Que on the level of GSH (m. mol/mg/ protein) of male rats.

	Control	CL	QU	TAA	TAA+CL	TAA+QU	TAA+CL+QU
GSH (m. mol/mg/ protein)	6.49±.04	6.26±.055	5.66±.005	1.47±.018[a]	2.10±.10[a]	3.25±.108[ab]	4.51±.101[b]

Values are presented as mean ±SE

a: Statistically significant compared to corresponding value in controls (control, clomid and quercetin groups (group A)).

b: Statistically significant compared to corresponding value in TAA group.

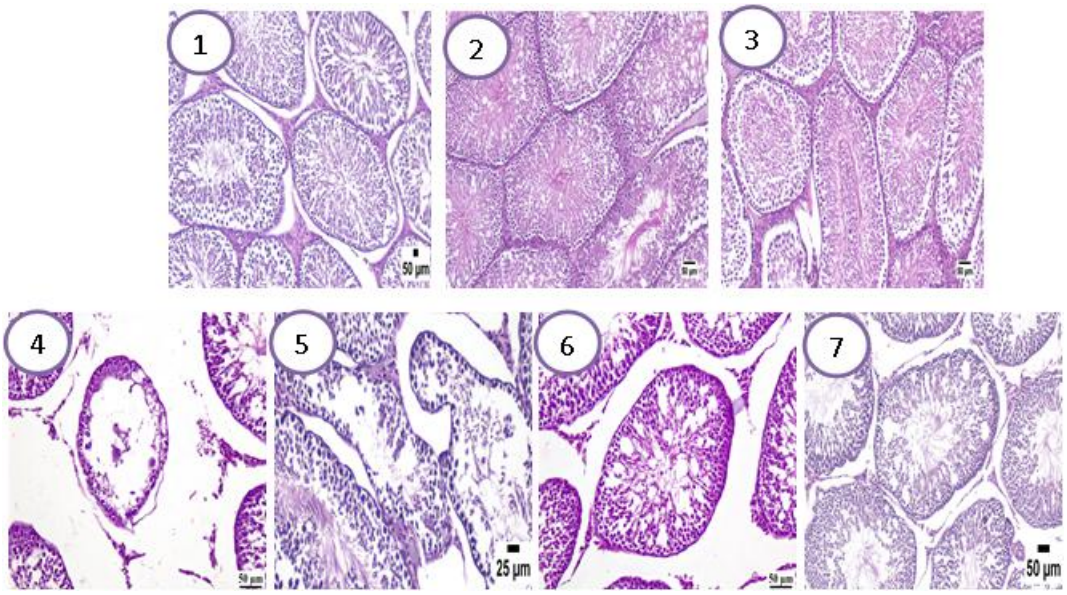
3.4. Histological study

Microscopic examination of testes from the **control groups** (control, clomid and quercetin groups (group A)) (**Fig. 1-3**) revealed normal histology of seminiferous tubules that appeared packed by increased numbers of spermatogonial cells and Sertoli cells with presence of numerous spermatids.

Testes of **group 1B** (**Fig. 4**) showed increase in histopathological observed lesions. The vacuolation was frequently detected within and between Sertoli cells in the affected seminiferous tubules. Increased number of atrophied seminiferous tubules was frequently detected in the testicular tissue that characterized by marked decrease in spermatogenesis. Exfoliation of germ cells was less frequently detected that illustrated by sloughing of germ cells into the lumen of seminiferous tubules.

Group 2&3b (**Fig. 5-6**) showed mild histopathological changes that revealed numerous apparently normal seminiferous tubules, meanwhile, fewer number of seminiferous tubules displayed vacuolation of germinal epithelium. Congested blood vessels were less commonly detected in the interstitial tissue.

Group 4B (**Fig7**) showed histopathological changes that revealed numerous apparently normal seminiferous tubules.



Figures, histological photographics showed: **Fig. (1,2,3)** Photomicrograph of testes, control group higher magnification showing normal seminiferous tubules (H&E). **(4):** Photomicrograph of testes, group 20 showing empty seminiferous tubule associated with wrinkled basement membrane (arrow) (H&E). (H&E×400). **(5,6)**: few vacuolated spermatogenic cells were detected with numerous intact testicular tissue in several sections **(7):** group showed apparently normal testicular tissue in numerous sections. Sporadic cases of mildly congested blood vessels were observed.

4. DISCUSSION

Infertility is a major public health issue affecting one out of five every married couples worldwide, with approximately 30% of the condition attributable to male factors **(Isidori *et al.*, 2006)**. It is on record that several factors can interfere with the process of spermatogenesis and reduce sperm quantity and quality with some of the identifiable causal factors being ischemic heart disease, diabetes mellitus, chronic liver diseases, cigarette smoking, agrochemical runoffs, air pollutants, and hypovitaminosis **(Thoma *et al.*, 2013**).

The present study indicated that the testes hormonal levels LH, FSH and testesterone decreased by TAA. adminsteration. Since it induced infertility, it shows the testes function, similar results were obtained by **(Yucel *et al.*, 2019).**

In the current investigation FSH and LH, testesterone increased after treatment with clomid or/ and quercetin. it could be attributed that quercetin as a free radicals scavenger maintains the membrane structure traces its function. These data were parallel with those obtained by **(Mirhoseini *et al.*, 2017).**

Earlier studies reported that TAA main mechanism induced infertility and oxidative stress that leads to the creation of ROS that directly interact with cell components including, lipid, proteins and DNA and alter their structure.

In the current study, TAA administration showed increment in the MDA and decrement in the antioxidant parameters including, GSH and catalase. The increment in MDA could be attributed to either the formation of ROS that interact with cell or to TAA attachement to lipid membrane causeing lipid peroxidation and leading to destruction of testes cells.

However, The decrease of GSH and SOD recorded may be attributed to ROS generation leading to inhibition to the antioxidant enzymes function **(Muratoglu *et al.*, 2019**).

Likewise, **(Olukole *et al.*, 2019)** mentioned that TAA. can react with thiol- containing proteins including glutathione- TAA leads to depletion of GSH and related antioxidants leads causing oxidative stress in the cells through the manufacturing of free radicals and raise the ROS.

This current study an increase of GSH and SOD with clomid treatment was recorded which may be attributed to ROS reduction in agreement with those results **Herbert *et al.* (2002),** noted that after insemination, a heavy accumulation of spermatozoa probably released from the cervical reservoir was at peak. Adequate sperm induction may be related to optimum motility, effective acrosomal mechanism and capacitation.

Bas & Naziroglu, (2019) informed that the antioxidant compounds, in quercetin that prevent the oxidative injury of cellular components indicated to exceptional electron donating capacity and its capability to remove reactive radicals targeting cell membrane.

The current study, demonstrated that quercetin administration produced a reduction in MDA and the increase in GSH and SOD levels, probably through its capability to scavenge free radicals. So, quercetin has a protective effect attributed to its antioxidant compounds. This result is an agreement with **(Semercioz *et al.*, 2017).**

The histological examination also afforded critical support for the biochemical results which revealed testicular congestion, degeneration, vocalization, necrosis, loss of spermatozoa and germinal cell in TAA group. These alterations are linked to the failure of testes functions may be attributed to that TAA improve production of ROS.

Preservation of the structure of spermatogenic germ epithelium is important in the evaluation of testicular tissue damage. This results are in agreement with **(Celik *et al.*, 2016 and Damilare *et al.*, 2021)** who proved that TAA caused significant injury to testicular tissues. According to the JTBS score, they observed impaired spermatogenic germ epithelium. In this group testis tissue, seminiferous tubules germinal epithelium were scattered, and atrophy was observed in some tubules. Among seminiferous tubules, vascular congestion, edema and vacuolization were reported in the areas close to the capsule.

The current study showed nearly normal structure of testis tissue after treatment of the TAA group with clomid and/or quercetin that is confirmed by improvment testes function parameters compared with non-treated TAA group. Such results may be attributed to the ability of quercetin to engraft into the injured cells and regenerate of the testes damage. Also, other studies postulated that quercetin administration reduced testes damage compared with TAA treated rats **(Wang *et al.*, 2019).** Quercetin antagonizes the TAA toxicity in rat testes by reducing oxidative stress and inhibiting autophagy. This study revealed the specific mechanism of quercetin against TAA and provided a new clue to reduce TAA poisoning **(Wang *et al.*, 2017 and Ling *et al.*, 2022).**

CONFLICT OF INTERESTS

There are no conflicts of interest.

Conflicts of Interest

The authors declare no conflict of interest.

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