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ARTEMISA HERBA-ALBA AMELIORATES CCl_4-INDUCED SPERMATOZOA TOXICITY THROUGH THE DOWNREGULATION OF ERCC1 EXPRESSION

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ABSTRACT

Testicular toxicity has been implicated as a remote cause of infertility in men. In this study, we aimed at investigating the ameliorative potentials of *Artemisa herba-alba* against calcium tetrachloride (CCl_4) induced toxicity in rats and its influence on ERCC1 gene expression. Four groups of twenty male Wistar rats (n =5) were created at random. Group I was the untreated control group. Group II was given a dose of 0.4 ml/200g CCl_4 orally every other day for 3 weeks. Group III was given a dose (500 mg/ kg b.w) of Artemisia herba-alba (ART) extract orally every other day for 3 weeks. Group IV, received an oral dose of an extract of Artemisia herba-alba at a dose level of (500 mg/ kg b.w) alternated every other day with 0.4 ml/200g of CCl_4 at a dose for 3 weeks. Bodyweight, relative kidney weight, serum testosterone, tissue oxidative stress, the expression of the ERCC1 gene, and testis histology were accessed. Our results showed that the administration of CCl4 to rats led to a decrease in body weight, tissue GSH, serum testosterone, increase in lipid peroxidation, an upregulation of ERCC1 gene expression, and modification of testicular histology. However, *Artemisa herba-alba* treatment following CCl_4 administration to rats resulted in the restoration of testicular histology and the downregulation of ERCC1 gene expression in addition to modestly maintaining the body weight of rats and serum testosterone level. More studies with a prolonged treatment duration are, therefore, required to establish ART as a potential therapeutic for its use in testicular toxicity.

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Introduction

The testes are a pair of organs located externally in the groin region of the male body and are a major organ of reproduction in males [1]. They are responsible for the production of the male reproductive cells (sperm cells), hormones (androgen), and the transfer of genetic material to the female reproductive cells [2].

Testicular toxicity is one of the major causes of infertility among males whose incidence has continued to increase in recent times thereby constituting a major health concern. The microenvironment of the testes is characterized by low oxygen tensions and can become affected by oxidative stress caused by unregulated levels of reactive oxygen species [3]. Over the years, the male reproductive organs have been negatively affected by a series of factors like drugs, and environmental and occupational exposure to different elements which results in its toxicity and thereby disrupting its function [4].

Carbon tetrachloride (CCl_4) is a colorless, sweet-smelling, manufactured chlorinated hydrocarbon that was used in the past as a cleaning agent and a degreaser. It was also used in fire extinguishers and as a precursor of refrigerants and propellants. CCl_4 is a very toxic compound and exposure to a high concentration can lead to damage to organs like the kidney, liver, and lungs. It can also affect the central nervous system [5, 6]. When humans are exposed to a certain concentration of CCl_4 through the oral, inhalation, or skin routes, the intoxication leads to high production of free radicals in different organs of the body. When the CCl_4 binds to the liver's cytochrome P450, it produces free radicals that start the oxidation of membrane lipids. When the secondary metabolic radicals of CCl_4 react with lipids or proteins, the permeability of the mitochondria, endoplasmic reticulum, and plasma membrane is changed, which can cause cell injury [7-9].

Artemisia herba-alba (Desert wormwood) is a plant that belongs to the genus *Artemisia* and contains about 400 species. It is a perennial shrub with short, linear-striped, bi-pennate leaves that are greenish-silvery, and hairy [10, 11]. This plant can be found in North Africa, India, the Middle East, Spain, the Northwestern Himalayas, and in the deserts of the Sinai Peninsula

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[12]. It is worth mentioning that this plant has found wide usage in folk and ancient medicine for the treatment of various types of diseases. For example, numerous ethnopharmacological and phytopharmacological studies have revealed that this plant possesses different medicinal properties including anti-diabetic, antimicrobial, antioxidants, antifungal, antihypertensive, neurological, immune-modulatory, antimalarial, and anti-spasmodic properties [10]. Studies on the phytochemical constituent of *Artemisia herba* alba have shown that this plant contains a lot of important and beneficial compounds such as sesquiterpene lactones which are important for their anti-inflammatory, antioxidant, anti-malaria, anti-cancer, and antibacterial effects [11, 13].

The Excision Repair Cross-Complementing 1 (ERCC1) gene holds an important position in the DNA damage repair system [14]. A functional ERCC1 is required for survival [14]. However, overexpression of the ERCC1 gene has been detected in testicular germ cells and correlates with the resistance to cisplatin-based chemotherapy [15]. Therefore, in this study, we examined the potential of *Artemisa herba-alba* to ameliorate the CCI_4-induced spermatozoa toxicity in rats and assessed its influence on ERCC1 gene expression.

Materials and Methods

Plant Material

The leaves of *Artemisia herba-alba* were obtained from an herbal and folk medicine market in Jeddah, Saudi Arabia. The leaves were air-dried and powdered followed by the dissolution of 10 g from this powder in 500 ml distilled water. The extract was filtered, concentrated to 8.5 mg/ml of *Artemisia herba-alba*, and kept till usage at 4 °C.

Animals

Male Wistar rats weighing 150-250 g were purchased from the King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. Animals were left to acclimatize to the lab ambiance for one week (12hr/12hr light off/on) and fed on a lab animal diet with freely available water. The King Abdulaziz University College of Medicine's Ethics Committee gave its approval to this animal experiment.

Chemicals

Carbon tetrachloride (CCl_4) was purchased from Sigma-Aldrich, (Missouri, United States) and diluted in olive oil (1:10 v/v). All other chemicals were of analytical grade.

Experimental Design

Rats were randomly placed into four groups (n = 5) after acclimatization and given the following treatment:

Group I (Control): the control group, received no treatment.

Group II (CCl_4): This group received an oral dose of CCl_4 in olive oil (1:10), at a dose of 0.4 ml/200g on alternate days for 3 weeks.

Group III (ART): Animals in this group received an extract of *Artemisia herba-alba* (ART) orally at a dose level of (500 mg/ kg b.w) on alternate days for 3 weeks.

Group IV (CCl_4 + ART): This group received an oral dose of an extract of *Artemisia herba-alba* at a dose level of (500 mg/ kg b.w) on alternate days with CCl_4 at a dose of 0.4 ml/200g for 3 weeks.

At the end of the three weeks experimental periods, food was withdrawn from the animals overnight and they were later euthanized under diethyl ether anesthesia. Following this, blood was drawn from the aorta in the abdomen and the testes were removed, rinsed in normal saline, and weighed. Parts of the testes were either stored in 10% buffered formalin for histological analysis or kept at -80˚C for extraction of RNA. The other half was homogenized in 100 mM phosphate buffer pH 7.4 at 14,000 rpm for 30 min.

Biochemical Analysis

For this, the manufacturer's instructions were followed when measuring the amounts of glutathione (GSH) and malondialdehyde (MDA) in the supernatant collected after centrifugation at 14,000 rpm using a commercial kit (MyBioSource, California, USA).

Hormonal Assay

An enzyme-linked immunosorbent assay (ELISA) kit (Diagnostic System Laboratories Inc., USA) was used to measure the serum levels of testosterone.

RNA Extraction and Real-time quantitative PCR (RT-qPCR):

According to the manufacturer's recommendations, total RNA was extracted from the testes using a (QIAgen RNeasy mini kit, cat # 74104). Next, 200ng of the extracted RNA was used in cDNA synthesis by the use of the M-MLV Reverse Transcriptase System (Promega, USA), and the qPCR reaction was made of the following components: cDNA, 3 mL; right and left primers, 0.5 mL (500 nM); purified water, 1 mL; SYBR Green Master Mix (Applied Biosystems, USA). In order to assess the relative mRNA expression, the $2^{-\Delta\Delta CT}$ method was applied and normalized to the expression of (GAPDH).

Table 1. Primer sequences

Isoforms	Primers sequence (5`-3`)
ERCC1 - left	5'-AAG GCG TAT GAG CAG AAG C-3'
ERCC1 right	5'-TCC AAA TGT AGT GAG GAG GGT-3'
GAPDH - left	5'-GAT GGT GAA GGT CGG TGT G-3'
GAPDH -right	5'-ATG AAG GGG TCG TTG ATG G-3'

Histopathology

Testes fixation was carried out in 10% buffered formalin, dried out in ethanol, and then embedded in paraffin wax for a day at room temperature. To evaluate histopathological alterations, hematoxylin, and eosin (H&E) were used to stain the sections of tissue blocks that were cut into thin sections. Light microscope images of stained sections taken at a 400x magnification

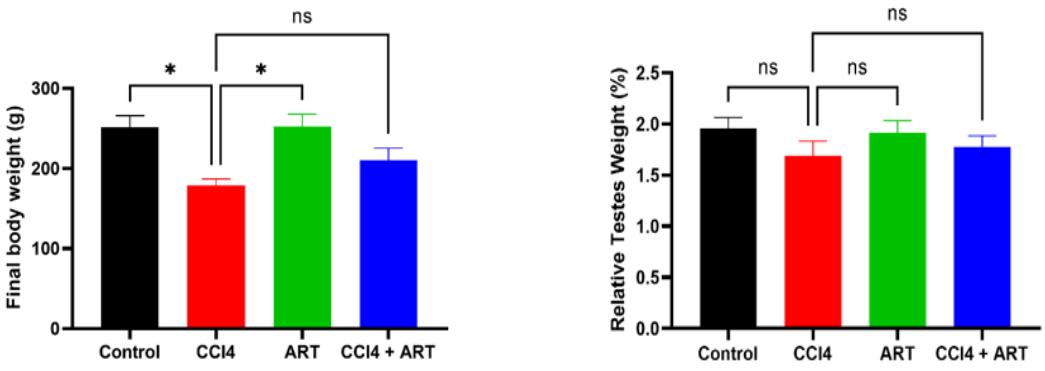
Statistical Analysis

The statistical analyses for this study were conducted using one-way ANOVA, and the data are presented as mean SEM. Means were compared using Dunnett's multiple comparisons test, and a significance threshold of $p < 0.05$ was selected.

Results and Discussion

Effects of CCl_4 and ART on Final Body Weight and Relative Testes Weight

The toxicity of CCl_4 has been established by several previous studies. Here, we firstly examined the effects of CCl_4 on final body weight and relative kidney weight of rats. The administration of CCl_4 to rats significantly ($p < 0.05$) resulted in a decrease in the body weights of rats when compared to the untreated animals in the control group **(Figure 1a)**. In addition, animals administered with ART showed no difference in body weight in comparison to the animals in the control group. However, animals administered with CCl_4 and treated with ART showed a 17% improvement in body weight as compared to the CCl_4-only administered rats **(Figure 1a)**. Furthermore, although there was a 16% decrease in the relative testes weights in the CCl_4 treated group in comparison the animals in the control group, this decrease was not up to a significant level. The relative testes' weights did not differ much in the CCl_4 administered group treated with ART and the CCl_4-only administered group **(Figure 1b)**.

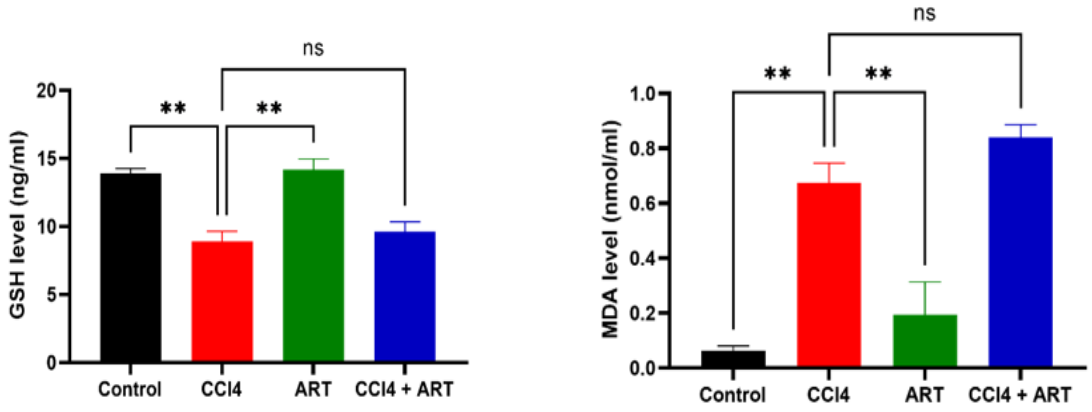


a) b)

Figure 1. Effects of CCl_4 and ART on final body weight and relative testes weight. a) final body weight. b) relative testes weight

Effects of CCl4 and ART on serum GSH and MDA levels

Next, we examined the effects on CCl_4 and ART on the tissue antioxidant content. Rats administered with CCl_4 differ significantly ($p < 0.01$) in having a lower GSH contents when compared to the animals in the control group and the ART-only-administered rats. In addition, the GSH contents did not differ much between the ART-administered rats and the control group. Also, ART treatment in rats after CCl_4 administration led to an 8% increase in the GSH content when compared to the CCl_4-only although not to a significant level **(Figure 2a)**. Furthermore, and as expected, CCl_4 led to a noticeable rise in the MDA content in comparison to the animals in the control group and the group that received ART only **(Figure 2b)**. The administered of ART to CCl_4 treated rats showed no effect as compared to the CCl_4-only treated rats **(Figure 2b)**.

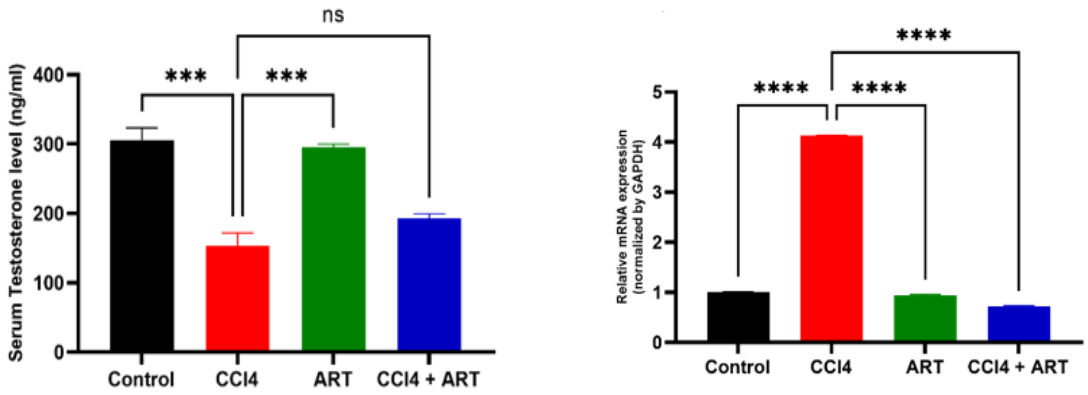


a) b)

Figure 2. Effects of CCl4 and ART on GSH and MDA levels. a) Serum glutathione (GSH) level. b) Serum malondialdehyde (MDA) level.

Effects of CCl4 and ART on Testosterone Levels and ERCC1 Gene Expression

Next, we accessed the effects of CCl_4 and ART on serum testosterone levels and the ERCC1 gene expression in testis tissues. As shown in **Figure 3a**, rats administered with CCl_4 led to a substantial decrease ($p < 0.001$) in the levels of serum testosterone levels when compared to the rats in the ART-only treated group and control group. The serum levels of testosterone in the control group and the ART group remained the same. Moreover, ART administered to the CCl_4 group led to a 26% increase in testosterone levels as compared to the CCl_4 group even though this was not up to a significant level. Furthermore, there was a significant upregulation in the expression of ERCC1 in the testes of rats administered with only CCl_4 when compared with both ART-only treated group and the control group. However, the expression levels of ERCC1 in the control group and the ART-treated group remained the same. Interestingly, ART treatment to CCl_4 administered to rats showed a significant ($p < 0.0001$) down-regulation in the expression of the ERCC1 gene in comparison to its level in the testes of rats administered with only CCl_4 **(Figure 3b)**.

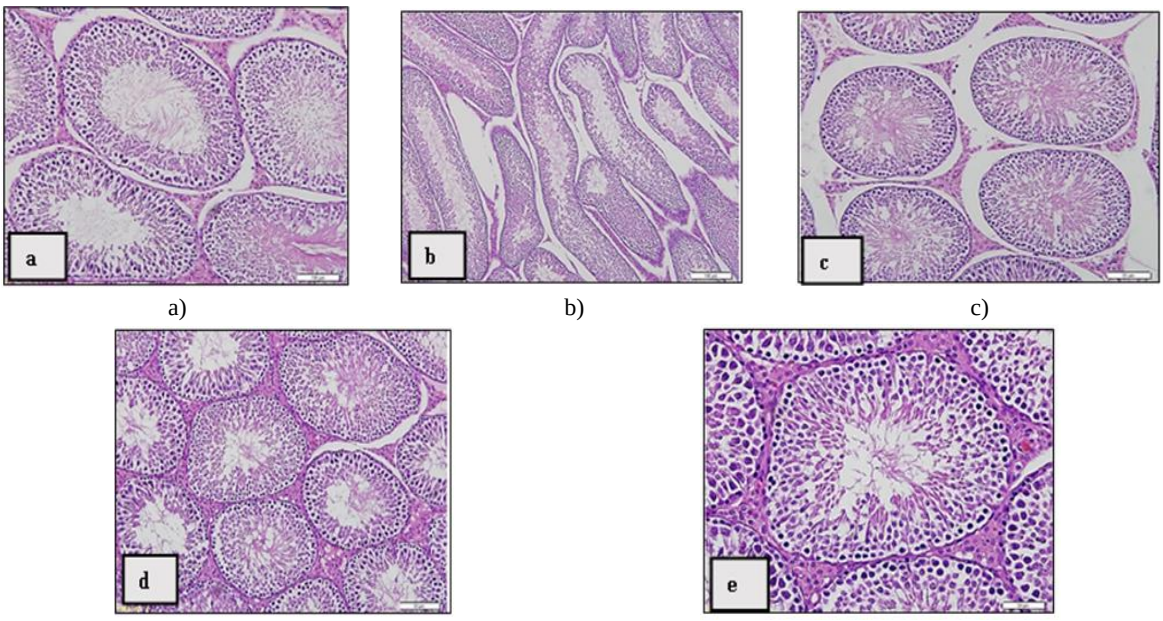


a) b)

Figure 3. Effects of CCl_4 and ART on testosterone levels and ERCC1 gene expression. a) serum testosterone level. b) Relative ERCC1 gene expression.

Effects of CCl4 and ART on Testicular Histology

Finally, we accessed the effects of CCl_4 administration and treatment with ART on the histological architecture of the testes using H & E staining. The histological analysis showed that CCl_4 administration to rats disrupted the seminiferous tubules and caused degeneration of the testis's membrane in comparison to the testes of the animals in the control group which showed normal membrane and spermatozoa. In addition, CCl_4 also resulted in an abnormal epithelium as compared to the histology of the testes in of the animals in the control group and the group that received ART only **(Figures 4a-4c)**. However, ART treatment following CCl_4 administration restored the testes architecture to near normal and improved the number of both the primary and the secondary spermatocytes **(Figures 4d and 4e)**.



a) b) c)

d) e)

Figure 4. Effects of CCl_4 and ART on testicular histology. a) Histology of the testes in the normal control group showing normal epithelium and seminiferous tubules. b) Histology of the testes of the animals in the CCl_4-administered group showing abnormal testis membranes and seminiferous tubules. c) ART-administered group, showing normal testes architecture. d-e) CCl_4 + ART group showing improved testis architecture to near normal with visible epithelial height and spermatogonia.

Previous studies have reported that the increase in the production of free radicals and/or a decrease in the antioxidant defense capacity results in oxidative stress [16]. Therefore, to maintain normal cellular functions, it is important that the concentration of the reactive oxygen species (ROS) and the antioxidants are kept at equilibrium [17]. An increase in the concentration of ROS results in a decrease in the activities of important cellular antioxidant defense systems [18]. Different enzymes and non-enzymes in mammalian cells help to fight against reactive oxygen species and free radicals. However, there are events where the strength of the antioxidants against ROS is not enough [19]. Due to the effect of excess production of ROS in the cells, recent studies have focused on plants with antioxidant potentials that can help fight against the oxidative stress caused by ROS [20].

This study examined the ameliorative potentials of *Artemisia herba-alba* on CCl_4-induced testicular toxicity. *Artemisia herba-alba* is a plant that possesses antioxidants, antimicrobial, neurological, antimalarial, and immune-modulatory properties [10]. The results of this study showed that CCl_4 caused a decrease in the final body weight and resulted in the relative testes weight of the CCl_4 treated group being decreased by 16%, which is marginally consistent with earlier studies conducted by Hashem [17] who reported a substantial decrease in the testes weight and body weight ratio in the CCl_4-administered rats compared to the control group; largely due to the toxicity of CCl_4. The group treated with both CCl_4, and ART showed a 17% non-significant improvement in the final body weight.

Furthermore, a decrease in the tissue GSH and an elevation in the MDA concentrations by CCl_4 signify an oxidative stress and can cause testicular toxicity which can negatively affect spermatogenesis and steroidogenesis [21]. In this study, CCl_4 caused a significant decrease in tissue GSH levels in the testes of rats which agrees with a previous study on the prevention of CCl_4-induced toxicity [19]. The treatment of the CCl_4 group with ART showed a non-significant increase of 8% in the GSH levels unlike the effect of *Launaea procumbens* on the GSH concentration where there was a significant increase [22]. The increase in malondialdehyde (MDA) caused by the administration of CCl_4 agrees with the findings of Ojo *et al*. [21] compared to the control group. However, the treatment of animals with ART following CCl_4 administration showed no effect on the elevated MDA levels **(Figure 2b)**. This might likely be due to the short treatment duration.

The significant decrease in the serum testosterone level which conforms with what was observed by Shareen *et al*. [23] signifies a direct effect of CCl_4 on Leydig cell concentration or an indirect effect as a result of oxidative stress caused by a disturbance in the hormonal microenvironment. The effect of CCl_4 on the serum testosterone level can inhibit the production of the male sperm cell. Although there was a 26% increase in testosterone in the group treated with both ART and CCl_4 when compared to the CCl_4 only group, this increase was not significant.

The ERCC1 gene is a DNA excision repair protein that assists in the repair of DNA damaged by chemicals and helps in maintaining chromosome stability [24, 25]. Soares *et al*. [26] studied the relationship between ERCC1 rs3212986 polymorphism and clinical side effects of chemotherapy and radiation for cervical cancer and reported a link between this gene and the late gastrointestinal toxicity onset. In addition, previous studies have shown the correlation between the ERCC1 status and toxicity and patient-reported quality of life [27].

In this study, a substantial elevation in the ERCC1 gene expression in the testes was noted in comparison to the control group and the group that received ART only **(Figure 3)**, which according to Jacobsen *et al*. [28] strongly indicates that there is a genomic alteration and instability which could be a result of the administered CCl_4 [26]. The group administered with CCl_4, and ART showed a decrease in the ERCC1 gene expression compared to the CCl_4 group.

The testicular histology showed that the administration of CCl_4 resulted in a disrupted seminiferous tubule, abnormal testis membrane, and a reduction in spermatogonia. Interestingly, the treatment of rats with ART after CCl_4 administration attenuated the changes in the testicular histology caused by CCl_4 administration. Our results agree with previous studies which showed changes in the seminiferous tubules and a reduction in spermatogenesis following CCl_4 administration to rats [18, 21, 29].

Conclusion

In this study, we examined the ameliorative potentials of *Artemisa herba-alba* against CCl_4-induced testicular toxicity in rats. We should that the administration of CCl_4 to rats led to a decrease in body weight, tissue GSH, serum testosterone, upregulation of ERCC1 gene expression, and modification of testicular histology. In addition, CCl_4 also results in an elevation of tissue MDA. However, *Artemisa herba-alba* treatment following CCl_4 administration to rats resulted in the restoration of testicular histology and the downregulation of ERCC1 gene expression in addition to modestly maintaining the body weight of rats. More studies with a prolonged treatment duration are, therefore, required to establish ART as a potential therapeutic for the treatment of testicular toxicity.

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Conflict of interest: None

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Ethics statement: None

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