# Effect of Cinnamaldehyde on the Toxicity of Cyclophosphamide in the Testes of rats: Influence of Pre- and Post-Treatment Schedule

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**Abstract:** The current study was designed to investigate the possible protective effects of cinnamaldehyde (CIN) on cyclophosphamide (CP)-induced testicular damage in rats. CP-induced male Wistar rats were either pre-treated or concurrently supplemented with 20 mg/kg body weight CIN for 7 days. CP administration induced severe testicular damage evidenced by the histopathological alterations including extensive interstitial hemorrhage in-between seminiferous tubules, complete separation between different stages of spermatogenic cells, vacuoles within spermatogenic cells and disorganization of spermatogenic cells. CIN supplementation potentially alleviated the histological architecture of CP-induced rats. Lipid peroxidation, a convenient marker of oxidative stress, showed a significant elevation in testicular tissue of CP-induced rats. On the other hand, reduced glutathione content and activity of superoxide dismutase and glutathione peroxidase were significantly declined in testes of CP-induced rats. CIN markedly decreased testicular lipid peroxidation and ameliorated the antioxidant defenses. In conclusion, CIN has protective effect against CP-induced testicular injury in rats probably mediated through its ability to attenuate oxidative stress.

[Eman S. Abd EL-Reheem; Ayman M. Mahmoud; Abeer M. Abd El-Hameed and Hanan A. Soliman. **Effect of Cinnamaldehyde on the Toxicity of Cyclophosphamide in the Testes of rats: Influence of Pre- and Post-Treatment Schedule.** *Nat Sci* 2016;14(5):44-51]. ISSN 1545-0740 (print); ISSN 2375-7167 (online). http://www.sciencepub.net/nature. 6. doi:10.7537/marsnsj14051606.

**Key words:** Cinnamaldehyde, cyclophosphamide, oxidative stress, testicular injury.

## 1. Introduction

Therapeutic effectiveness of anticancer drugs is associated with severe side effects due to their adverse toxicity. Cyclophosphamide (CP) is one of the widely used anticancer drugs for its therapeutic efficacy against a variety of cancers and disorders like systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis (Perini *et al.,* 2007). It is extensively used to treat a wide range of cancers, as an immunosuppressive agent following organ transplants and for treating various autoimmune disorders (Fleming, *et al.,* 2001). CP undergoes a metabolic activation by hepatic microsomal cytochrome P450 mixed functional oxidase system to produce the two metabolites, phosphoramide mustard and acrolein, which are highly toxic (Kern *et al.,* 2002) and have the potential to generate superfluous reactive oxygen species (ROS) (Ahmady *et al.,* 2008). The principal alkylating metabolite, phosphoramide mustard, is responsible for the therapeutic activity. It may also provoke a wide range of adverse effects including testicular toxicity in humans and animals (Fraiser *et al.,* 1991).The cytotoxic effects of CP and other chemotherapeutic drugs result in part from their interaction with DNA leading to defective DNA, abnormal cell function, and cell death (Lee and Schmitt, 2003).The cytotoxicity of CP to rapidly dividing cells makes the testes a target for its damaging effects. In rodents, administration of CP leads to decreased testicular weight, decreased DNA synthesis in spermatogonia, transitory oligospermia and protein synthesis in spermatids (Elangovan *et al.,* 2006).

Cyclophosphamide has shown to produce severe testicular damage, which is characterized by spermatogenic damage, germ cell apoptosis, Leydig cell dysfunction and testicular steroidogenic disorder. It has also been reported that cyclophosphamide administration induces oxidative stress and the generation of toxic reactive oxygen species (ROS) which may be responsible for its testicular toxicity (Jianpingqiu, *et al.,* 1995). Animal studies have revealed that the treatment of rats/mice with CP led to biochemical and histological alterations in the testis and epididymis (Kaur *et al.,* 1997). The mechanism by which CP causes testicular injury is unknown; however, numerous studies have shown that CP exposure can disrupt the redox balance of tissues to suggest that biochemical and physiological disturbances may result from oxidative stress (Haque *et al.,* 2001; Das *et al.,* 2002; Ghosh *et al.,* 2002). Human body has different methods of mitigating oxidative injuries, either by repairing injuries or by directly diminishing the occurrence of oxidative stress via enzymatic and/or non-enzymatic antioxidant systems. Non-enzymatic antioxidants such as natural compounds can also protect the body against oxidative

stress via their antioxidant activities. Considering the importance of CP in the clinical practice, there arise a necessity of an agent, which can ameliorate the side effects of this alkylating agent, without compromising on its therapeutic benefits.

Many reports demonstrate that natural compounds can reduce lipid peroxidation (Altuntas *et al.,* 2002). It is therefore important to continue the search for an effective model compound that will protect against CP-induced oxidative stress and reduce toxicity issues associated with chemotherapy. In the light of this, several chemo-preventive strategies have been presented, which have beneficial impacts on the CP-experimental model of testicular injury (Selvakumar *et al.,* 2004 and 2006). These studies suggest that natural compounds with antioxidative properties may have the potential to ameliorate CP-mediated testicular injury. In the past decade, the bioactivities of natural compounds on human health have given rise to much attention, especially the antioxidant activity (Zhang, 2005). Biological compounds with antioxidant properties may protect cells and tissues from deleterious effects of reactive oxygen species (ROS) and other free radicals generated during CP exposure (Das *et al.,* 2002; Ghosh *et al.,* 2002). Considering the importance of this drug in the clinical practice, there arise a necessity of an agent, which can ameliorate the side effects of this alkylating agent, without compromising on its therapeutic benefits.

Cinnamaldehyde (CIN) has been in public use since 1900. It is a yellowish liquid with a strong pleasant fragrance derived from the bark of Cinnamomum cultivated trees. The occurrence of cinnamaldehyde is noticed in several brands of cinnamon breads, cereals, cookies, puddings, applesauces and fruit juices (Friedman *et al.,* 2000). Cinnamon, scientifically named *Cinnamomum spp*, is a plant with many uses as an herbal medicine, containing mucilage, tannin, sugar, resin, and essential oil, among which the essential oil is the most important part, a substantial portion of which is made up of cinnamaldehyde or cinnamic aldehyde (Barceloux, 2009). In traditional medicine, various therapeutic uses have been proposed for this plant (Ziment and Yick, 2003; Verspohl *et al.,* 2005; Bandara *et al.,* 2011); besides, it has been found to have high antioxidant (Shan *et al*., 2005), anti-bacterial (Lopez *et al*., 2005), and anti-inflammatory (Chao *et al*., 2005) activity which also play a role in tissue repair (Kamath *et al*., 2003). Therefore, the present study was designed to investigate the efficacy of CIN against CP-induced testicular injury and oxidative stress in rats.

## 2. Materials and methods

### Chemicals

CP (Endoxan) was supplied as vials from Baxter Oncology (Dusseldorf, Germany). Cinnamaldehyde (CIN), reduced glutathione (GSH), thiobarbituric acid (TBA), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and pyrogallol were purchased from Sigma (USA). All other chemicals were of analytical grade and obtained from standard commercial supplies.

### Animals and experimental design

Male Wistar rats weighing 100–120 g, obtained from animal house of the National Research Centre (El-Giza, Egypt) were included in the present investigation. The animals were housed in plastic well-aerated cages at normal atmospheric temperature (25 ± 2°C) and normal 12-h light/dark cycle. Rats had free access to water and were supplied daily with laboratory standard diet of known composition. Rats were kept under observation for 1 week before the onset of the experiment for acclimatization and to exclude any intercurrent infection. All animal procedures were undertaken with the approval of Institutional Animal Ethics Committee of Beni-Suef University (Egypt).

To study the protective effect of cinnamaldehyde against CP-induced testicular damage and oxidative stress, thirty rats were randomly allocated into five groups having six in each as follows:

Group 1 (control): received the vehicle 0.5 % carboxymethyl cellulose (CMC) by oral gavage for 15 days and a single intraperitoneal (ip) injection of saline at day 7.

Group 2 (CIN): received 0.5 % CMC by oral gavage for 7 days and a single ip dose of saline at day 7 followed by oral administration of 20 mg/Kg (CIN) (Anand *et al*., 2010) for 7 consecutive days.

Group 3 (CP): received 0.5 % CMC by oral gavage for 7 days and a single ip dose of 150 mg/kg CP at day 7 (Mahmoud and Al Dera, 2015)

Group 4 (CP+CIN): received 0.5 % CMC by oral gavage for 7 days and a single ip dose of 150 mg/kg CP at day 7 followed by oral administration of 20 mg/kg CIN suspended in 0.5 % CMC by oral gavage for 7 consecutive days.

Group 5 (CIN+CP): received 20 mg/kg CIN suspended in 0.5 % CMC by oral gavage for 7 consecutive days and a single ip dose of 150 mg/kg CP at day 7.

The doses of CIN were balanced consistently as indicated by any change in body weight to keep up comparable dosage for every kg body weight over the entire period of study.

### Blood and tissue sampling:

At the end of the experiment ($15^{th}$ day), animals were sacrificed under ether anesthesia and samples were collected. Testes samples were immediately excised and perfused with ice-cold phosphate-buffered saline (PBS). Frozen samples (10% w/v) were homogenized in chilled PBS, and the homogenates

were centrifuged at 3000 rpm for 10 min. The clear homogenates were collected and used for subsequent assays. Other testes samples were immediately excised, perfused with ice-cold PBS, and fixed in 10% formalin for histological processing.

**Biochemical assays:**

**Assay of oxidative stress and antioxidant defense system:**

Lipid peroxidation content was assayed in testes homogenates by measurement of malondialdehyde (MDA) formation according to the method of Preuss *et al.* (1998). Reduced glutathione (GSH) content and activities of the antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPx), were measured according to the methods of Beutler *et al.* (1963); Marklund and Marklund (1974) and Matkovics *et al.* (1998), respectively.

**Histopathological study:**

The testes samples were flushed with PBS and then fixed in 10 % buffered formalin for 24 h. After fixation, the specimens were dehydrated in ascending series of ethanol, cleared in xylene, and embedded in paraffin wax. Blocks were made, and 4-µm-thick sections were cut by a sledge microtome. The paraffin-embedded sections were deparaffinized, washed with PBS, and stained with hematoxylin and eosin (H&E). The stained slides were examined under light microscope.

**Statistical analysis**

Statistical analysis was performed using SPSS v.20. Results were expressed as mean ± standard error (SEM), and all statistical comparisons were made by means of the one-way ANOVA test followed by Tukey's test *post hoc* analysis. A P value <0.05 was considered significant.

## 3. Results

**Effect of CIN on oxidative stress and antioxidant markers:**

CP administration produced a significant ($P$<0.001) increase in levels of the lipid peroxidation marker MDA when compared with the corresponding control rats (Table 1). Pretreatment of the CP-administered rats with CIN significantly ($P$<0.001) ameliorated the altered levels of MDA. Similarly, CIN administered concurrently produced a significant ($P$<0.001) decrease in testicular MDA content. CIN produced a more pronounced effect when supplemented after CP administration.

Conversely, GSH levels in the testes of CP-administered rats showed a significant ($P$<0.001) decrease when compared with the control group, as depicted in Table 1. Oral supplementation of CIN significantly ($P$<0.001) prevented the decline in GSH levels in testes tissue of the CP-administered rats. Administration of CIN post CP-administration produced a significant ($P$<0.05) increase in GSH level when compared with CIN supplemented prior CP.

Similar to GSH, activity of the antioxidant enzymes SOD and GPx showed a significant ($P$<0.001) decrease in testes of the CP-administered rats when compared with the control group, as illustrated in Table 1. Oral supplementation of CIN, either pre- or with CP, significantly ($P$<0.001) increased the activity of GPx and SOD when compared with the CP group.

**Table 1: Effect of Cinnamaldehyde on oxidative stress and antioxidant defense system in testes tissue of control and cyclophosphamide-administered rats.**

| Parameters / Groups | MDA (nmol/100 mg tissue) | GSH (nmol/100 mg tissue) | GPx (U/g tissue) | SOD (U/g tissue) |
|---|---|---|---|---|
| Control | 7.52± 0.17 | 10.15 ± 0.10 | 11.13 ± 0.53 | 7.71 ± 0.06 |
| CIN | 7.37 ± 0.31 | 10.97 ± 0.42 | 9.53 ± 0.33 | 8.00 ± 0.24 |
| CP | 15.00± 0.35$^{***}$ | 2.75 ± 0.03$^{***}$ | 3.73± 0.24$^{***}$ | 4.17 ± 0.40$^{***}$ |
| CP + CIN | 6.90 ± 0.34$^{+++}$ | 10.58 ± 0.11$^{+++}$ | 9.09± 0.45$^{+++}$ | 8.01 ± 0.24$^{+++}$ |
| CIN + CP | 7.58 ± 0.13$^{+++\#}$ | 9.45 ± 0.10$^{+++\#}$ | 9.28 ± 0.14$^{+++}$ | 8.19 ± 0.24$^{+++}$ |

*** $P$<0.001 versus Control. $^{+++}P$< 0.001 versus CP and $^{\#}P$< 0.05 versus CP + CIN. Data are M ± SEM (N = 6)

**Histopathological study:**

Histopathological examinations of H&E- stained testis sections from control and cinnamaldehyde supplemented control rats demonstrated normal seminiferous tubules lined with stratified epithelium formed of different stages of spermatogenic cells and Sertoli cell. Seminiferous tubules are separated by interstitial space contains cells of Leydig (Figures 1A & 1B). On the other hand, sections from the testis of CP-induced rats showed extensive interstitial hemorrhage in-between seminiferous tubules. Some tubules showed complete separation between different stages of spermatogenic cells, some show vacuoles within spermatogenic cells while others show disorganization of spermatogenic cells (Figure 1C). Sections from cinnamaldehyde pretreated CP-induced

rats showed normal seminiferous tubules with normal spermatogenic and Sertoli cells except for some appearance of vacuoles within the seminiferous tubule. Seminiferous tubules are separated by interstitial space also looks normal (Figure 1D). CP-induced rats received concurrent administration of cinnamaldehyde showed irregular seminiferous tubules, vacuoles within spermatogenic epithelium, decreased thickness of spermatogenic epithelium and congested interstitial blood vessels (Figure 1E).

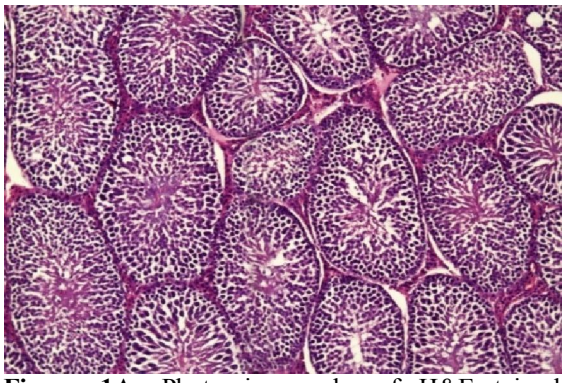

**Figure 1A:** Photomicrographs of H&E-stained testes sections of control rats showing normal histological structure.

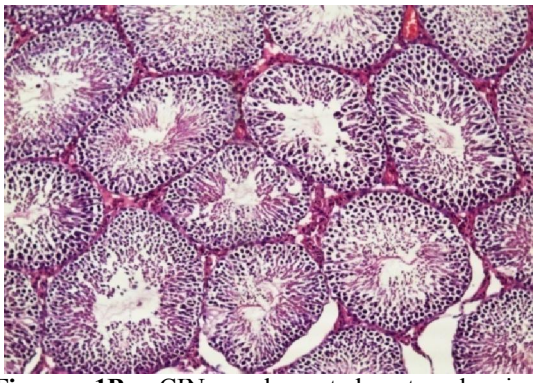

**Figure 1B:** CIN-supplemented rats showing normal histological structure.

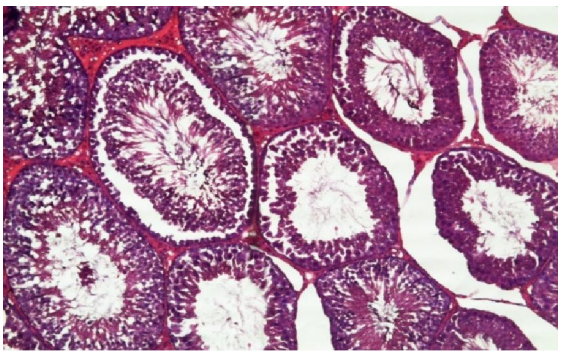

**Figure 1C:** CP-induced rats showing extensive interstitial hemorrhage in-between seminiferous tubules, complete separation between different stages of spermatogenic cells, vacuoles within spermatogenic cells and disorganization of spermatogenic cells.

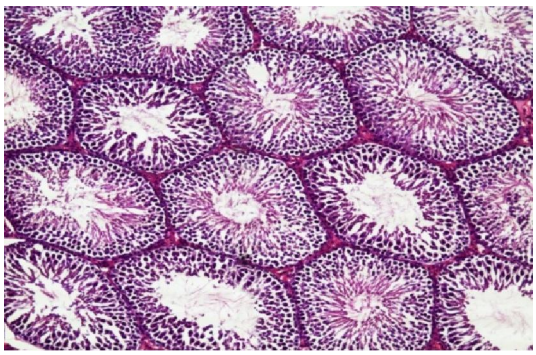

**Figure 1D:** CP-induced rats pretreated with CIN showing normal seminiferous tubules with normal spermatogenic and Sertoli cells except for some appearance of vacuoles within the seminiferous tubule.

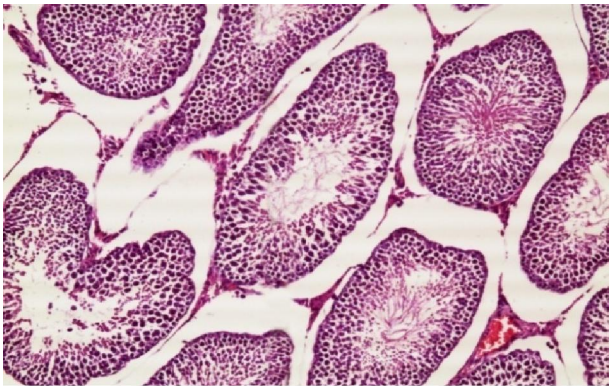

**Figure 1E:** CP-induced rats concurrently treated with CIN showed irregular seminiferous tubules, vacuoles within spermatogenic epithelium, decreased thickness of spermatogenic epithelium and congested interstitial blood vessels.

**4. Discussion**

In the present study, we investigated the protective effects of cinnamaldehyde against CP-induced testicular toxicity in male rats. Our results showed that a single intraperitoneal dose of CP resulted in significant testicular toxicity, as evidenced by the histopathological alterations and induced oxidative stress. Cinnamaldehyde treatment showed a protective effect against the testicular toxicity induced by CP. CP, a cytotoxic alkylating agent, is used extensively as an antineoplastic agent for the treatment of various cancers; however, its full clinical utility is limited because of several adverse effects including reproductive toxicity in humans and experimental animals (Anderson *et al.,* 1995). The biochemical basis of CP toxicity is believed to be related to free radicals generated in these tissues. The cellular mechanisms by which CP causes testicular injury are poorly understood; however, numerous studies have shown that CP treatment is associated with induction of oxidative stress by the generation of free radicals and ROS (Das *et al.,* 2002; Ghosh *et al.,* 2002; Bhatia, 2003). Oxidative stress occurs when oxidative substances disturb the oxidant–antioxidant balance in human body and thus causing oxidative damages to deoxyribonucleic acid, proteins, and lipids (Bartsch and Nair, 2000; Chandrasekara and Shahidi, 2011). There is a general agreement that male reproductive organs are particularly susceptible to the deleterious effects of ROS and lipid peroxidation (Williams *et al.,* 1998). Previous studies showed that oral antioxidants treatment reduced the percentage of testicular toxicity (Huang *et al.,* 2009). Thus, it is concluded that ROS and lipid peroxidation may be involved in CP-induced testicular toxicities in rats.

The current findings showed that CP administration induced marked increase in the lipid peroxidation marker MDA in testicular tissue. Lipid peroxidation is the fundamental parameter of oxidative stress and MDA is considered to be an index of lipid peroxidation. MDA is the breakdown product of the chain reactions of polyunsaturated fatty acid oxidation and serves as a marker of lipid peroxidation in tissues (Poli*et al.,* 1989). Testicular toxicity may occur due to an increase in the intracellular levels of ROS, which are toxic at high levels and can interact with macromolecules. Our results are supported by the finding of Chabra *et al.* (2014) and Bhargavan *et al.* (2015) who reported that CP treated rats had a significant increase in testes lipid peroxidation, suggesting the presence of free-radical toxic stress within the testes. Pretreatment and post treatment of animals with cinnamaldehyde for 7 consecutive days significantly reduced the MDA levels and depleted lipid peroxidation in the testicular tissue of rats. This study demonstrated that cinnamaldehyde had potential protective effects against the testicular toxicity induced by CP in male rats. The post-treatment with cinnamaldehyde showed more significant decrease of MDA when compared with the pretreatment. However, it is still not clear how cinnamaldehyde mitigate the cellular oxidant state but recent reports by Jang *et al.* (2007), Chao *et al.* (2008) and Babu *et al.* (2014) hypothesize that the antioxidant potential of cinnamaldehyde is strongly dependent on its free radical-scavenging activity. On the other hand, it is well documented that polyphenols act as reactive oxygen and nitrogen species scavengers, redox-active transition metal chelators and enzyme modulators (Lee *et al.,* 2004).

In the present study, activities of antioxidant enzymes SOD and GPx in tests tissue was examined in addition, GSH content was determined. As compared to control group, the administration of cyclophosphamide in rats produced significant differences in the activities of SOD and GPx and in GSH content. We have demonstrated decreased activity of the enzymatic and non-enzymatic antioxidant defenses in the testes of CP-intoxicated rats which was associated with CP-induced oxidative stress. SOD and GPx play a crucial role in protecting the body against the deleterious effects of ROS and free radicals (Wei *et al.,* 2011). The observed depletion of GSH in the testicular tissue of CP-administered rats could be resulted by the direct conjugation of CP metabolites with GSH (Yousefipour *et al.,* 2005). The studies of Chabra *et al.* (2014) and Bhargavan *et al.* (2015) demonstrated that CP treated rats and mice had a significant drop in antioxidant levels SOD and GSH suggesting the presence of free-radical toxic stress within the testes The present investigation revealed that the administration of cinnamaldehyde significantly reverted back the altered levels of antioxidants enzymes SOD and GPx, which in turn reveals the antioxidant potential of this compound (Devipriya *et al.,* 2007; Babu *et al.,* 2014). In line with Jing *et al.* (2011) cinnamaldehyde induced higher activities of antioxidant enzymes GPx and SOD that in turn lowered the levels of MDA. Similar observations were also recorded by Hu *et al.* (2010) and Moselhy and Junbi (2010).

The major histopathological findings observed in the CP group included extensive interstitial hemorrhage in-between seminiferous tubules. Some tubules showed complete separation between different stages of spermatogenic cells, vacuoles within spermatogenic cells while others showed disorganization of spermatogenic cells. CP has been reported to induce germ cell damage and several sperm abnormalities along with severe histomorphological changes in the testes of humans and experimental animals (Matsui *et al.,* 1995). The

observed histopathological alterations may be considered a direct or indirect effect of CP, which later induces lipid peroxidation capable of disrupting the function of testis and epididymis. However, the incidence and severity of testicular in rats pre- and co-treated with cinnamaldehyde was considerably ameliorated when compared to that in the CP group.

**In conclusion,** the present investigation proves that cinnamaldehyde is able to reduce the testicular toxicity induced by CP in male rats through antioxidant activity, free radical-scavenging and anti-inflammatory properties. With regard to the low toxicity related to its consumption and high level of antioxidant activity, cinnamaldehyde is a good candidate to help defend the body against side effects induced by hazardous chemical agents.

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4/6/2016