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Protective Potential of Thymoquinone on Cyclophosphamide-Induced Hepatotoxicity in Rats

Adana M.Y., Opabunmi E.T., Sunmonu, O.E., Bello A.A., Onigbolabi O.G., Imam A., Ajao M.S.

Department of Anatomy, College of Health Sciences, University of Ilorin, Ilorin Nigeria

ABSTRACT

Background: Cyclophosphamide (CYC) has been known as an anticancer drug with several side effects on various organs such as the liver. In this study, the hepatoprotective properties of thymoquinone (TQ) were tested to decrease the damaging effects of Cyclophosphamide on the liver.

Objective: To study and observe the possible ameliorative effect of thymoquinone on cyclophosphamide-induced hepatic toxicity in Wistar rats.

Methods: Thirty Wistar male Adolescent (8 – 9 weeks old) rats weighing 70g – 150g were divided into Six groups; 5 rats per group (n=5) and treated orally for 21 consecutive days. Group A served as the control, and the rats in this group received normal saline. Group B was treated with 20mg/kg of cyclophosphamide. Group C was treated with 5mg/kg of Thymoquinone. Group D was treated with 10mg/kg of Thymoquinone. Group E was treated with 20mg/kg of cyclophosphamide and 5m/kg of Thymoquinone while Group F animals were treated with 20mg/kg of

Cyclophosphamide and 10mg/kg of Thymoquinone.

Results: The Cyclophosphamide treated group showed significant decreases in the body and liver weight, distorted hepatic cytoarchitecture, and reduction in hepatic function whereas co-treatment with TQ, showed protective effects on Cyclophosphamide-induced hepatotoxicity damage.

Conclusion: These results suggest that the administration of Thymoquinone may have a protective potential on Cyclophosphamide induce hepatotoxicity.

Keywords: Cyclophosphamide, Thymoquinone, Hepatotoxicity

Corresponding Author:

Name: Misturah Yetunde Adana
Address: Department of Anatomy, College of Health Sciences
University of Ilorin, Ilorin, Nigeria
Email: misturahadana@gmail.com, adana.my@unilorin.edu.ng
Contact: 08033727625

INTRODUCTION

Hepatotoxicity is defined as injury or liver damage caused by exposure to drugs or other non-pharmacological agents (1). It is an adverse drug reaction that may be uncommon but serious, and therefore, have a considerable impact on health (2, 3). Hepatotoxicity contributes to between 1/600 and 1/3500 of all admissions into the hospital (4). Overall, over 900 drugs, toxins, and herbs have been reported to cause hepatotoxicity and drugs contribute to 20-40% of all instances of fulminant hepatic failure (5). The hepatotoxicity of drugs is the main cause of drug withdrawal from the pharmaceutical market and interruption of the development of new molecules. Liver damage is so diverse that it reproduces almost all non-iatrogenic liver diseases (6). Acute hepatitis is the most common manifestation, accounting for more than 90% of drug-induced liver disease (6).

Cyclophosphamide is a synthetic alkylating agent used in chemotherapy and as an immunosuppressive agent. As a chemotherapeutic agent, it is used to treat a variety of cancers. As an immune suppressor, it is used in nephrotic syndrome, granulomatosis with polyangiitis, and following organ transplant among other conditions (7). Despite being considered the commonest chemotherapeutic agent used in the treatment of several human cancers, it is known also to induce marked hepatic toxicity by causing severe cellular damage (8). Thymoquinone, the main constituent of *Nigella sativa* possesses strong antioxidant properties that protect against oxidative damage resulting from free radical-generating agents. It was reported to have anti-inflammatory actions, an analgesic, and a protective effect against chemical-induced carcinogenesis (9). This research work aims to evaluate the possible ameliorative effect of Thymoquinone on

Cyclophosphamide induced toxicity in the liver of the Wistar rat model.

MATERIALS AND METHODS

The experimental work of the present study was conducted at the Experimental Animal house of the Department of Anatomy, University of Ilorin, Ilorin, Kwara State, Nigeria.

Chemicals

Thymoquinone was purchased from Sigma-Aldrich and cyclophosphamide was purchased at Aromokeye Pharmacy, Ilorin, Kwara State. TQ and CYC were dissolved in Normal saline, the solution was then given orally.

Experimental Animals

Thirty 8-week-old male Wistar rats weighing between 67-186g were obtained and bred in the animal house of the College of Health Sciences, University of Ilorin, and are used for research. The rats were randomly assigned into six groups of 5 rats per group. Animals were housed in clean animal cages under natural light and proper ventilation at room temperature. Animals in all groups fed on normal rat pellets grower mash and had free access to water. The nutrient content of the feed is as follows: 10% crude fiber, 15% crude protein, 7% fat, 1% calcium, 0.3% phosphorus, and 2,550 kcal/kg of metabolized energy as stated in the pack. The animals were kept for a week for acclimatization to their new environment and their weights were taken twice in a week. This research work was approved by the University of Ilorin ethical review committee (UERC) (UERC/ASN/2019/856).

Experimental Design

Animals were divided into Six groups; 5 rats per group (n=5) and treated orally for 21 consecutive days. Treatment groups were as

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follows:

- Group NS: Served as a control group receiving normal saline (20mls/kg/day).
- Group CYC: received Cyclophosphamide (20mg/kg body weight/day)
- Group 5TQ: received Thymoquinone (5mg/kg body weight/day) (10)
- Group 10TQ: received Thymoquinone (10mg/kg body weight/day) (10)
- Group C5TQ: received both Cyclophosphamide (20mg/kg body weight/day) and lower dose of Thymoquinone (5mg/kg body weight/day)
- Group C10TQ: received both Cyclophosphamide (20mg/kg body weight/day) and higher dose of Thymoquinone (10mg/kg body weight/day).

At the end of the experiment, the rats were weighed and then euthanized under anesthesia (ketamine 20mg/kg body weight). The liver of each animal was excised. Blood samples were withdrawn directly from the heart of each animal using standardized laboratory techniques.

Statistical Analysis

The obtained data were analyzed using Graph Prism 9.0. Results are presented as mean $\pm$ SEM, and all statistical comparisons were performed using a one-way analysis of variance (ANOVA). The test of significance was also performed to test any significant differences between different treatments (the level of significance was set at $P < 0.05$).

RESULT

To assess the effects of treatment on the overall well-being of the animals, the weights of the experimental animals across the groups were compared. The animals in the cyclophosphamide group show a significant decrease in weight when compared with the control groups. The decrease in weight experienced by the animals in the CYC group was also significant compared to the animals in the TQ group. Animals that were administered CYC and TQ had a much less decline in weight compared to the CYC group. This was also observed in the relative hepatic weight (table 1).

Biochemical Analysis

This was carried out to help explicate the alteration of markers which reflect the liver disease. The assessment of liver enzymes and expression of Proliferative Cell Nuclear Antigen (PCNA).

The liver total protein file showed no statistically significant difference in rats across the various groups [figure 1]. In the Alanine transaminase assay, Group A has a significantly lower ALT compared to B and C ($P < 0.05$). Group B has the highest level of ALT [figure 3]. The Aspartate transaminase assay showed that the value in Group A is significantly lesser compared to Group C, D, and E ($P < 0.05$) [figure 3]. In the alkaline phosphate assay, a rise in the values was observed in Groups C, D, and E when compared to Group A ($p < 0.05$). Group A significantly reduced compared to

Group F ($***=p < 0.05$). Group B significantly reduced compared to Group C, D, and E ($***=p < 0.05$). In lactate dehydrogenase, Group A is significantly reduced compared to Group B and F ($***P = < 0.05$). Group B is significantly increased compared to Group D and Group E ($**P = < 0.05$) [figure 5].

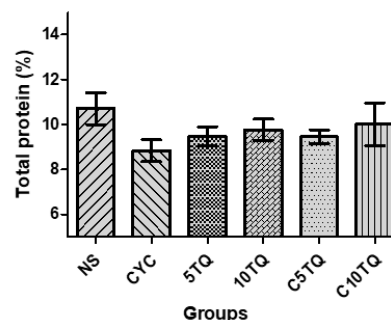


Fig 1. showing the changes in the levels of the total protein in the liver of rats across the groups. NS=control, CYC=Cyclophosphamide (20mg/kg), 5TQ=Thymoquinone (5mg/kg), 10TQ=Thymoquinone (10mg/kg), C5TQ=Cyclophosphamide (20mg/kg) + Thymoquinone (5mg/kg), C10TQ=Cyclophosphamide (20mg/kg) + Thymoquinone (10mg/kg).

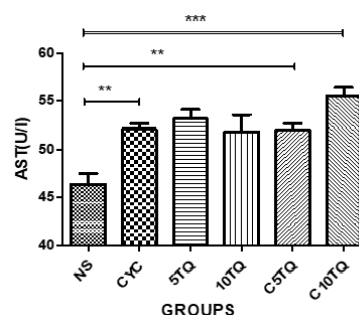


Fig 2. Changes in the levels of AST in the liver of rats across the groups. NS=control, CYC=Cyclophosphamide (20mg/kg), 5TQ=Thymoquinone (5mg/kg), 10TQ=Thymoquinone (10mg/kg), C5TQ=Cyclophosphamide (20mg/kg) + Thymoquinone (5mg/kg), C10TQ=Cyclophosphamide (20mg/kg) + Thymoquinone (10mg/kg) *, **, *** represents difference at $p < 0.05$, $p < 0.01$ and $p < 0.001$ between the group and NS group respectively.

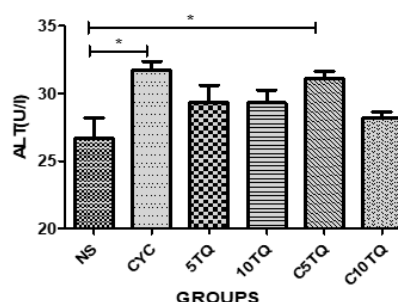


Fig 3. Levels of ALT in the liver of rats across the groups. NS = Control, CYC = Cyclophosphamide (20 mg / kg), 5TQ=Thymoquinone(5mg/kg), 10TQ=Thymoquinone(10mg/kg), C5TQ=Cyclophosphamide(20mg/kg) + Thymoquinone(5mg/kg), C10TQ=Cyclophosphamide(20mg/kg) + Thymoquinone (10mg/kg)

*, represents difference at $p < 0.05$, between the group and NS group.

Table 1. Body weights, weight gain, and relative hepatic weight

Group	IBW	FBW	WG	RHW
NS	103.2 $\pm$ 12.23	121.6 $\pm$ 20.74	18.40 $\pm$ 5.244	4.65 $\pm$ 0.41
CYC	112.4 $\pm$ 15.86	100.8 $\pm$ 21.45	-12.80 $\pm$ 6.028 ^a	3.77 $\pm$ 0.24
5TQ	118.4 $\pm$ 15.28	145.8 $\pm$ 18.19	27.40 $\pm$ 6.779 ^b	4.21 $\pm$ 0.25
10TQ	142.8 $\pm$ 14.29	175.4 $\pm$ 10.45	32.60 $\pm$ 13.96 ^b	3.81 $\pm$ 0.94
C5TQ	176.0 $\pm$ 4.195	199.6 $\pm$ 13.08	23.60 $\pm$ 9.714 ^b	4.00 $\pm$ 0.16
C10TQ	147.4 $\pm$ 6.282	138.0 $\pm$ 6.075	-9.600 $\pm$ 3.866 ^a	3.78 $\pm$ 0.25

NS = Control, CYC = Cyclophosphamide (20 mg / kg), 5TQ=Thymoquinone(5mg/kg), 10TQ=Thymoquinone(10mg/kg), C5TQ=Cyclophosphamide(20mg/kg) + Thymoquinone(5mg/kg), C10TQ=Cyclophosphamide(20mg/kg) + Thymoquinone (10mg/kg), IBW=Initial body weight, FBW=Final body weight, WG=weight gain, RHW=relative hepatic weight

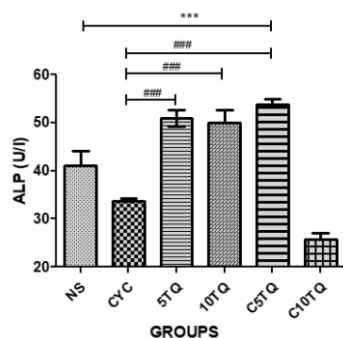


Fig. 4. Graph showing the changes in the levels of ALP in the liver of rats across the groups. NS=control, CYC=Cyclophosphamide (20mg/kg), 5TQ=Thymoquinone(5mg/kg), 10TQ=Thymoquinone(10mg/kg), C5TQ=Cyclophosphamide (20mg/kg) + Thymoquinone(5mg/kg), C10TQ=Cyclophosphamide(20mg/kg) + Thymoquinone (10mg/kg). *** represents significant difference between the group and NS, ### significant difference between CYC and other groups represents at $p < 0.001$.

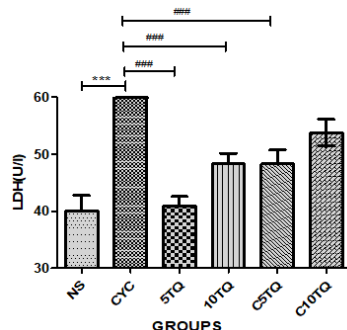


Fig. 5. Levels of LDH in the liver of rats across the groups. NS=Control, CYC= Cyclophosphamide (20mg/kg), 5TQ= Thymoquinone (5mg/kg), 10TQ= Thymoquinone (10mg/kg), C5TQ= Cyclophosphamide (20mg/kg) + Thymoquinone (5mg/kg), C10TQ= Cyclophosphamide (20mg/kg) + Thymoquinone (10mg/kg).

Histological Observation

In the study, cyclophosphamide administration caused marked deterioration of the histopathological architecture of the liver tissue when compared to normal control group. The liver tissues revealed low density of hepatocytes, vacuolations, and scattered area of inflammatory cell aggregates in between the hepatocytes (figure 6).

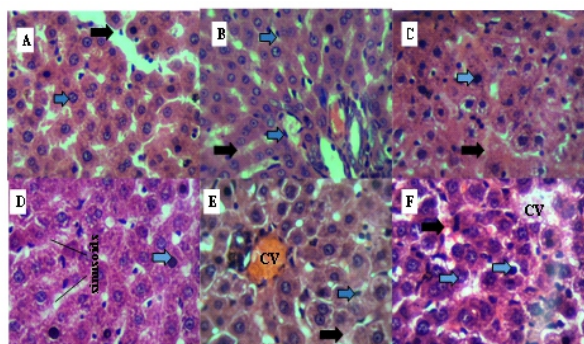


Fig. 6. Representative photomicrograph of Hematoxylin and Eosin-stained Liver sections of animals in groups A-F (400x magnification). A (NS) =control, B (CYC) = cyclophosphamide (0.02mg/kg), C (5TQ) = thymoquinone (5mg/kg), D (10TQ) = thymoquinone (10mg/kg), E (C5TQ) = cyclophosphamide (20mg/kg) + thymoquinone (5mg/kg), F (C10TQ) = cyclophosphamide (20mg/kg) + thymoquinone (10mg/kg). Black arrow= Liver sinusoids, blue arrows=Hepatocytes, CV=central Vein.

Immunohistochemical Observation

The response to proliferative cell nuclear antigen (PCNA) was significantly reduced in the CYC group when compared to the other groups except for C10TQ. The few stained cells revealed a milder response to the antigen when compared to the other groups (see figures 7(a) and (b)).

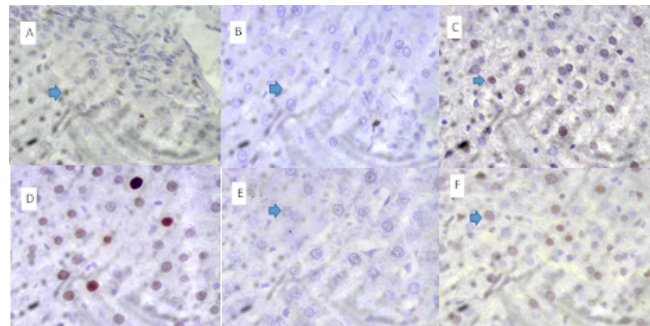


Fig. 7(a). Representative photomicrograph of PCNA immunostaining, showing the cytoarchitecture of the liver of experimental animals at 400x magnification. A (NS) =control, B (CYC) = cyclophosphamide (0.02mg/kg), C (5TQ) = thymoquinone (5mg/kg), D (10TQ) = thymoquinone (10mg/kg), E (C5TQ) = cyclophosphamide (20mg/kg) + thymoquinone (5mg/kg), F (C10TQ) = cyclophosphamide (20mg/kg) + thymoquinone (10mg/kg). Blue arrows shown in A, B, D, E and F the hepatic cells.

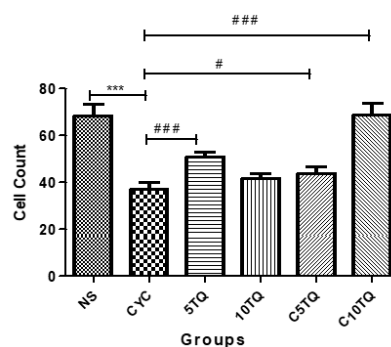


Fig. 7(b). Hepatic Proliferative cell counts for groups NS=control, CYC=cyclophosphamide(0.02mg/kg), 5TQ= thymoquinone(5mg/kg), 10TQ= thymoquinone (10mg/kg), C5TQ= cyclophosphamide(20mg/kg) + thymoquinone(5mg/kg), C10TQ= cyclophosphamide (20mg/kg) + thymoquinone(10mg/kg). Blue arrows shown in A, B, D, E and F the hepatic cells. A shows a normal liver cytoarchitecture. *, *** represents difference at $p < 0.05$, and $p < 0.001$ between the group and NS group respectively.

DISCUSSION

Cyclophosphamide, despite being considered the commonest chemotherapeutic agent used in the treatment of several human cancers, is known also to induce marked hepatic toxicity by causing severe cellular damage (8).

Treatment with cyclophosphamide had significant physical and morphological effects on the animals. During the treatment period, animals lost weight rather than gain weight like the animals in other groups. Co-administration with thymoquinone was unable to totally protect against this loss in weight as the control animals had a much higher weight gain. Although treatment with a higher dose of thymoquinone, significant decrease in body weight was observed. This can be a result of cyclophosphamide-induced disruption of umami taste functions and taste epithelium in the animals (11). This will contribute to loss of appetite and reduce consumption of feeds. Cyclophosphamide use has also been noted for the continuous decline in body weight associated with

severe nausea and vomiting (12, 13).

The hepatic functions have also been shown to be adversely impaired by the cytotoxic effects of cyclophosphamide (14, 15) levels of hepatic enzymes were altered in the cyclophosphamide-treated groups. The LDH and ALT levels were significantly elevated in the CYC-treated groups. Co-administration with thymoquinone was able to restore levels comparable to that of the control group as reported by prior studies (16).

AST is an enzyme that is found mostly in the liver, and also in muscles. Elevation in serum levels is an indication of liver damage or injury. In this study, the levels of AST were elevated in all groups when compared to the control. This suggests that both CYC and thymoquinone impact negatively on the liver cells. Increases in the level of AST with the use of thymoquinone were also reported by previous researchers (17).

Abnormal levels of alkaline phosphatase in the blood could indicate problems relating to the liver, gall bladder, or bones. Surprisingly, in this study, there was a significant decrease in the ALP levels in the CYC and C10TQ groups and the levels of total protein showed no significant difference across the animals in the groups. The low level of ALP is suggestive of severe malnutrition which was observed in the CYC-treated group (18, 19).

Histopathological examinations of the liver revealed a distorted cellular layout in the CYC-treated groups. Cyclophosphamide led to damage to liver tissues showing the low density of hepatocytes, vacuolations, and scattered areas of inflammatory cell aggregates in between the hepatocytes. Also seen were dilated central veins and congested portal veins. The extent of distortion in the CYC+TQ treated groups was not as much.

Proliferating cell nuclear antigen (PCNA) is an antigen that is expressed in the nuclei of cells during the DNA synthesis phase of the cell cycle where it acts as a platform to recruit proteins involved in DNA replication, DNA repair, chromatin remodeling, and epigenetics (20). To quantitatively assess damage to the liver tissue, a cell count of PCNA expression in the liver was done. This revealed that the proliferating cell count in the CYC group was significantly reduced when compared to the other groups. Coadministration of CYC+TQ restored this drop in a dose-dependent fashion.

The beneficial effect of TQ was attributed to the strong antioxidant, anti-inflammatory, and antifibrotic effects (21). Cotreatment of thymoquinone leads to restoration of the normal architecture of hepatocytes with normal central nuclei. Regeneration of hepatocytes can also be observed by the presence of binucleate cells and minimal collagen fibers deposition around the central vein. This finding agrees with the study of Alenzi and his colleagues who studied the effects of TQ on hyperbilirubinemia and liver toxicity caused by cyclophosphamide (22). A treatment regimen with potential antioxidant agents could be an approach to ameliorate chemotherapeutic-induced hepatotoxicity (23).

CONCLUSION

The use of Thymoquinone, an extract of *Nigella Sativa* may reduce the morphological alterations and damage associated with Cyclophosphamide-induced toxicity in the liver. Therefore, the coadministration of cyclophosphamide and thymoquinone could offer clinical benefits in hepatoprotective and therapeutic interventions against damage that are associated with the

administration of cyclophosphamide.

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