ORIGINAL ARTICLE

CYCLOPHOSPHAMIDE RELATED HEPATOTOXICITY AND PROTECTION BY THYMOQUINONE, A HISTOLOGICAL VIEW

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ABSTRACT

Background: Cyclophosphamide (CP), used to cure cancer, causes hepatotoxicity. Hepatoprotection, brought by thymoquinone (TQ) found in kalonji. Study was performed to prove it.

Objectives: To observe thymoquinone restorative effect on the hepatic toxicity by cyclophosphamide.

Methods: It is Experimental randomized control trial. Adult albino rats were 24, put in 3 groups, each having 8. control Group A, given 2 ml Phosphate Buffer Saline (PBS) for a week by gastric gavage, 2 intraperitoneal injection (i.p) 2 days apart, gastric gavage for 1 week again. Experimental Group B, two i.p doses of CP as 200 mg/kg, on day 1, 4. Group C, given TQ (10 mg/kg/day) by gastric gavage for 1 week, 2 doses of CP, given on day 8 and 10, again TQ for 1 week. 24 hours after the experiment, livers were isolated from rats after dissection and processed. Gross and histopathological changes were observed.

Results: Weight of rats of both experimental groups (B, C) was decreased in comparison to A group (p-value <0.001), reduced from (190.4 g-136.9 g) for group B, (177 g-147g) for group C rats, weight increased in group A rats (184.9 g-223.1g). RTWI, more in both groups (4.38 in B and 3.55 in C) in comparison to control (2.59), B versus A (p-value <0.001), B versus C (p-value 0.012) and C versus A (p-value 0.004). Hepatic lobules shape difference was statistically significant in experimental groups when compared to the control (p-value <0.001).

Conclusion: Study proved hepatotoxic impact of CP on liver histology, shows ameliorative effect of TQ.

Keywords: Cyclophosphamide (CP), Thymoquinone (TQ), Phosphate Buffer Saline (PBS), Intra-peritoneal (i.p)

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INTRODUCTION

Cyclophosphamide is useful in systemic lupus erythematosus (SLE), adult and paediatric multiple sclerosis, is used clinically for chemotherapy and bone marrow transplantation.[1]

After break down of CP, phosphoramide mustard formation in cells with low level of aldehyde dehydrogenase (ALDH). Alkyl group is coupled to DNA bases, resulting in formation of tiny particles and DNA synthesis and RNA transcription is affected.[2]

Alkylating drugs harm nucleic material but its effect is accentuated by addition of PARP (poly ADP ribosome polymerase) inhibitors. Metronomic dosing, a new concept to use cytotoxic medicines, when CP is used for long term, as in advanced ovarian cancer, especially for patients where health status is deteriorated to tolerate toxicity. In 35 patients with solid tumors and lymphomas, a Phase I trial was demonstrated to record impact of metronomic CP with veliparib. CP 50 mg was given daily with maximum tolerated dose of veliparib 60mg, given orally once a day for 1 week, 14 days or 3 weeks. A phase II trial was established to compare CP alone with combination therapy CP/veliparib in mBRCA (breast cancer gene) patients with

primary peritoneal, fallopian tube or high-grade ovarian cancer. This comparative study showed no betterment in results by the combination of veliparib to metronomic CP.[3]

Kalonji is super herb of the century, known as N. sativa Linn, of family Ranunculaceae. It is splendid seasoning, can be obtained from local market.[4]

Molecular formula of TQ is $C_{10}H_{12}O_2$.[5] Chemical structure is 2-Isopropyl-5-methyl-1,4-benzoquinone.[6]

N. Sativa is indicated to use in headache and back pain, diabetes mellitus, renal stones and hypertension, infections, asthma and allergies.[7] NSO with active component TQ has anti-oxidative activity. During inflammation, it retards the release of 5-lipoxygenase related products.[8]

Blood brain barrier is crossed by TQ due to low molecular weight and lipid solubility. It is similar to coenzyme Q, important component of electron transport chain. After oral ingestion, its bioavailability is less, due to less water solubility and fractionation rate.[9]

A study proved that TQ assisted stoppage of G1 (cell cycle), stimulation of tumor suppressor p53 gene, caused cell necroptosis in breast cancer lines. In context of liver cancer, i.v TQ 10 mg/kg for one month and 5 doses in a week, it was reported that TQ has quality to reduce oxidative stress, by avoiding cell decline and accentuating regeneration in hepatic tissue.[10]

The study aims to prove amelioration by TQ on the liver toxicity by CP.

Study Rationale: To measure dose related cyclophosphamide toxicity, in liver of male albino rats, main side effect of chemotherapy. It is in common use for the cure of amyloidosis. Female rats have estrous cycle and due to release of stress hormones, study results can be affected, so not used.[11] To observe the hepatoprotective effect of thymoquinone or Kalonji, commonly used edible. So, the inference of research will be fruitful for mankind.

Objective: To observe thymoquinone restorative effect on the hepatic toxicity by cyclophosphamide.

METHODS

Place of experimental study was Department of Anatomy, Shaikh Zayed Postgraduate Medical Institute, Lahore. 8 male albino rats (2-3 months age), average weight 123-178 gm were allocated each in all 3 groups by lottery method. Animals were fed throughout the day at optimum temperature 26-30 ºC and 30%-70% relative humidity[12] according to season.

By using power and precision 3.0 software with effect size of 1.33 and error SD of 4.0, the size of sample was estimated as 4. The sample size is raised to 8 in each group, keeping in view the target histological parameter.

CP was dissolved in sterile water, given by i.p injection. TQ was in crystalline form, mixed with Dimethyl sulphoxide (DMSO) solution and dissolved in phosphate buffer saline (PBS) which is water-based solution and given to rats.

Group A (Healthy Control): It received PBS by gastric gavage for a week in dose of 10 ml/kg b.w/day, then 2 injections of same reagent, 1st on day 8 and 2nd on day 11, followed by gastric gavage for a week in a single dose/day for total eighteen days. On day eighteen, animals were sacrificed.

Group B (Experimental): It received CP (i.p) by total two injections. 200 mg/kg/injection as a first dose on day one and then on day fourth. On fifth day, animals were sacrificed.

Group C (Experimental): It received TQ through gastric gavage and the dose was 10mg/kg for a week, two injections of CP as 200 mg/kg/injection two days apart (on day 8th and 11th day).[13] 6 hours after this TQ, at a dose of 10mg/kg was given for a week.[14] Animals were sacrificed at 18th day.

Liver was dissected out by giving midline incision to rat,[15] weighed and 10% formaldehyde solution was used to fix it. 5µm section was cut and for staining, Haematoxylin and Eosin (H & E) was used for detailed histological study.[16]

Statistical Analysis: Statistical Package for Social Sciences (SPSS) version 28 was used for study data to be entered, tabulated, compared and analyzed. Mean of observations and standard deviation (SD) for the values were being calculated. By using mean, ± standard deviation, data for all quantitative variables like body weight, liver weight, relative tissue weight index (RTWI) was presented and comparison among groups by using one way analysis of variance (ANOVA). Tukey's test was used for post-hoc analysis. Qualitative variable, shape of hepatic lobule was described by using frequency and percentage of each group. Comparison among groups was made by using Chi-square test. P-value ≤0.05 was considered statistically significant.

Body weight of the Rats (g): At the start of experiment, the mean body weight of rats recorded was 184.9 ± 13.9 g, 190.4 ± 14.7 g, 177 ± 11.1 g for all 3 groups control A, experimental groups B and C. Difference among 3 groups was insignificant statistically with p- value 0.156 (Table 1). The mean weight of rats was 223.1 ± 19.1 g, 136.9 ± 28.0 g, 147.9 ± 18.5 g for groups A, B and C, at the end of experiment. The difference was statistically significant with p-value <0.001 (table 1), among all three groups.

Table 1: Body weight of rats (g) at start and end of experiment among control and experimental groups

Group	Body weight of rat before experiment		Body weight of rat after experiment	
	Mean ± SD	p-value	Mean ± SD	p-value
Group A	184.9 ± 13.9 (day 1)		223.1 ±19.1 (day 18)	
Group B	190.4 ± 14.7 (day 1)	0.156	136.9 ± 28.0 (day 5)	< 0.001 *
Group C	177.0 ± 11.1 (day 1)		147.9 ± 18.5 (day 18)	

A Control groups, **B** Experimental group
C Experimental group
***** Significant difference (p value < 0.05) Post hoc

Weight of Liver of Rats (g): The mean liver weight was 5.79 ± 0.86 g for control group A, and for group B and C were 5.88 ± 0.91 g and 5.31 ± 1.19 g respectively. Liver

weight difference among 3 groups was statistically insignificant with p-value 0.493. One way ANOVA test was applied to compare the liver weight among groups.

Relative tissue weight index: When relative tissue weight index was measured, for liver, it appeared that control group was having tissue weight index of 2.59 ± 0.40, while group B had 4.38 ± 0.64 and group C was having 3.55 ± 0.49. Difference among all 3 groups was significant statistically with p-value <0.001(table 2).

Table 2: Relative tissue weight index of liver in control and experimental groups

	Relative Tissue Weight Index	
Group	Mean ± SD	p-value
Group A	2.59 ± 0.40	
Group B	4.38 ± 0.64	< 0.001 *
Group C	3.55 ± 0.49	

Key
A Control groups, **B** Experimental group,
C Experimental group
*Significant difference (p value < 0.05)

Shape of Hepatic Lobule: Hepatic lobule shape is hexagonal, centrilobular vein has central position and portal triad occupying periphery of hexagon. In the portal triad, hepatic portal vein, terminal smaller hepatic artery branches and bile duct are present. When there is comparison among three groups in relation to shape of hepatic lobule, all 8 (100%) of control group A were hexagonal which is normal shape (fig. 1) and all 8 (100%) rats of both experimental group B (abnormal) (fig. 2) and C (oversized) had distorted lobules (fig. 3) and difference proved significant statistically with p-value 0.001 (table 3).

Two experimental groups B and C were compared with control and proved that both were having difference statistically significant with p-value <0.001. When comparison was performed between two experimental groups B and C, no statistically significant difference was found with p-value >0.999 (table 3)

Table 3: Multiple comparisons for shape of hepatic lobules of rats among control and experimental groups

Group		p-value
	Group B	< 0.001 *
Group A	Group C	< 0.001 *
Group B	Group C	> 0.999

* Significant difference (p value < 0.001)

DISCUSSION

Cyclophosphamide (CP) is used directly or via its active ingredient (phosphoramide mustard) to treat multiple cancers because it is hyroxylating cytotoxic drug.[17] Nigella Sativa has immunoregulatory and biotherapeutic properties in encephalomyelitis and colitis, peritonitis, arthritis and oedema.[7]

Figure 1: Microscopic structure of liver of adult albino rat of control Group A showing a classical hepatic lobule, having Centrilobular Vein (CV) occupying center and Portal Triad (PT) at the edges and Hepatic Capsule (CAP). (H & E, 4x).

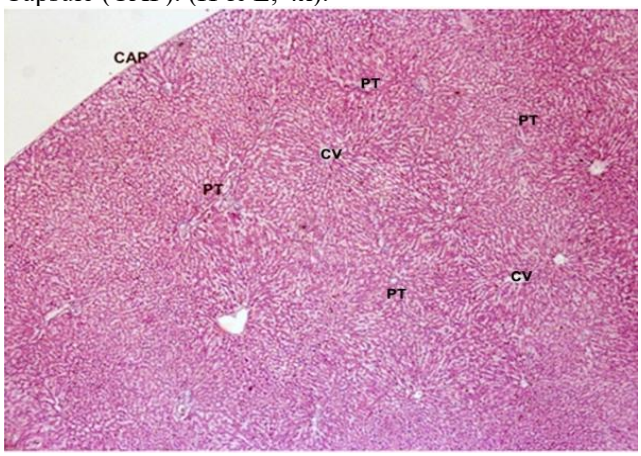


Figure 2: Microscopic structure of liver of adult albino rat of experimental Group B showing a distorted hepatic lobule, having congestion in Centrilobular Vein (CV) and portal vein in Portal Triad (PT). Hepatic Capsule (CAP). (H & E, 4x).

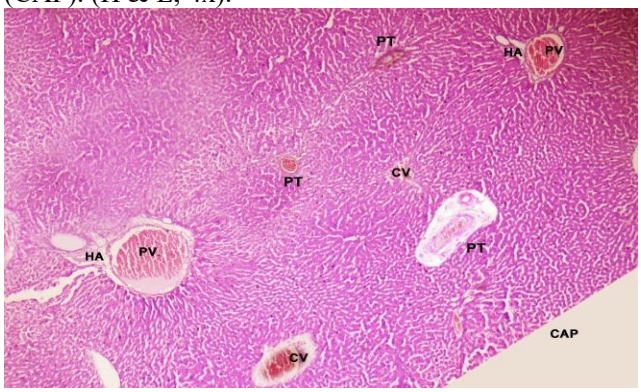
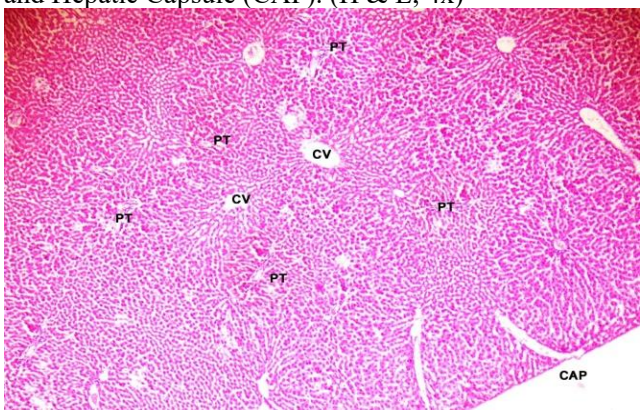


Figure 3: Microscopic structure of liver of adult albino rat of experimental Group C showing a normal looking Hepatic lobule, having Centrilobular Vein (CV) occupying the center and Portal Triad (PT) at the edges and Hepatic Capsule (CAP). (H & E, 4x)



Weight of control group A was more because animals gained weight on routine diet. There was significant decline in mean body weight of rats in group B and it also reduced in group C. Comparison among all three groups was significant with p-value <0.001. Rats in group C, which were treated with CP and TQ, gained weight as compared to group B (p-value 0.592). Weight loss in group B was due to effects of CP like loss of appetite leading to anemia and neutropenia[18] but not documented. Although TQ was given in C group and it improves GIT, immune and defense system of body,[4] but gain in body weight was not significant because given for relatively shorter time period of 2 weeks. This result follows data by Alenzi who observed much loss of weight of CP treated wistar rats via i.p injection as 2 doses of 200 mg/kg on day 1 and 4 and slight decline in rat body weight on TQ, CP plus TQ for 12 days in dose of 10 mg/kg.[13] A study, followed present research, CP treated rat with i.p 200 mg/kg drug showed loss of body weight due to decrease in muscle mass.[19] In a study on female Wistar rats, a treatment group received i.p 0.5 mg/kg CP for 4 weeks, other three treatment groups received 0.5 mg/kg CP with p.o NSO 200 mg/kg, 400 mg/kg, and 800 mg/kg for 21 days. Results showed significant increase in body weights among all groups. Reason is that NSO did not reduce appetite of animals.[20]

Mean liver weight of albino rats in recent study revealed liver weight was 5.79 g in control group, 5.88 g in group B and 5.31 g in group C. Gain in liver weight in group B animals is due to oedema fluid collection in tissue and less weight in group C animals tissue is due to healing process by adding TQ to animal diet. Insignificant difference was found among all three groups. (p-value 0.493).[21,22]

Another research coincides that liver weight gain in rats by using single i.p dose of CP 200 mg/kg was due to inflammatory response in body.[19] A study opposed that weight was reduced by p.o CP in dose of 20 mg/kg in wistar rats and mild rise in tissue weight in group on CP + TQ in dose of 10 mg/kg. This is due to ROS produced by CP use and liver enzymes function were improved by using TQ.[21] In a 21 days study in female Wistar rats, 1st treatment group was given i.p CP 0.5 mg/kg, other 3 groups received CP+ p.o NSO in 200mg, 400mg, 800 mg/kg. Results showed significant increase in organ weights across all groups. Both the control and all treatment groups showed increase in mean organ weight. Reason shows organ toxicity at higher doses of NSO.[20]

RTWI was higher in both groups B and C in comparison to control group. Analysis revealed in group B, more because of liver congestion, liver weight was more but body weight was declined due to CP caused loss of appetite and anemia when compared to control group A (p-value <0.01). In group C, it was less because liver weight was comparatively less, might be due to healing induced by TQ but body weight was improved due to better digestion in animals[23] than that of group B but was more than the control group A (table 2). Collateral use of TQ in CP treated albino rats in group C resulted in mild rise in body weight but statistically significantly less than control group A. Similarly, in group C, it is significant when compared with other two groups. In recent work, there was least change in liver weight after experiment. These results counter a study that showed decrease in relative weight of liver after CP use and increase by oral TQ 10 mg/kg in a 3 week experiment. TQ was given after p.o CP oral at 20 mg/kg. Reason is CP caused decline in liver and animal weight both, but TQ caused development in both parameters, so relative weight was increased.[21]

Another study followed that relative hepatic weight was increased in CP male rats as single injection of 200 mg/kg due to peroxidative mechanism.[19] In a study, a treatment group was given i.p CP 0.5 mg/kg to female Wistar rats, other three groups were given NSO p.o 200 mg, 400 mg, 800 mg/kg in a month study. No significant changes in relative tissue weights, in all groups. Result differs, reason being both the animal and liver weight was raised in all treatment groups.[20]

In present research, hepatic lobule shape was compared among all 3 groups. Difference between control group A (hexagonal) and experimental groups B (distorted) given CP, was statistically significant with p-value <0.001 (table 3). Group C animals were compared with that of control group A, and difference was significant statistically with p-value <0.001. Both groups B and C were compared, and insignificant difference was found (p-value >0.999, table 3). So, CP caused deterioration of liver lobules shape. CP and TQ both were given in experimental group C and TQ caused healing but was just in process due to short period of time and lobules remained just enlarged.

CONCLUSION

Thus, hypothesis is proved that a dose of 200 mg/kg/day of cyclophosphamide by intra-peritoneal injection induced hepatic toxicity in male albino rats and oro–gastric injection of thymoquinone in 10 mg/kg/day dose protected the liver of animals on morphological and histological basis.

Limitation Of Study: The study duration was short due to time restriction. Animal based studies and models had constraints about portability, monotony and credibility of researching data to general human illness.

Future Recommendations: Cyclophosphamide has extensive noxious effects on tumor and normal cells both. It is need of time to invent cancerous cells specific new compounds. As compared to normal cells, TQ has selective specific action against human glioblastoma cells. Further research is required to explore capacity of TQ to treat human diseases as acid peptic disease, AIDS and cancer. Incomplete data is available about effect on macrophages, APCs, dendritic cells and modulatory impact on Th1 and Th2 related immune inflammatory processes.

Bioavailability is still under research and proper study is lacking in this regard. Its use for site specific selective cancers will be given preference, whenever a consensus

appears. Laboratory is focusing to understand its mechanism of action on molecular basis to be able to design analogs with more benefits and least side effects. Based on new research methodologies, coming years will show us astonishing facts into the therapeutic indications of the TQ.

ETHICAL APPROVAL

Ethical approval of article was granted by the Institutional Review Board of Federal Postgraduate Medical Institute, Shaikh Zayed Hospital, Lahore IRB No 1433, Dated: September 21, 2016.

AUTHOR'S CONTRIBUTIONS

SL: Conceived idea, manuscript writing, data collection
SI: Manuscript writing, review of literature
FS: Manuscript writing,
YB: Review of literature, proof reading
LS: Manuscript writing, revision
MS: Critical review, data analysis & interpretation
All Authors: Approval of the final version of the manuscript to be published

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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