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DAN damage analysis in liver of male albino rats treated with different doses of cyclophosphamide

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Abstract: *Cyclophosphamide is a synthetic alkylating agent chemically related to the nitrogen mustards widely used as an anticancer and immunosuppressive drug.This study was to investigate the damage in the DNA liver of male albino rats treated with low and high dose of cyclophosphamide.Use for this purpose 30 of the strain Sprague Dawley albino rats and divided into three groups, each group consisted of 10 rats; Group 1 (control) was injected with physiological solution (normal saline 0.9%), Group 2 (low dose) received 50mg/kg/b.w/weekly of drug and Group 2 (high dose) received 80mg/kg/b.w/weekly of the drug for 10 weeks.At the end of dosage, duration, the rats were dissected and the liver was excised and washed with normal saline.DNA damage analysis by using comet assay according comet assay kit. This result of comet assay showed that damage in DNA of liver cells at 10 weeks of treatment with cyclophosphamide high and low dose in four parameters comet high, comet medium, comet low and no damage. With low and high dose groups increase in comet high, comet medium percentage, decrease in comet low, and no damage percentage. The results of the present study indicated that high and low dose of Cyclophosphamide able to induce DNA damage in the liver of male albino rats.*

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I.Introduction

Cyclophosphamide (CP) popularly known as Cytoxan or Endoxan is an extensively used drug to treat a wide range of various neoplastic diseases such as Hodgkin's disease, non-Hodgkin's lymphoma, many types of leukemia, multiple myeloma, retinoblastoma, neuroblastomas, carcinomas of the ovary, breast, and endometrium, and certain malignant neoplasms of the lung.[1, 2, 3] Cyclophosphamide, which is a non-active cyclic phosphamide ester of mechlorethamine causes cross-linking of DNA strand which leads to an increase in inhibition of DNA polymerase activity and thus prevents cell division. Although CP has proved to be a promising and effective chemotherapeutic agent, the International Agency for Research on Cancer (IARC), 1991, designated it as carcinogenic to humans. [1, 2, 3]Cyclophosphamide has been reported to produce genotoxicity and oxidative stress in mice [4] and early lung injury in rats.[5] Numerous studies have shown that CP exposure can disrupt the redox balance of tissues and that these biochemical and physiological disturbances resulted from oxidative stress may be implicated in disorders like hemorrhagic cystitis, testicular gametogenic and androgenic disorders, liver and kidney disorders, inhibition of ovarian steroidogenesis, etc.[6–10]The aim of the present study was to investigate the damage in the liver DNA of male albino rats treated with different doses of cyclophosphamide.

II. Material and methods

Preparation of cyclophosphamide drug

Cyclophosphamide (Endoxan) produced by (Baxter international company U.S), was used by administering 50 mg/kg of body weight/week as low dose and 80mg/kg of body weight/week as high dose. It injected with animal intravenously for 10 weeks.

Experimental Animal

The experimental animals used in this study were male albino rats, Rattus norvegicus weighing (225 – 250 g), and at the age of (14 – 16) weeks. The animals were purchased from Pharmaceutical control of the Ministry of Health in Baghdad. Animals were given food and water ad libitum. Rats were maintained in a friendly environment with a 12 h/12 h light-dark cycle at room temperature (22 °C – 25 °C). Rats were acclimatized to laboratory conditions for 7 days before commencement of the experiment.[11]

Experimental Design

On present investigation, 30 male rats divided into three groups, each group consisted of 10 rats as follows:

- **Group I (control)**Injected orally with normal saline.
- **Group II (low dose)** injected intravenously with CP(50mg/kg/b.w) one time/week for 10 weeks.

- **Group III (high dose**) injected intravenously with CP (80mg/kg/b.w) one time/week for 10 weeks.

Collection of organ

The male albino rats were fully anesthetized by diethyl ether for several minutes. The rats were dissected and the liver was excised and washed with normal saline (0.9 % NaCl).

Comet assay

Comet assay in order to determine DNA damage. The Oxiselect comet assay kit was used to perform the test.[12]

Tissue Preparation

Blood rich organs (liver), chop tissue into large pieces (1-2 mm), let settle for 5 minutes then aspirate and discard medium. Add 1-2 ml of ice cold 20 mM EDTA in 1X PBS (Ca++ and Mg++ free), mince the tissue into very small pieces and let stand for 5 minutes. Recover the cell suspension, avoiding transfer of debris. Count cells, pellet, and resuspend at $1x10^5$ cells/ml in ice-cold 1x PBS (Ca++ and Mg++ free).

Assay Protocol

The electrophoresis conditions used will determine the sensitivity of the assay. Neutral Comet Assaywill detect double-stranded DNA breaks, whereas Alkaline Comet Assaywill detect single and double-stranded DNA breaks, and the majority of a basic site as well as alkali labile DNA adducts (*e.g.*phosphoglycols, phosphotriesters).

Alkaline Comet Assay

1. Lysis Solution was prepared and cooled at 4°C for at least 20 minutes before use.

2. LM Agarose was melted in a beaker of boiling water for 5 minutes, with the cap loosened. The bottle was placed in the 37°C water bath for at least 20 minutes to cool.

3. Cells were combined at 1 x 10^5/mL with molten LM Agarose (at 37°C) at a ratio of 1:10 (v/v) and immediately pipetted 50 µl onto Comet Slide. If necessary, side of pipette tip was used to spread agarose/cells over the sample area to ensure complete coverage of the sample area. If the sample is not spreading evenly the slide was warmed at 37°C before application.

4. Slides flat was placed at 4°C in the dark (*e.g.* Place in refrigerator) for 10 minutes. A 0.5 mm clear ring appears on edge of the Comet Slide area. Increasing gelling time to 30 minutes improves adherence of samples in high humidity environments.

5. Slides were immersed in 4°C Lysis Solution for 30-60 minutes. For added sensitivity or convenience it should be incubated overnight at 4°C.

6. Excess buffer was drained from slides and immersed in freshly prepared Alkaline Unwinding Solution, pH>13. Gloves should be worn when preparing or handling this solution.

7. Comet Slide was immersed in Alkaline Unwinding Solution for 20 minutes at room temperature or 1 hour at 4°C, in the dark.

8. For the Comet Assay electrophoresis(ES-unit), ~850 mL 4°C Alkaline Electrophoresis Solution was added, slides were placed in an electrophoresis slide tray (slide labeled adjacent to black cathode) and covered with Slide Tray Overlay. The power supply was setted to 21 volts and voltage was applied for 30 minutes.

9. The excess electrophoresis solution was drained, gently immersed twice in dH_2O for 5 minutes each, then in 70% ethanol for 5 minutes.

10. Samples were dried at 37°C for 10-15 minutes. Drying brings all the cells in a single plane to facilitate observation. Samples must be stored at room temperature, with desiccant prior to scoring at this stage.

11. 100 µl of diluted SYBR Green was placed onto each circle of dried agarose and stained 30 minutes (room temperature) in the dark. The slide was gently tapped to remove excess SYBR solution and rinsed briefly in water. Slides were dried completely at 37°C.

12. Slides were viewed by fluorescence microscopy. (SYBR Green's maximum excitation/emission is 496 nm/522 nm. Fluorescein filter is adequate).

Fifty randomly selected cells were counted per sample to quantify the comet cell. The scored was calculated from the ratio of (L/W) comet to determine the Comet Index (CI). Scored range from 1.2 to 2 considered low DNA damage (LD), from 2.1 to 3 medium DNA damage (MD), and up to 3 high DNA damage (HD).[13]

Statistical Analysis

The Statistical Analysis System-SAS (2012) program was used to determine the effect of different factors in study parameters. All the values were statistically analyzed by analysis of variance (ANOVA) -Least significant difference (LSD) test. Data are expressed as the mean ± SD. $P < 0.05$ was considered as significant difference.

III. Result

Comet Assay parameters The results of the present study in thetable (1) showed that damage in DNA of liver cells at 10 weeks of treatment with cyclophosphamide high and low dose in four parameters comet high, comet medium, comet low and no damage.

Comet high percentage

The results in (table 1) show a significant ($P < 0.05$) increase in comet high of cyclophosphamide low dose group (50 mg/kg b.w) was (19.267±2.104) %, and CP high dose group (80 mg/kg b.w.) was (30.100±2.524) % compared with the control group was (7.673±1.134) %. (Figure 1 C).

Comet medium percentage

The results in (Table 1) show a significant ($P < 0.05$) increase in the comet medium of cyclophosphamide low dose group (50 mg/kg b.w) was (50.333±8.501) %, and CP high dose group (80 mg/kg b.w.) was (78.000±6.578) %, compared with the control group was (21.333±3.615) %. (Figure 1 B).

Comet low percentage

The results in (Table 1) show a significant ($P < 0.05$) decrease in comet low of cyclophosphamide low dose group (50 mg/kg b.w) was (24.860±1.786) %, and CP high dose group (80 mg/kg b.w.) was (17.380±1.664) %, compared with the control group was (36.610±1.198) %. (Figure 1. A2).

No damage percentage

The results in (Table 1) show a significant ($P < 0.05$) decrease in no damage of cyclophosphamide low dose group (50 mg/kg b.w) was (26.130±2.024) %, and CP high dose group (80 mg/kg b.w.) was (18.827±1.407) %, compared with the control group was (42.063±3.553) %. (Figure 1. A1).

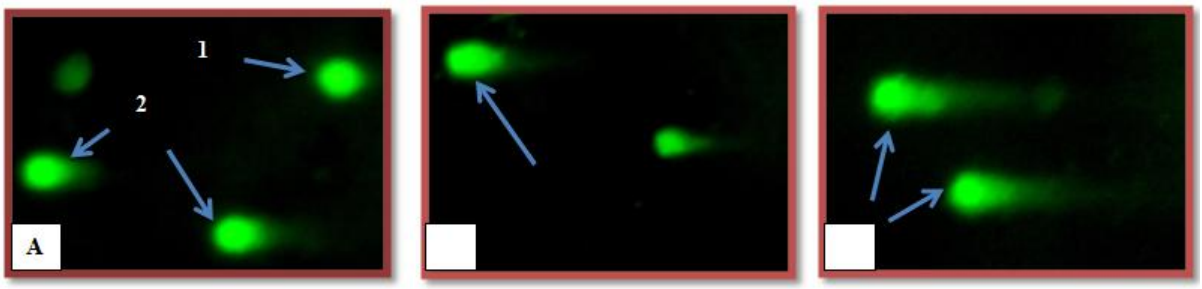

Figure (1): Threeexamples of scoring categories for the comet assay (A1: No damage, A2: Comet low; B: Comet medium; C: Comet high in liver cell of male albino rats.

Table (1): Eeffect of Cyclophosphamide on DNA of Liver.

Groups (Treatments)	Mean±SD (stander deviation)			
	Comet high %	Comet medium %	Comet low %	No damage %
Control	7.673±1.134	21.333±3.615	36.610±1.198	42.063±3.553
Low dose (50mg/kg)	19.267±2.104	50.333±8.501	24.860±1.786	26.130±2.024
High dose (80mg/kg)	30.100±2.524	78.000±6.578	17.380±1.664	18.827±1.407
LSD	3.26*	4.31*	5.06*	4.09*
* ($p<0.05$)				

IV. Discussion

Cyclophosphamide isaneffectivechemotherapeuticdrugwidelyusedasanimmunosuppressantinthetreatment of various human cancers and rheumatoid arthritis as well [14]; however, its serious and evident toxicity on target organs and common cells is worrisome.[15] Thus, there is urgency for an efficacious chemopreventive agent or food supplement which can decrease the toxicity of CP therapy. The purpose of the present study was to find the damage in the DNA liver of male albino rats.

The results presented here show DNA damage analysis in liver of male albinorats treated with low and high doses of cyclophosphamide intravenously for 10 weeks by using the comet assay.In a low dose group show significantly increase in comet high percentage (high damage) and the comet medium percentage (medium damage) and show significantly decrease in comet low percentage (low damage) and no damage percentage in the DNA of the liver compared with the control group.In the high dose group show, significantly increase in comet high and medium percentage, and show significantly decrease in comet low and no damage percentage in the DNA of the liver compared with low, and control group.

This results show as DNA damage of liver with high dose of Cp more than low dose of Cp.Cyclophosphamide metabolically activated with the help of hepatic mixed-function oxidases. It is an alkylating agent and shows its cytostatic effects by forming covalent DNA adducts.[16]Cyclophosphamide cytotoxicity is mediated by alkylation of DNA at the N7 position of guanine and by the formation of DNA–DNA cross-links, DNA–protein

cross-links, and single-strand breaks, and double-stranded breaks.[17]Many in vivo studies have been conducted to test the genotoxicity produced by Cyclophosphamide. It was clearly indicated that genotoxicity may increase with repeated dosage or chronic administration of CP.[18]

Cyclophosphamide oxidative stress plays a part in many pathological cycles in the body, excessive amounts of free radicals arising in these processes are counterbalanced by the antioxidant system; if this balance is not maintained, and tissue injury occurs.[19]It was reported that these radicals lead to peroxidation and modification by oxidizing carbohydrates, lipids, proteins and DNA in the cell, exerting a quite a toxic effect.[20]

Cyclophosphamide, which is a non-active cyclic phosphamide ester of mechlorethamine causes cross-linking of DNA strands which leads to an increase in inhibition of DNA polymerase activity and thus prevents cell division. Cytotoxic impacts result from the responsive metabolites that alkylate DNA and form an assortment of DNA adducts that adequately modify DNA structure or capacity, prompting to the arrangement of chromosomal deviations and micronuclei development. [21]

V. Conclusion

The results of the present study indicated that high and low dose of Cyclophosphamide able to induce DNA damage in the liver of male albino rats. High dose of Cp (80mg/kg)should be not used because ofits more negative effects on the liver DNA compared with low dose of Cp (50mg/kg).

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