

## RESEARCH ARTICLE

# Effect of Ifosfamide on corneal genetic material and the role of lecithin and quercetin

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### ABSTRACT

The present study aimed to investigate the possible roles of lecithin and quercetin either singly or combined against Ifosfamide-induced DNA damage in corneas. Healthy female *Rattus norvegicus* rats weighing  $180 \pm 10$  g were divided into seven groups each comprised of ten rats. Group I served as control and intraperitoneally injected with 0.25 ml of physiological saline (0.9% NaCl) for 5 days. Groups II and III were orally administered lecithin at a dose of 100 mg/kg body weight or quercetin at a dose of 50 mg/kg body weight suspended in distilled water, respectively for six days; whereas group IV was used as positive control and intraperitoneally injected with Ifosfamide at a dose of 80mg/kg body weight for five days. Groups V and VI were injected with either quercetin or lecithin along with Ifosfamide by the same treatment regimens, respectively. Group VII received a combination of both protective agents along with Ifosfamide and treated similarly. Corneas from all groups were used for comet assay.

**Results:** The lecithin and quercetin treated groups were characterized by normal comet parameters compared to control. After Ifosfamide injection, all comet parameters were significantly increased ( $p < 0.05$ ). No changes in comet parameters were observed for lecithin-Ifosfamide treated group as well as for quercetin-Ifosfamide one. Co-administration of lecithin and quercetin indicate significant increase ( $p < 0.05$ ) in all comet parameters (group VII). All together led to conclude that Ifosfamide causes DNA damage of rat cornea due to its oxidative stress and, co-administration of lecithin and quercetin was contradicting each other while using these antioxidants separately has beneficial effects on corneal genetic material.

**Key Words:** Ifosfamide, cornea, lecithin, quercetin, comet assay, DNA

### Introduction

Systemic drug-induced ocular side effects are increasing because of the vast numbers of new drugs being introduced. Reports of drug-induced ocular toxicity must be well documented, and the other causes of these side effects ruled out to help establish causality. Systemic anticancer therapies can produce acute and chronic organ damage, but the eye is usually considered a protected site<sup>1</sup>. Nonetheless, the oculo-visual system has a potentially high

degree of sensitivity to toxic substances. Ocular toxicity induced by cancer chemotherapy includes a broad spectrum of disorders, reflecting the unique anatomical, physiological and biochemical features of the eye. Understanding the ocular side effects will assist the ophthalmologist and oncologist to recognize them early and intervene before blindness occurs.

Ifosfamide (Ifo) is an alkylating agents that directly damage DNA and prevent cancer cell from being

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reproduced. It is mainly metabolized through CYP 3A4 and CYP 2B6 enzymes. Ifosfamide is used in the treatment of a variety of solid tumors including those of the cervix, endometrium, lung, ovary, testes and thymus as well as in sarcoma and in the treatment of Burkitt's lymphoma<sup>2</sup>.

Lecithins (Lec) are usually phospholipids, composed of phosphoric acid with choline, glycerol acid or other fatty acids usually glycolipids or triglycerides. Glycerophospholipids in lecithin include phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and phosphatidic acid<sup>3</sup>.

Quercetin (Que) is a flavonol, a plant derived flavonoid used as a nutritional supplement found in fruits and vegetables. Quercetin is thought to have potent antioxidant, antidiabetic, anti-tumor, antiviral, and anti-inflammatory benefits<sup>4</sup>.

The present study is aiming to investigate the possible protective roles of Lec and Que either singly or combined against Ifo-induced DNA damage in corneas of female rats.

## Materials and methods

### Chemicals

All chemicals utilized were obtained from Sigma Company (St. Louis, MO, USA) with the highest purity commercially available.

### Animals

Healthy seventy female *Rattus norvegicus* rats weighing 180±10g were divided into seven groups, each containing ten rats. The first group named group I served as control, and received 0.9% NaCl vehicle; 0.25ml intraperitoneally for 5 days. Groups II and III were orally administered Lec at a dose of 100 mg/kg bwt<sup>5</sup> or Que at a dose of 50 mg/kg bwt<sup>6</sup> suspended in distilled water, respectively for six days. Group IV was used as positive control and injected with Ifo at a dose of 80 mg/kg bwt<sup>7</sup> intraperitoneally for five days. Groups V and VI were administered either Que or Lec along with Ifo by the same treatments regimens, respectively. Group VII received a combination of both protective agents along with Ifo and treated similarly.

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## Comet assay procedure

### Preparation of slides

Crushed corneas were transferred to 1ml ice-cold PBS (phosphate buffer saline, pH 7.9). This suspension was stirred for 5 min and filtered. Cell suspension (100µl) was combined with 600µl of low melting agarose (0.8% in PBS), where 100µl of this mixture was spread on the slides. The coated slides were immersed in lyses buffer (0.045 M TBE, Tris borate EDTA pH 8.4, containing 2.5% SDS) for 15 min.

### Electrophoresis of Slides

Slides were positioned on the horizontal gel box and were completely enclosed with fresh electrophoresis buffer (pH>13) for 20 min to unwind of DNA and the expression of alkali-labile damage. The power supply was turn on to 1 volt/cm and adjusts the current to 100 m amperes for 25 minutes then gently lift the slides from the buffer and were coated with neutralization buffer for at least 5 minutes. Slides were then stained with 80 µL of 1X Ethidium Bromide (EtBr), leaved for 5 min and then dipped in distilled water to remove excess stain. Finally, drain process was done by keeping the slides for 20 min in cold 100% ethanol and place them in an oven at 500°C for 30 min and repeat staining with EtBr.

### Evaluation of DNA Damage

For visualization of DNA damage, observations are made of EtBr-stained DNA using a 40x objective on a fluorescent microscope. A Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK) linked to a charge-coupled device (CCD) camera were used to assess the extent of DNA damage in the cells through measuring the length of DNA migration and the percentage of migrated DNA. Finally, the program calculates tail moment<sup>8</sup>.

### Statistical analysis

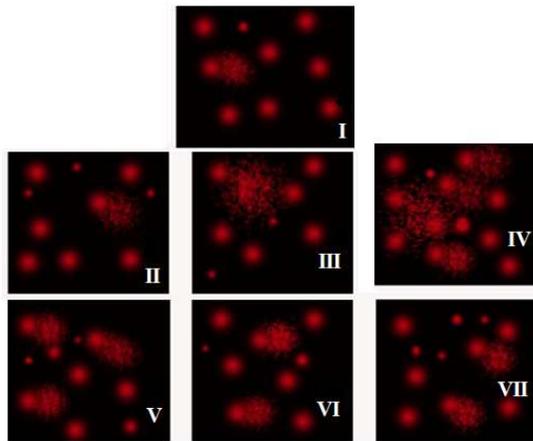
Results were displayed as the mean ±SD. In order to get a comparison between groups, investigation of fluctuation (ANOVA) was done by using commercially available software program (SPSS-11 for windows, SPSS Inc., Chicago, IL, USA), where the significance level was set at p<0.05.

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## Results

Figure (1) represents comet assay images stained with ethidium bromide for all studied groups denoted by numbers I to VII and are discernible for control, Lec, Que, Ifo, Lec-Ifo, Que-Ifo and Lec+Que-Ifo, respectively. At the molecular level, the formation of “comets” in the DNA of cells upon genotoxic insult can be visualized through the method of gel electrophoresis and indicates DNA strand breaks, as the damaged DNA migrates at a different rate than non-damaged DNA during electrophoresis. In the comet assay, when a damaged DNA-containing single cell suspension embedded in low melting agarose is electrophoresed, the damaged DNA migrates away from the undamaged DNA-containing nucleoid body, resembling the structure of a comet, hence the name comet assay. In the comet structure, the undamaged DNA nucleoid part is referred to as the “head” and the trailing damaged DNA streak is referred to as the “tail”.



**Figure (1):** Comet assay images of corneal DNA from all groups. I: the control group, II: lecithin group, III: quercetin group, IV: ifosfamide group, V: lecithin-ifosfamide group, VI: quercetin- ifosfamide group and VII: lecithin+quercetin - ifosfamide group.

The percentage of DNA in the tail is directly proportional to the percentage of DNA damage that has occurred in a particular cell. Thus, by counting a representative sample of ~100–300 cells per tissue it is possible to get the average percentage of DNA damage accumulated in a particular tissue due to genotoxic stress.

The Tail Moment has been suggested to be an appropriate index of induced DNA damage in considering both the migration of the genetic material as well as the relative amount of DNA in the tail. Percentage tail DNA calculated by the equation  $\text{Tail DNA}\% = 100 \times \text{Tail DNA}$

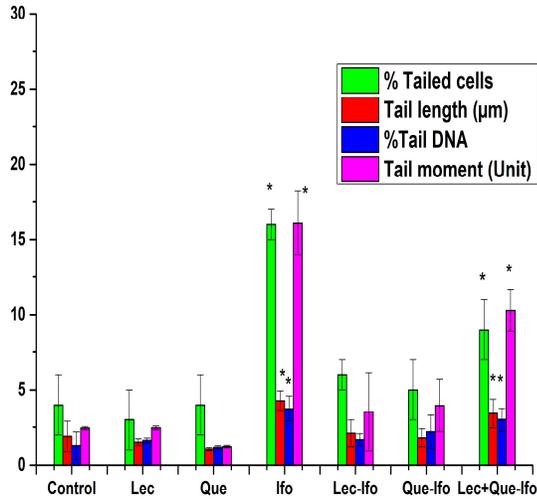
Intensity/Cell then DNA tail moment can be measured using the following methods:  $\text{Extent Tail Moment} = \text{Tail DNA}\% \times \text{Length of Tail}$ . Analysis of comet images were summarized in figure (2) to reflect the comet parameters resulted from Komet program which are percentage tail cells, tail length in  $\mu\text{m}$ , percentage tail DNA and tail moment. The values of these parameters for control group were  $4 \pm 2\%$ ,  $1.89 \pm 1.00 \mu\text{m}$ ,  $1.29 \pm 0.9 \%$  and  $2.44 \pm 0.1$  units, respectively as shown in table (1). Lec and Que groups indicate a normal comet parameters compared to control. After Ifo injection, all comet parameters were significantly increased ( $p < 0.05$ ). No changes in comet parameters were observed for Lec-Ifo group and Que-Ifo group. Treatments with both lecithin and quercetin (Lec+Que-Ifo) indicate significant increased ( $p < 0.05$ ) in all comet parameters (group VII).

## Discussion

Ocular toxicities are a common side-effect of systemic chemotherapeutic drugs<sup>9</sup> and have also emerged as an important clinical concern for newer molecularly-targeted agents entering standard oncology practice<sup>10</sup>.

Ifosfamide has a broad spectrum of activity against tumors in both animals and humans, and appears to be particularly active against testicular cancer and sarcomas. Like cyclophosphamide, ifosfamide has been reported to cause reversible blurred vision and conjunctivitis<sup>9</sup>.

Phospholipids are constituents of all cell membranes and are present in food from plant and animal sources. Soy lecithin is a mixture of naturally occurring phospholipids, phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol, and is considered as an excellent source of choline (essential nutrient) for nutritional supplement<sup>11-12</sup>. Antioxidants with flavonoid functionality -as quercetin- are low-molecular weight polyphenolics which occur in a variety of vegetables and fruits<sup>13</sup>. The antioxidant properties of flavonoids are responsible for the protective effect disease<sup>14</sup>.



**Figure (2):** Histograms pattern of % tailed cells, tail length, % tail DNA and tail moment for all studied groups.

Single cell gel electrophoresis, or the comet assay, was considered as a sensitive technique for detecting DNA strand breaks, at the level of individual cell<sup>15</sup>. Tail moment calculated from comet image is an indicator to DNA damage and the severity of damage. Degree of DNA damage happened in control group explained that about 10,000 oxidation hits to DNA per cell have been expected to occur per day in the human body, and more than 35 forms of oxidized bases are found in DNA. Effective DNA-repair enzymes can repair that damage, but some damage escapes repair, leading to stable damage. The significant increase ( $p < 0.05$ ) of tail moment, tail length, % tailed DNA and % tailed cells in cornea of rats injected with Ifo reflect the damage in cornea and this may be due to the oxidative stress resulting from injection of Ifo. During cancer chemotherapy treatment, drug-induced oxidative stress can limit therapeutic efficiency and cause a number of side effects. Excess reactive oxygen (ROS) and nitrogen species (RNS) lead to oxidative stress and oxidation of cellular structure especially membrane lipids and proteins. Also they lead to mutation of mitochondria and damage to DNA<sup>16</sup>.

**Table (1):** Percentage of tailed cell, tail length, tail DNA and tail moment of all studied groups compared to control group.

\*statistical significant ( $p < 0.05$ )

Sample	Tailed %	Tail length	Tail DNA %	Tail Moment
		µm	%	Unit
Group I Control	4±2	1.89±1.0	1.29± 0.9	2.44±0.10
Group II Lec	3±2	1.51±0.21	1.62±0.16	2.45±0.13
Group III Que	4±2	1.05±0.10	1.16±0.1	1.218±0.09
Group IV Ifo	16±1*	4.29±0.63*	3.75±0.84*	16.08±2.12*
Group V Lec- Ifo	6±1	2.10±0.9	1.68±0.4	3.53±2.6
Group VI Que-Ifo	5±2	1.80±0.6	2.20±1.1	3.96±1.74
Group VII Ifo+ Lec-Que	9±2*	3.42±0.96*	3.01±0.77*	10.29±1.36*

During cancer chemotherapy treatment, drug-induced oxidative stress can limit therapeutic efficiency and cause

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a number of side effects. Excess reactive oxygen (ROS) and nitrogen species (RNS) lead to oxidative stress and oxidation of cellular structure especially membrane lipids and proteins. Also they lead to mutation of mitochondria and damage to DNA<sup>16</sup>. The primary site of ROS/RNS generation is the cytochrome P450 monooxygenase system of hepatic microsomes. Enzyme systems such as the xanthine-xanthine oxidase system, and non-enzymatic mechanisms, such as Fenton reaction, also play a role in creating excess oxidative stress throughout chemotherapy. Role of Lec or Que separately in protection from oxidative damage due to Ifo injection is cleared in results of tail moment, tail length and % tailed DNA that mimic the control. The combination of two treatment indicated significant increase ( $p < 0.05$ ) in all parameters compared to control that indicate contradicting effect on the cornea.

## Conclusion

Ifosfamide causes DNA damage of rat cornea due to its oxidative stress. Co-administration of Lecithin and quercetin with intraperitoneally injection of Ifo was contradicting each other while, using these antioxidants separately with Ifo was found to have beneficial effect corneal genetic material.

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