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Research paper

Ifosfamide toxicity to the retina and the possible roles of lecithin and quercetin in albino rats

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Abstract

Background: During cancer chemotherapy, drug-induced oxidative stress can limit therapeutic efficiency and cause a number of side effects. **Objectives:** Our study aimed to characterize the side effects of an alkylating agent chemotherapy ifosfamide to the retina and if the supplementation of lecithin and or quercetin can diminish its oxidative stress by means of comet assay and FTIR.
Methods: Seventy female albino rats divided as control, rats given orally quercetin or lecithin, rats injected with ifosfamide, rats given quercetin or lecithin and in combination of them with ifosfamide injection.
Results: Lecithin and quercetin groups indicate a normal comet parameters and distribution of protein secondary structure components content of β-turn, α-helix and β-sheet. After Ifosfamide injection, all comet parameters and β-Turns content were significant increase ($p<0.05$) with the same context significant decrease ($p<0.05$) of α-helix was observed. Lecithin or quercetin reduces the effect of ifosfamide injection in tail length and percentage tailed DNA. Combined treatment gives more protection against DNA damage. Lecithin role is cleared in returning the normal distribution of β-turn, α-helix, β-sheet and lack of protective effect of quercetin regarding the protein secondary structure of retina was observed.
Conclusion: We suggest using lecithin and quercetin in combined treatment to reduce the oxidative stress due to ifosfamide.

Keywords:*Chemotherapy; Ifosfamide; Lecithin; Quercetin; Retina.*

1. Introduction

Ocular toxicity by chemotherapy incorporates a broad range about disorders, reflecting those interesting anatomical, physiological and biochemical characteristic of the eye. Understanding the visual side impacts will aid the ophthalmologist Furthermore oncologist with distinguish them promptly what's more intercede previous to visual deficiency happens. Also expectation of different treatment-related toxicities might give the chance to pharmacists with create mediation methodologies that might minimize side effects [1].
Ifosfamide ($C_7H_{15}Cl_2N_2O_2P$) are chemotherapeutic agents utilized frequently in the medication of sarcomas and hematologic malignancies. It is nitrogen mustard alkylating agents that attach an alkyl group to DNA, avoiding replication enzymes from enough entrance the template strand [2]. It is strongly affect neoplastic cells due to its affinity to proliferate faster than normal cells. Ifosfamide has recognized to cause bone marrow inhibition as well as hemorrhagic cystitis, nephrotoxicity, and neurotoxicity [3]. Ifosfamide may be the reason for central nervous system toxicity [4]. It can also cause glomerular and tubular toxicity that is characterized by Fanconi syndrome and hypophosphatemicricket [5]. Ifosfamide has been documented to affect blurring of vision and florid conjunctivitis [6]. Nutrients that have antioxidants action may help in decreasing the neurotoxicity of chemotherapy in different organs damage as Lecithin and Quercetin [7], [8].
Lecithin (Lec) has a molecular structure $C_{12}H_{24}NO_7P$ and found in most mammalian membranes living organisms especially in nervous tissue and brain [9]. It is available from sources such as soybeans, eggs, milk, cottonseed, and sunflower. Researches indicated that lecithin increase good cholesterol and decrease bad ones [10], [11]. Miranda et al., [12] found that daily lecithin supplementation increased macrophage activity which can engulf cancerous cells in the body by 29% on rats that suggest increase the immune function. Stremmel et al., [13] advises that the emulsifying activity of lecithin improves mucus in the intestine, protecting the gastrointestinal lining. Najafi et al., [14] cited the positive effect of Lecithin in improve quality-related variables in ram semen.
Quercetin ($C_{15}H_{10}O_6$)[16] is plant derived flavonoid used as a nutritional supplement found in fruits and vegetables. Quercetin (Que) is thought to have potent antioxidant, antidiabetic and anti tumour, and antiviral, anti inflammatory benefits [15]. Dong et al., [16] demonstrated that Que reduced doxorubicin-induced cardiotoxicity in vitro and in vivo by decreasing oxidative stress by up-regulation of Bmi-1 expression. Altintas et al., [17] concluded that Que prevent docetaxel-induced testicular damage in rats.Quercetin diminishes chronic paclitaxel-induced neuropathic pain by making the mast cell membrane stable, which inhibited the extreme histamine release [18].
Due to the various ocular side effects of anticancer chemotherapeutic agents, the present study will evaluate possible protecting effects of Lec and Que on ifosfamide side effects on the rats'retina.

2. Materials and methods

2.1. Chemicals

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Haloxan vials contain 1g Ifosfamide in powder form (Baxter, Germany) and dissolved in saline solution (0.9% NaCl) before injection. Quercetin was obtained from Sigma-Aldrich Chemical (St. Louis, MO). Phosphatidylcholine (lecithin) from soybean was obtained from Lewis Laboratories International Ltd. (Westport, CT). All chemicals used were of high analytical grade.

2.2. Animals grouping

Animals experiment was performed with approval from the local ethics committee. Adult female healthy albino rats were supplied by National organization of drug control and research, Egypt (NODCAR). Rats of seven weeks old weighing 180– 200 g, were kept under controlled environmental conditions (El-Nasr Chemical Co., Cairo, Egypt).

The rats were separated into seven groups, each consisting of ten animals. The experimental design included one control and six experimental groups as follows:

1) Group I served as control (Con): Rats injected daily with 0.9% NaCl (0.25 ml) intraperitoneally for 5 days.
2) Group II (Lec): Rats supplemented orally with (100 mg kg^{-1} body weight) Lec daily for 6 days. The Lec dose was adopted from a study by Lee et al., [8].
3) Group III (Que): Rats supplemented orally with (50 mg kg^{-1} body weight) Que daily for 6 days. The dose was adopted from a study by Francescato et al., [19].
4) Group IV (Ifo): Rats were injected Ifo (80 mg kg^{-1} body weight) intraperitoneally daily for 5 days. Ifo dose was adopted from a study by Chen et al., [20].
5) Group V (Lec+Ifo): adjunct supplementation of Lec with Ifo where rats supplemented with the pervious lecithin dose for 6 days and then received the selected previous dose ifosfamide daily for 5 days.
6) Group VI (Que+Ifo): Rats supplemented with the previous Que dose and then received the selected dose of ifosfamide daily for 5 days.
7) Group VII (Lec+Que+Ifo): Rats were administered Ifo just as the Ifo group, except that they supplement orally with 100 mg kg^{-1}Lec plus 50 mg kg^{-1} Que, respectively. After 5 days of Ifosfamide injection, retina from each eye of all groups was used for comet and FTIR.

2.3. Comet assay steps

2.3.1. Preparation of slides

Crushed samples were transferred to 1 ml ice-cold PBS (phosphate buffer saline, pH 7.9). This suspension was stirred for 5 min and filtered. Cell suspension (100 µl) was combined with 600 µl of low melting agarose (0.8% in PBS), where 100 µl 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.

2.3.2. Electrophoresis of Slides

- Slides were positioned on the horizontal gel box.
- Slides 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 lifted the slides from the buffer and coated the slides with neutralization buffer for at least 5 minutes.
- Slides were stained with 80µL 1X Ethidium Bromide (EtBr), leaved for 5 min and then dipped in distilled water to remove excess stain.
- Slides drain process were done by keeping them for 20 min in cold 100% ethanol and place them in an oven at 500 ^{0}C for 30 min and repeat staining with EtBr.

2.3.3. 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 [21].

2.4.FTIR spectroscopy

The retinas from all animal's groups were weighed separately, and then crushed to powder by mortar. The resulted powder was freeze-dried for 24 h then mixed with potassium bromide (KBr) powder (95 mg KBr:5 mg retina) in order to prepare the KBr disks that will be used for the FTIR measurement using Thermo Fisher Scientific Inc, USA spectrometer. For enhancement of the signal, hundred readings were recorded and baseline corrected and smoothed. The spectra that belong to all groups were averaged using OriginPro 9 software to obtain the final average spectrum then curve fitting, to determine the underlying peaks to the contour of amide I band (1750-1600 cm^{-1}). The number of the underlying peaks was established by the second derivative of the subgroup spectrum [22].

2.5. 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 $p<0.05$.

3. Results

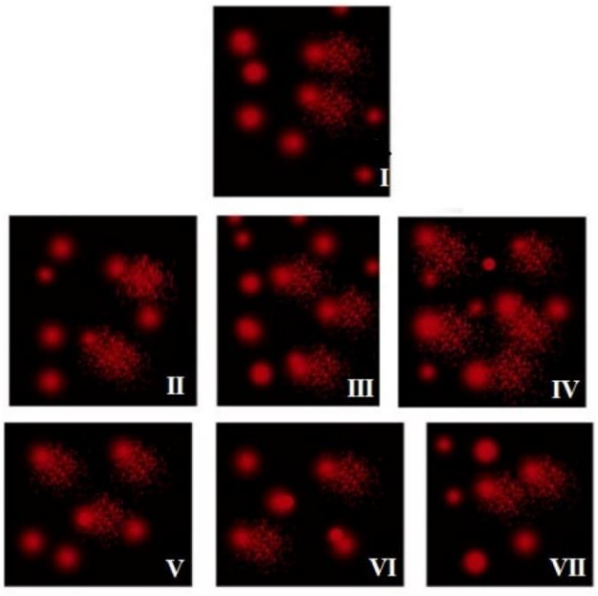


Fig. 1: Comet Assay Image to All Groups. I is the Control Group, II is Lecithin Group, III Is Quercetin Group, IV is Ifosfamide Group, V is Lecithin-Ifosfamide Group, VI is Quercetin- Ifosfamide Group and VII is Lecithin+ Quercetin- Ifosfamide Group.

Figure (1) represented comet assay images stained by ethidium bromide for all studied groups denoted by numbers I to VII for control, Lec, Que, Ifo, Lec-Ifo, Que-Ifo and Lec+Que-Ifo, respectively. Analysis of comet images was summarized in figure (2) to indicate the comet parameters results from Komet program, which are percentage tail cells, tail length in µm, percentage tail DNA and tail moment.

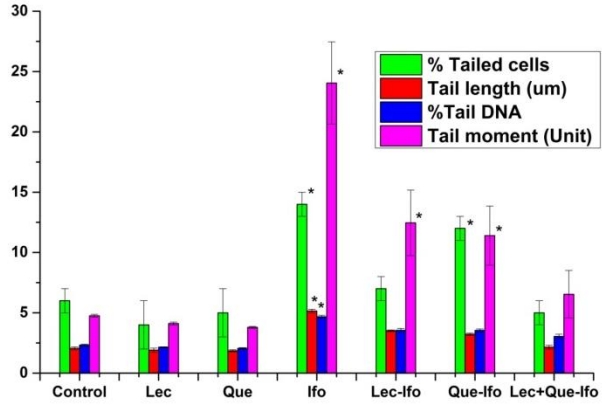


Fig.2: Histograms Pattern for percentage Tailed Cells, Tail Length, % Tail DNA and Tail Moment to All the Studied Groups.

The values of these parameters for control group were 6±1 %, 2.05±0.13 µm, 2.32±0.08% and 4.76±0.12 unit, respectively. Lec and Que groups indicate a normal comet parameters compared to control. After Ifo injection, all comet parameters were significant increase ($p<0.05$). Lec-Ifo group indicates significant increase ($p<0.05$) in tail moment only and no significant changes observed for the rest parameters. Que-Ifo group indicates significant increase ($p<0.05$) in the percentage tail cells and tail moment. Both treatments of Lec+Que-Ifo revealed no changes in comet parameters.

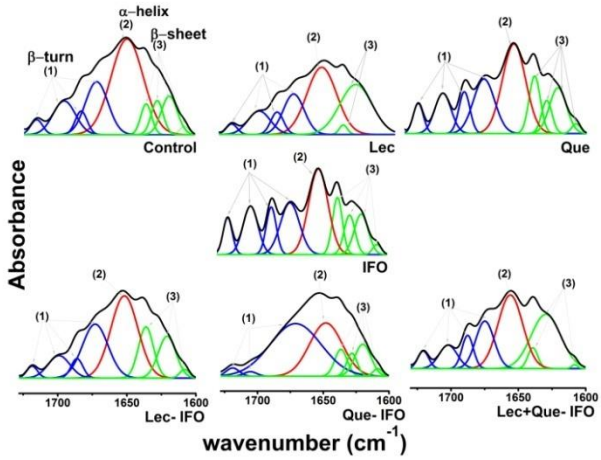


Fig. 3:FTIR Spectra to Amide I in the Range 1730-1600 Indicating (1) B-Turn, (2) A-Helix and (3) B-Sheet for All Groups.

Figure (3) showed amide I region of the retinal FTIR spectra in the range 1730-1600 cm^{-1} and the curve enhancement procedure to all studied groups. After fitting analysis, the control group spectra revealed nine peaks that assignment as 4 peaks to β-turn, 1 peak to α-helix and 4 peaks for β-sheet. The assignment of the bands was indicated by Fuller et al., [23].

The distribution of protein secondary structure components were calculated as the area percentage relative to the total area for all groups and illustrated in table (1). The area percentage for control group were 34.83±2% for β-Turns, 40.51±9 % for α-helix and 24.66±6 % for β-Sheet. The content of different protein secondary structure components for Lec and Que group revealed the same range of control group. Significant increase of β-Turns and in the same context decrease of α-helix was observed for Ifo group and also in Que-Ifo group. No changes in the content of protein secondary structure components were observed for Lec-Ifo group or Lec+Que-Ifo group.

4. Discussion

Our study aimed to characterize the side effects of an alkylating agent chemotherapy ifosfamide to the retina and if the supplementation of lecithin and or quercetin can diminish its oxidative stress by means of comet assay and FTIR.

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 [24]. 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 retina of rats injected with Ifo reflect the damage in retina and this may be due to the oxidative stress resulting from injection of Ifo. During cancer chemotherapy drug-induced oxidative stress can limit therapeutic efficiency and cause a number of side effects. Excess reactive oxygen (ROS) and nitrogen (RNS) species lead to oxidative stress and oxidation of cellular structure especially membrane lipids and proteins. In addition, they lead to mutation of mitochondria and damage to DNA [25]. 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 and Que in protection from oxidative damage due to Ifo injection is cleared in results of tail length and % tailed DNA that mimic the control. Tail moment is still significant increase ($p<0.05$) compared to control but it is better than Ifo group. The combination of two treatment indicated better results in all comet parameters that indicate Lec and Que are the key factor to balance the DNA damage in retina due to Ifo injection.

Infrared spectroscopy is known to test the structure and functions of tissues to give direct approaching into the biochemical mechanism responsible for their abnormal working under the effect of disease and toxicity. The amide I band, arises mainly from absorption of the function group C=O which is a stretching vibration with slight contributions from the out-of-phase CN stretching vibration, the CN deformation and the NH in plane bend. The Amide I band is suggesting a conformational change in α-helixes [26]. Table 1 indicated that Ifo group and also Que-Ifo group results that related to amide I band proved that proteins lose their structure because of the significant decrease observed due to ROS after Ifo injection. The contact of proteins to ROS induces secondary structural changes, since secondary structure is become stable by hydrogen bonding of peptide backbone. Proteins are organized into α-helixes, but the hydrogen bond is broken, so the chains open and are more sensitive to ROS, leading to the change of α-helix. The content of α-helix decreased from 40.51±9 to 26.37 ±2 percentage associated with increase of β-turn content from 34.83±2 to 45.39±3 percentage in Ifo group reflects that retina proteins become aggregated due to Ifo injection. In addition, the same phenomena were observed for Que-Ifo group in which α-helix content was decreased and β-turn content was increased. This indicates the lack of protective effect of Que regarding the protein secondary structure of retina. βeta-turns are the smallest type of protein secondary structure, joining other elements of secondary structure such as α-helix and β-sheets and rapidly change the direction of the polypeptide chain and may dictating the folding of longer polypeptide chains. β-turns are also general conformations that enable protein to adopt globular structures, and supplya nucleation site for folding of proteins. Oxidative stress may be the cause of protein changes, leading to mitochondrial injure and another suggestion mechanism of toxicity may be due to disruption of cell membrane of tissue.

Table 1: The Content of Different Protein Secondary Structure Components Expressed As Area Percentage Relative to the Total Area of Rats Retina.

Groups	β -turn	α -helix	β -sheet
Gr.I Control	34.83±2	40.51 ±9	24.66±6
Gr.II (Lec)	32.56±2	36.65±3	30.79±3
Gr.III (Que)	39.63±4	31.81±5	28.56±2
Gr.IV (Ifo)	45.39±3†	26.37 ±2 †	28.24±4
Gr.V (Lec-Ifo)	36.85±2	36.81±4	26.34±2
Gr.VI Que-Ifo	65.53±2†	7.68±5†	26.79±3
Gr.VII Lec+Que-Ifo	33.83±1	32.93±4	33.24±5

† Statistical significant ($p<0.05$).

Ifosfamide metabolism is a compound process involving the exchange to active and inactive metabolites. A balance between two CYP-mediated metabolic pathways: a 4-hydroxylation pathway which leads to drug activation and the less wanted N-dechloroethylation pathway leading to ifosfamide deactivation and the subsequent neurotoxic metabolite, chloroacetaldehyde [27].
Dietary antioxidants Lec reflect shifting the balance of oxidative stress to more physiological level. Lecithin includes a substance called phosphatidylcholine that is responsible for its therapeutic effects. Phosphatidylcholine is a main part of the membranes adjacent our cells. However, when you consume this substance it is broken down into the nutrient choline rather than being carried directly to cell membranes, Choline promotes methylation. It is also used to make acetylcholine, a nerve chemical necessary for accurate brain function.

5. Conclusion

Ifosfamide causes DNA damage and affect the secondary structure of protein of rat retina due to its oxidative stress. Lecithin and quercetin supplementation in the same time is effective strategy and may provide a protection against ifosfamide side effects. Several investigations in this active area of researches were needed to evaluate a combination of different agent's treatments in order to minimize chemotherapy side effect. In patients treated with ifosfamide, a regimen of follow up to the retina is important.

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