Histopathological Evaluation of Erythropoietin's Protective Effect Against Doxorubicin-Induced Hepatotoxicity

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Abstract:

Doxorubicin is an effective broad-spectrum anticancer drug; however, hepatotoxicity limits its clinical usage. Erythropoietin, a glycoprotein hormone primarily recognized for its crucial function in promoting erythropoiesis. Recently, it has been shown that it possesses significant cytoprotective effects in several organs, including the liver, heart, and brain.

The objective of this study was to assess the hepatoprotective properties of erythropoietin in the context of doxorubicin-induced liver injury.

Thirty-six male Wistar rats were separated into six groups: a negative control group and an induction group administered with doxorubicin. Additionally, there were three groups that received pretreatment with erythropoietin at three different doses (1000, 3000, and 6000 IU/kg), and a group that received conventional treatment and was pretreated with silymarin (100mg/kg). Liver samples were then collected on day 9 of the experiment from each group for histopathological examination.

Results showed that the induction group, treated with doxorubicin, revealed signs of doxorubicin-induced hepatotoxicity that include hepatocellular necrosis, severe zonal hepatic degeneration, congestion, and inflammation. On the other side, groups pre-treated with erythropoietin in all three doses revealed pronounced improvement in liver morphology especially at the higher doses used. In conclusion, erythropoietin pre-treatment can mitigate the hepatotoxicity induced by doxorubicin in a manner that is dependent on the dose, and it was more effective than the conventional therapy silymarin especially at the highest dose used.

Key words: Doxorubicin, Erythropoietin, hepatotoxicity.

عنوان البحث: التقييم النسيجي المرضي للتأثير الوقائي للإريثروبويتين ضد التسمم الكبدي الناجم عن الدوكسوروبيسين

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الخلاصة

دوكسوروبيسين هو دواء فعال للغاية مضاد للسرطان واسع النطاق، ومع ذلك فإن تطبيقه السريري مقيد بسبب آثاره الجانبية الخطيرة التي تشمل السمية الكبدية. الإريثروبويتين، وهو هرمون بروتين سكري معروف في الأصل بدوره الهام في تحفيز تكون كريات الدم الحمر، تبين مؤخرًا أن له تأثيرات وقائية خلوية كبيرة في الأنسجة المختلفة، بما في ذلك الكبد والقلب والدماغ.

الهدف من هذه الدراسة هو تقييم التأثير الوقائي للإريثروبويتين ضد التسمم الكبدي الناجم عن الدوكسوروبيسين في الجسم الحي.

تم تقسيم ستة وثلاثين ذكرًا من جرذان ويستار إلى ست مجموعات؛ مجموعة تحكم سلبية، مجموعة حث سمية الكبد بالدوكسوروبيسين، ثلاث مجموعات معالجة بالإريثروبويتين في ثلاث جرعات مختلفة (1000، 3000 و6000 وحدة دولية/كغ)، ومجموعة علاج تقليدية معالجة بالسيليمارين (100 ملغ/كغ). ثم تم جمع عينات الكبد في اليوم التاسعمن التجربة من كل مجموعة لفحصها النسيجي.

أظهرت النتائج أن المجموعة المستحثة، التي عولجت بالدوكسوروبيسين، كشفت عن علامات تسمم الكبد الناجم عن الدوكسوروبيسين والتي تشمل نخر الخلايا الكبدية، وانحطاط الكبد المناطقي الشديد، والاحتقان، والالتهاب. على الجانب الآخر، أظهرت المجموعات المعالجة مسبقًا بالإريثروبويتين في جميع الجرعات الثلاث تحسنًا واضحًا في شكل انسجة الكبد خاصة عند الجرعات الأعلى المستخدمة. كشفت هذه الدراسة، يمكن للمعالجة المسبقة بالإريثروبويتين أن تخفف من التسمم الكبدي الناجم عن الدوكسوروبيسين بطريقة تعتمد على الجرعة، وكانت أكثر فعالية من العلاج التقليدي بالسيليمارين خاصة عند أعلى جرعة مستخدمة.

الكلمات المفتاحية:دوكسوروبيسين, إريثروبويتين, التسمم الكبدي.

Introduction

Chemotherapy is a vital component of cancer treatment, but its effectiveness is frequently compromised by the unintended side effects of hepatotoxicity. The hepatotoxic effects of anticancer chemotherapy pose a significant hindrance to the broader utilization of these life-saving agents (1).

Doxorubicin (DOX) is an often-used chemotherapeutic agent for cancer therapy. It is currently greatly used in the treatment of several malignancies as ovarian cancer, thyroid cancer, breast cancer and leukemia with good clinical outcomes (2).

Despite the wide range of anticancer efficiency, DOX leads to varied side effects, including cardiomyopathy, renal, hepatic, pulmonary, testicular, and hematological toxicities (3,4). While cardiotoxicity is a preferential target of DOX treatment, hepatotoxicity remains one of the well-established negative effects of DOX (5). The liver is the chief detoxifying tissue; therefore, it is the target of excessive amounts of genotoxic composites and anticancer drugs including DOX. Approximately 40% of patients on DOX suffer from liver injury (6,7).

The primary cause of DOX-induced liver damage is mostly attributed to the generation of reactive oxygen species (ROS) by the medication during its metabolism in the liver. Oxidative stress arises from this mechanism, leading to reduced amounts of defensive enzymes, cellular demise, inflammation, and impaired mitochondrial function (8,9). Consequently, concurrent interference of oxidative stress, inflammation, and apoptosis must be a potent strategy to target DOX-induced hepatotoxicity.

Erythropoietin (EPO), a circulating glycoprotein hormone stimulates red blood cells production within bone marrow(10). Chronic renal failure or chemotherapy patients receive recombinant human erythropoietin (rhEPO) for anemia treatment (11). However, EPO and its receptors (EPORs) have been found in different organs and tissues such as brain, heart, kidney, and liver (12). Also, scientific studies proved that EPO has anti-apoptotic; antioxidant and anti-inflammatory features (13,14).

Regarding this study, the idea that EPO might be able to protect the liver from DOX damage is a significant new advantage. Through its actions, as its influence on oxidative stress and anti-apoptotic pathways, EPO is a very

hopeful option for reducing liver damage (15).

Therefore, the aim of this study is to evaluate the potential protective effect of EPO on tissue histology in a male rat model of DOX-induced liver damage.

Materials and Methods

Animals and Experimental Design

The experiment started after approval of the scientific committee in the department of pharmacology and toxicology at Mustansiriyah University/College of Pharmacy.

This study utilized thirty-six, non-previously treated, adult male Wistar rats, with weights ranging from 200 to 220g. The rats were allocated to six groups in a random manner, with six rats in each group. They were housed in plastic cages in a well-ventilated environment. The cages had woodchip bedding, and the rats had free access to food and water.

Group 1(n=6): Negative control, rats was given distilled water intraperitoneally (I.P) every day for 7 days.

Group 2(n=6): The induction, rats in this group was injected by distilled water I.P every day for 6 days. On day 7 of the experiment, a single I.P injection of DOX at a dose of 20 mg/kg was given to each animal.

Group 3(n=6): Pre-treatment group, rats in this group was injected with 1000 IU/kg/day of EPO I.P for 6 days. On the seventh day, a single I.P injection of DOX at a dose of 20 mg/kg was given to each animal.

Group 4(n=6): Pre-treatment group, rats was injected with EPO 3000 IU/kg/day I.P for 6 days. Then on day 7, a single I.P injection of DOX at a dose of 20 mg/kg was given to each rat.

Group 5(n=6): Pre-treatment group, rats in this group was injected by EPO 6000 IU/kg/day I.P for 6 days. On day 7 of the experiment, a single I.P injection of DOX at a dose of 20 mg/kg was given to each animal.

Group 6(n=6): Positive control, rats in this group was pre-treated by I.P injection of 100mg/kg/day of silymarin that was dissolved in distilled water and Tween 80, for 6 days. On the seventh day, a single I.P injection of DOX at a dose of 20 mg/kg was given to each rat.

Hepatotoxicity was induced on the 7^{th} day through I.P injection of DOX at a dosage of 20 mg/kg according to previous studies (16). The DOX was prepared by dissolving 50 mg of the substance in 25 ml of distilled water, in accordance with the instructions provided by the manufacturer. The injection was administered to all groups, except for group 1, which received an I.P injection of distilled water.

Animal handling

Each rat was held by the left arm in an upright position. After wiping the abdomen with alcohol spray and gauze, the needle was gently inserted into the right lower part of the rat's abdomen, trying to avoid hitting the bladder, liver, or other internal organs. As the animal injected, a gentle aspiration to check if the needle hit an internal organ or not, if no blood incoming in the aspiration that means no damage had been occurred and then slowly the desired drug was injected I.P.

Liver Sampling

On the ninth day of the experiment, after 48 hours of DOX therapy, rats were euthanized after anesthesia induction with an intramuscular injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The rat's abdominal cavity was opened using forceps and the liver was harvested immediately and washed with distilled water and was preserved for histopathological study in 10 %

formalin to protect the tissue structure from autolysis caused by tissue lysosomal enzymes.

Assessment of Histopathological Changes

To prepare the liver tissues for microscopical inspection, the conventional procedure; "the paraffin-embedded method", was utilized in this investigation. The steps included: **I**. Fixation of liver tissues: Tissues were immersed in neutral buffered formalin (NBF). **II.** Processing of liver tissues: Phosphate-Buffered-Saline (PBS) was used to rinse liver tissue blocks until the fixative was fully gone. Then, using an automated tissue processor (LEICA TP1020-Germany), liver tissues were dehydrated by increasing concentrations of ethanol to remove water from tissues for the non-aqueous paraffin wax embedding media. It was followed by tissue clearance to retract the dehydration alcohol residues by substituting ethanol with xylene. A vacuum oven was then used for tissue infiltration where xylene facilitated infiltration of paraffin wax into cells since they were miscible with each other. **III**. Embedding of liver tissues: Liquid paraffin was utilized for the embedding of liver tissue blocks in cassettes. **IV**. Liver tissues sectioning: The tissue blocks were cut into sections which were placed on slides. **V.** Preparation and staining of slides: The tissue slides were heated in a dry oven to improve tissue attachment and soften the paraffin. Hematoxylin and Eosin (H&E) stains were used to stain the slides. Hematoxylin colored the negatively charged cellular components blue, while Eosin stained the positively charged components red. **VI**. Permanent mounted sections preparation.

Results

The negative control group's histopathological results demonstrated normal hepatic lobule cytoarchitecture, which displayed normal hepatic cord, central vein, hepatic triad, hepatocyte, and sinusoidal arrangement, as shown in figure (1).

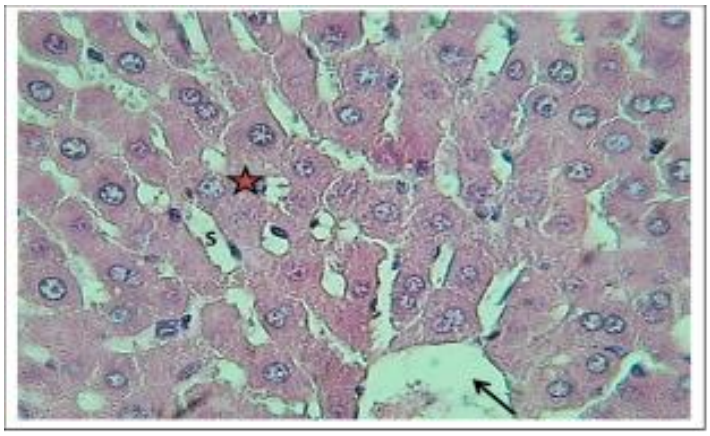

Figure (1): The liver histological structures within the negative control group shows: normal appearance of central vein (Cv), hepatocyte (asterisk), sinusoid (S) with Kupffer cells. H&E stain. x400

The sections of the induction group revealed marked disarrangement of hepatic cords, severe zonal hepatic degeneration (cellular swelling) with focal cellular necrosis of hepatocytes associated with moderate aggregation of mononuclear leukocytes (MNCs). Other figures revealed moderate pre-vascular lymphocytic cuffing at the portal triad with marked multiple figures of newly formed bile ductules. The hepatic

cords showed severe zonal cellular swelling of hepatocytes with necrosis (figure 2).

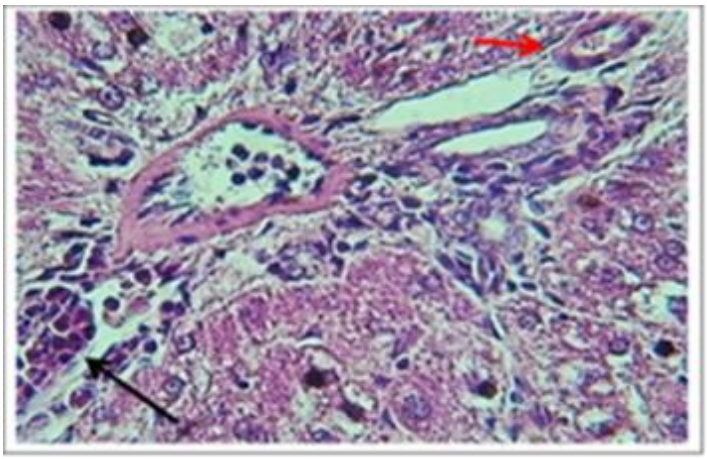

Figure (2): The liver histological structures within the induction group shows: pre-vascular lymphocytic cuffing (Black arrow) with proliferation of bile ductules (red arrows). H&E stain. x400.

The most histopathological figures of the study group pre-treated with 1000 IU/kg of EPO revealed moderate zonal and central hepatic degeneration (cellular swelling) with little focal cellular necrosis and dilation with congestion of central vein. Other figures showed mild congestion of portal vein with normal biliary ducts and mild zonal cellular swelling of hepatocytes without necrosis (figure 3).

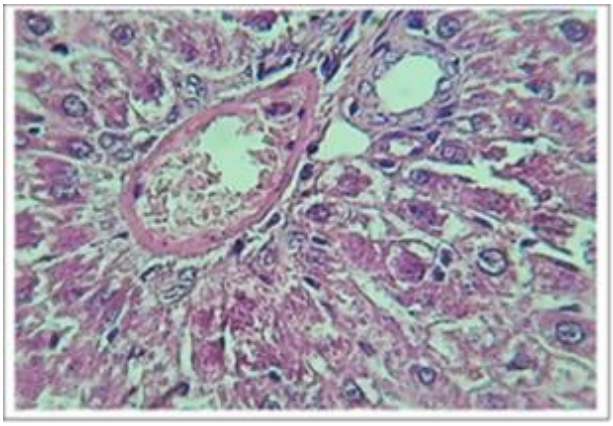

Figure (3): The liver histological structures within the EPO (1000 IU/kg) group shows: mild congestion of portal vein (arrow) (Red arrows). H&E stain. x400.

The histological findings of the group that received EPO 3000 IU/kg were mostly comparable to those of the negative control group. However, a small number of figures exhibited some vascular congestion in the central and portal triad (Figure 4).

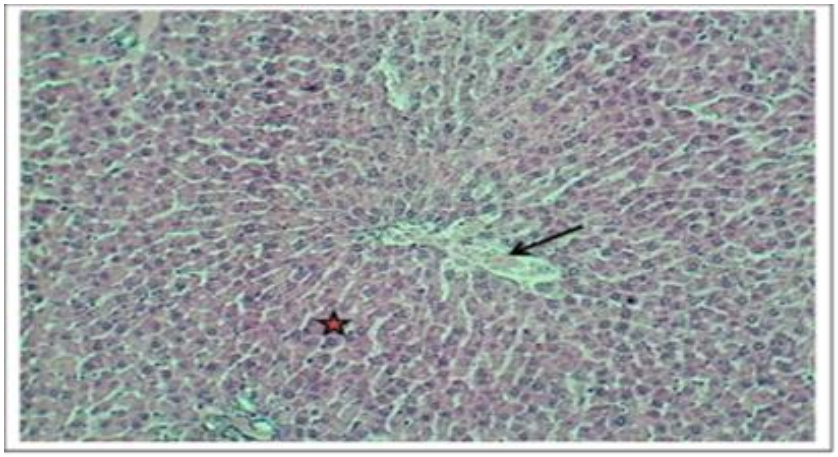

Figure (4): Histological structures of the livers in the EPO (3000 IU/kg) group shows: mild congestion of central vein (arrow) with normal hepatic cords (Asterisk). H&E stain. x100.

The histopathological results for the group that received EPO 6000 IU/kg were comparable to those of the negative control group, displaying normal hepatic cord arrangement with normal hepatocytes and sinusoids, as illustrated in figures (5).

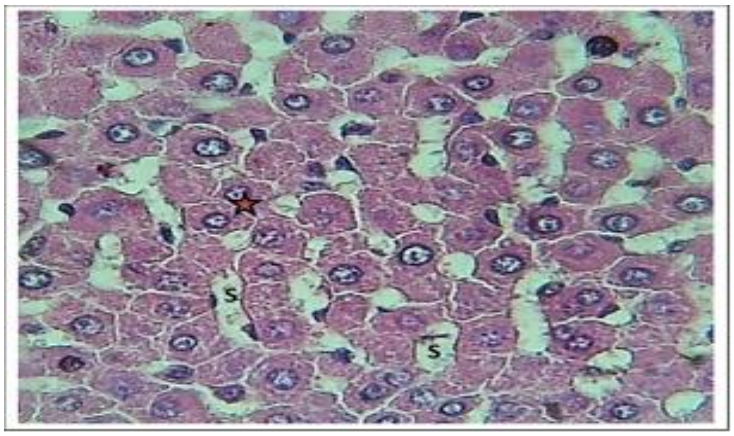

Figure (5): Histological structures of the livers in the EPO (6000 IU/kg) group shows: normal hepatocytes (asterisk) with normal sinusoids (S) H&E stain. x400.

The histological characteristics observed in the group that received the standard medication silymarin were comparable to those in the negative control group (figure 6).

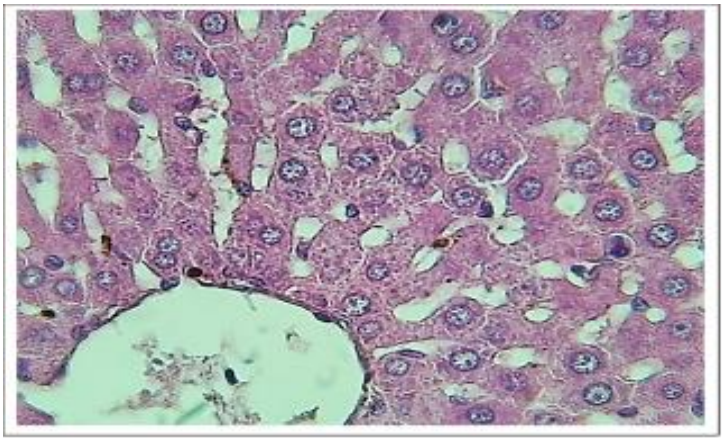

Figure (6): Histological structures of the livers in the Silymarin (100mg/kg) group shows: normal sinusoids, hepatic cords, and central veins. stain H&E. x400.

The following table shows the micropathological scores of hepatotoxicity, where scale 0 - 4 for grade and stage: 0: none, 1: minimal: rare spotty changes (can find 1 – 2), 2: mild: scattered, 3: moderate: confluent or clusters, 4: severe: generalized.

Table (1): The micropathological scores of hepatotoxicity.

Parameters	Negative control group	Induction (DOX) group	EPO (1000IU/kg) group	EPO (3000IU/kg) group	EPO (6000IU/kg) group	Silymarin (100mg/kg) group
Apoptosis	0	3	1	0	0	0
Sinusoidal dilation with congestion	0	3	1	0	0	0
Central vein dilation with congestion	0	3	1	1	0	1
Inflammatory infiltration	0	3	0	0	0	0
Degeneration	0	4	2	1	0	0
Bile duct hyperplasia	0	3	1	1	1	2
Edema	0	2	0	0	0	0
Disarrangement of hepatic cords	0	3	2	0	0	1
Parenchyma necrosis	0	3	1	0	0	0
Fibrosis	0	2	0	0	0	0

The negative control group showed a micropathological score of zero in all assessed parameters. In contrast, the DOX-induction group scored 4 for degeneration, 3 pertaining to apoptosis, sinusoidal dilation with congestion, central vein dilation with congestion, inflammatory infiltration, bile duct hyperplasia, disarrangement of hepatic cords and parenchyma necrosis. Also giving a score of 2 in the parameters of edema and fibrosis.

The group pre-treated with EPO 1000 IU/kg demonstrated degeneration and disarrangement of hepatic cords of score 2, and a score 1 pertaining to apoptosis, sinusoidal dilation with congestion, central vein dilation with congestion, bile duct hyperplasia and parenchyma necrosis.

The group pre-treated with 3000 IU /kg EPO had a score of 1 for central vein dilation with congestion, degeneration, and bile duct hyperplasia, while the group pre-treated with EPO 6000 IU/kg scored zero in all assessed parameters except for scoring 1 in the bile duct hyperplasia parameter.

The conventional treatment group pre-treated with silymarin scored 2 for bile duct hyperplasia, while it scored 1 for central vein dilation with congestion and disarrangement of hepatic cords parameters.

Discussion

Doxorubicin has several therapeutic uses and is a crucial part of the anticancer drug arsenal (2). However, its clinical applications are limited due to the induction of serious adverse effects(17). Despite its well-known ability to cause dose-limiting cardiotoxicity, DOX is toxic to other organs, including the liver (8,18). The current study investigated any potential defense of EPO against hepatotoxicity caused by DOX.

A histological investigation was performed, where the negative control group was found to have normal liver tissue without signs of hepatotoxicity. Liver sections from this group were used as references for healthy liver tissue.

The induction group receiving DOX showed signs of hepatotoxicity due to DOX, including hepatic necrosis, severe regional hepatitis, shrinkage, and inflammation, which have also been shown in other studies (16,19). Despite the increased detoxification capacity of the liver as compared with other tissues, it has been illustrated that the hepatocytes are unable to overcome acute or chronic toxic DOX exposures (20,21). In hepatocytes, the microsomal enzymes metabolize DOX to produce the more toxic metabolite doxorubicinol. The mechanisms of DOX-induced cytotoxicity result from its cellular accumulation and subsequent DNA damage, induction of oxidative stress, and activation of inflammatory signaling (22). Together, these actions might explain the failure of hepatocyte endogenous protective mechanisms, and the resultant disruption of tissue structure as illustrated in research data.

Histopathological analysis of the EPO-treated groups showed dose-dependent protection against DOX-induced hepatotoxicity. The group that received a lower dosage of EPO (1000 IU/kg) was assessed for any indications of protection against DOX-induced hepatotoxicity. Histological improvements included reduced necrosis, inflammation, and overall preservation of liver architecture compared to the induction group. EPO, through its anti-apoptotic and anti-inflammatory effects, mitigated the impact of DOX-induced oxidative stress and apoptosis, resulting in improved histological outcomes.

While the intermediate dose of EPO (3000 IU/kg) showed a dose-dependent response, with further improvements in liver morphology compared to the 1000 IU/kg group. This means that a higher dose of EPO enhances its protective effects, potentially through a more robust inhibition of apoptotic pathways and oxidative stress.

The group that received the highest dose of EPO (6000 IU/kg) had significant hepatoprotective effects. Histological examination of this group revealed a major reduction in hepatocellular damage, inflammation, and other indicators of hepatotoxicity. The highest dose of EPO exerted the most potent protective effects, significantly attenuating DOX-induced hepatotoxicity. Some previous studies also showed the same protective effects of EPO on liver tissue (15,23).

One explanation of these findings could be related to EPO's effect on modulation of multiple signaling pathways including phosoinositide-3 kinase (PI3K)/Akt and Nrf2/antioxidant response element (ARE). EPO's activation of PI3K/Akt promotes cell survival, counteracting DOX-induced apoptosis (24). Activated Akt can

phosphorylate and inhibit glycogen synthase kinase-3β (GSK3β), contributing to anti-apoptotic effects through downregulation of caspase 3 (25,26). Increasing the doses of EPO and especially the highest dose maximizes PI3K/Akt activation, providing the most potent anti-apoptotic effects. Continued inhibition of GSK3β contributes to the reduction in hepatocellular damage. Thus, inhibition of GSK3β and activation of PI3K/Akt contribute to the preservation of cellular structure and morphology of liver tissue. EPO's role in activation of Nrf2/ARE can enhance antioxidant defenses, mitigating DOX-induced oxidative stress, potentially reducing damage and inflammation in the liver (27,28).

Silymarin, a flavonoid with hepatoprotective properties, was used as the positive control group (29,30). Histopathological findings demonstrated protective effects, including reduced necrosis, inflammation, and overall preservation of liver architecture. Comparisons with this group provided insights into the relative efficacy of EPO in preserving liver tissue, which showed a higher level of protection compared to silymarin especially at the highest dose (6000 IU/kg).

Conclusion

The study revealed that pre-treatment with EPO can mitigate the hepatotoxicity caused by DOX. The response to EPO varies depending on the dosage, and it was more effective than the conventional therapy silymarin especially at the highest dose used (6000 IU/kg).

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