SCREENING THE HEPATOPROTECTIVE DOSE OF DEFERIPRONE AGAINST CONCANAVALIN A-INDUCED ACUTE HEPATOTOXICITY

BY

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Abstract

Iron overload is one of mechanisms by which hepatitis C virus causes oxidative stress that may contribute to fibrosis and carcinogenesis in the liver. Chronic hepatitis C (CHC) virus infection is a leading cause of progressive liver fibrosis, liver cirrhosis and hepatocellular carcinoma. Liver fibrosis with high mortality rate after diagnosis and limited successful treatment. The present study was designed to screen the potential hepatoprotective dose of deferiprone (DFP) and whether it can attenuate hepatotoxicity induced by concanavalin A (con A) in rats. Male Wister rats were randomized into 6 groups and treated with Con A (20 mg/kg, once, i.v) and/or deferiprone (5, 10, 25 or 40 mg/kg/day, one hour before con A). Liver enzymes levels were assessed in addition to histopathological examination of liver tissues. DFP (5 mg/kg) pre-treatment restored liver enzymes toward normal values. Moreover, histopathological examination confirmed the protective effect of this dose of DFP.

Key Words: Hepatotoxicity – deferiprone – concanavalin A.

Introduction

Iron overload has been suggested as a negative prognostic factor of chronic hepatitis C (CHC) **(Isom et al., 2009),** with possibly influences on the increase in aminotransferase activity, exacerbation of inflammation, progression of liver fibrosis and decrease in antiviral therapy effectiveness **(Bonkovsky et al., 1997).** Liver fibrosis results from an excessive accumulation of tough, fibrous scar tissue and occurs in most types of chronic liver diseases. The main causes of liver injury leading to fibrosis include chronic hepatitis virus infection, excess alcohol consumption and nonalcoholic steatohepatitis **(Hernandez-Gea et al., 2011).** Over time this process can result in cirrhosis of the liver, in which the architectural organization of the functional units of the liver becomes so disrupted that blood flow through the liver and liver function become disrupted which may require liver transplantation **(Sartori et al., 2010).**

Hepatic fibrosis was historically thought to be a passive and irreversible process due to the collapse of the hepatic parenchyma and its substitution with a collagen-rich tissue **(Imbert-Bismut et al., 2001).** However, that traditional view of liver disease as an irreversible process is obsolete and it is now evident that the development of liver fibrosis is a dynamic and potentially bidirectional process prior to the establishment of advanced architectural changes to the liver **(Hernandez-Gea et al., 2011).**

Deferiprone (DFP) is a bidentate iron chelator indicated for the treatment of patients with transfusional iron overload due to thalassemia **(Goel at al., 2008).** It was introduced about 20 years ago as the first oral iron chelator and such development of oral iron chelators intended to improve compliance **(Mazza et al., 1998).** There are mounting theoretical, experimental, and clinical evidences of the efficacy of therapy with DFP. Approval is based on a reduction in serum ferritin levels. DFP is a chelating agent with an affinity for ferric ion (iron III). Deferiprone binds with ferric ions to form neutral 3:1 (DFP : iron) complexes that are stable over a wide range of pH values **(Cappellini et al., 2009).** DFP is rapidly absorbed from the upper part of the gastrointestinal tract, appearing in the blood within 5 to 10 minutes of oral administration. Peak serum concentrations occur approximately 1 hour after a single dose **(Piga et al., 2010).** Although total iron excretion with DFP is somewhat less than other iron chelators, deferiprone may have a better cardioprotective effect due to its ability to penetrate cell membranes. Recent clinical studies indicate that oral DFP treatment is well tolerated **(Brittenham, 2003).** The present study aimed to screen the hepatoprotective dose of DFP in modulating acute hepatotoxicity induced immunologically by concanavalin A (con A) in rats.

Chemicals and drugs:

All chemicals used in this study were analytically pure and purchased from Sigma-Aldrich Co, St. Louis. MO, USA.

Animals:

Male albino rats weighing 110-130 g were obtained from the animal house of El Nile Co. for pharmaceutical industries, Cairo, Egypt. The animals were housed in air condition atmosphere at $25\pm2^{o}C$, and fed with a standard laboratory pellet diet and tap water *ad libitum.*

Experimental design:

Rats were randomized into 6 groups (6 animals each) and treated as follows:

Group 1: Control rats received single injection of phosphate-buffered saline (PBS).

Group 2: Rats received single dose of Con A (20 mg/kg, dissolved in PBS, intravenously) **(Nakano et al., 2010).**

Groups 3, 4, 5 and 6: Rats were administered orally different doses of Deferiprone (5, 10, 20, 40 mg/kg., respectively) at 1h after Con A injection.

After 24 h from Con A injection, blood samples were collected from the retro-orbital plexus and allowed to clot. Serum samples were immediately separated by centrifugation at 1000 g for 10 min and stored at -80°C. Then, rats were sacrificed and liver tissues were dissected, weighed and washed with ice-cold saline.

Specimens from the liver were fixed in 10% formalin for histopathological assessment.

Biochemical estimations:

Serum alanine aminotransferase ALT and aspartate aminotransferase AST were determined using commercial colorimetric Kits (Stanbio laboratory, Inc., San Antonio, TX, USA).

Histopathological assessment:

Autopsy samples were taken from the liver of rats in different groups and fixed in 10% formol saline for 24 hrs. Specimens were washed, dehydrated by alcohol, cleared in xylene and embedded in paraffin at 56 degree in hot air oven for twenty four hours. It was stained by hematoxylin and eosin (H&E) for histopathological examination as well as Masson trichrome stain for detection of the collagen fibers through the light electric microscope **(Banchroft et al. 1996)**.

Immunohistochemical assessment of Tumor necrotic factor alpha (TNF-α):

The antibody/antigen complex is labeled with an enzyme that can react with a suitable substrate to give a colored product. Proper fixation is crucial to successful staining. Formaldehyde fixation is often used as the routine initial method of choice for tissue and with the immobilized antigen to form an antigen-antibody complex. A second, biotinylated antibody specific for primary antibody reacts with the complex. Streptavidin conjugated to peroxidase reacts with the Ab-Ab-Ag complex immobilizing the peroxidase at the site of the antigen. Finally, the substrate is added causing a colored precipitate to form on the slide at the location of the antigen. This slide is analyzed using a light microscope **(Buchwalow et al., 2002)**.

Statistical Analysis:

Comparisons between different groups were carried out by one way analysis of variance (ANOVA) followed by Tukey as a post-hoc test using Graphpad Prism software version 6. The p values were at $p<0.05$ which means significance.

Results:

The acute study revealed that single injection of Con A could induce acute hepatotoxicity. Con A induced a significant increase in AST and ALT levels to be 161.25% and 155.42% respectively as compared to the control group. Pre-treatment of animals with DFP(5) and DFP(10) significantly reduced AST and ALT levels in comparison to con A group with DFP(5) giving better results. DFP significantly ameliorated AST and ALT levels to be 130.92% and 123.04% as compared to the control group. However, DFP(25) and DFP(40) pre-treatment didn't show any significant changes in AST nor ALT when compared to the con A group **(Table 1, Figures 1 and 2)**.

Liver sections from the control and DFP (5 mg/Kg) groups stained with H&E showed normal hepatocellular architecture. On the other hand, liver sections from the con A treated group showed fibroblasts proliferation in portal triad associated with portal inflammatory cells infiltration. Furthermore, there is congestion in central vein. So pre-treatment with 5 mg/kg of DFP screening different doses of DFP showed to be

the most hepatoprotective dose, evidenced by the significant amelioration of liver enzymes levels and by the histopathological examination **(Figure 3)**.

TNF-α was assessed immunohistochemically. Control rats showed a minimal immunostaining **(Figure 4 A)**. Con A induced an increase in the TNF-α levels in the liver tissues, which was evident from intense brown staining **(Figure 4 B)**. Pre-treatment of rats with DFP (5 mg/kg) showed TNF-α expression levels similar to the control group **(Figure 4 C)**. However, pre-treatment of rats with DFP (10, 25, 40 mg/kg) showed significant immunostaining, as compared to the control group **(Figures 4 D, 4 E, 4 F)**.

Table (1): Effects of pre-treatment with different doses of DFP(5, 10, 25, 40 mg/kg, oral) on serum levels of ALT and AST in rats subjected to acute Con A (20 mg/kg, i.v. 1 hour after DFP pre-treatment) hepatotoxicity:

Parameter	*ALT (U/L)*	*AST (U/L)*
Control	40.40±3.800	64.00±4.745
ConA	62.79±6.225[a]	103.20±11.48[a]
DFP(5) + ConA	49.71± 3.808[a,b]	83.79±7.857[a,b]
DFP(10) + ConA	51.02±5.919[a,b]	84.11±9.233[a,b]
DFP(25) + ConA	61.27±5.520[a,c,d]	106.4±8.402[a,c,d]
DFP(40) + ConA	66.47±5.647[a,c,d]	102.1±13.20[a,c,d]

Data are the mean ±SD, (N=6).

a,b,c,d Significantly different from control, Con A, DFP(5) & DFP(10) groups respectively at $P<0.05$ using one-way ANOVA followed by Tukey as a post-hoc test.
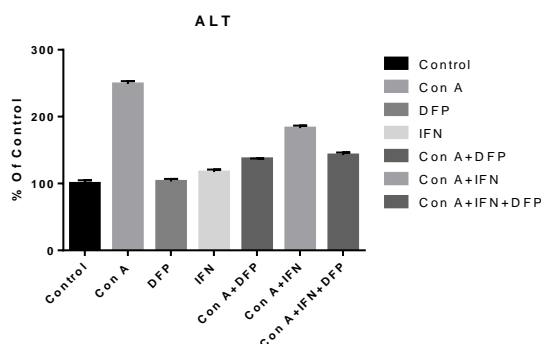

Figure (1): Effects of pre-treatment with different doses of DFP (5, 10, 25, 40 mg/kg, oral) on serum levels of ALT in rats subjected to acute Con A (20 mg/kg, i.v. 1 hour after DFP pre-treatment) hepatotoxicity. Values are percentage of control group (n=6). Comparisons between groups performed using one-way ANOVA followed by Tukey as post-hoc test. a,b,c,d: statistical significant difference from control, Con A, DFP(5) & DFP(10) groups respectively at $P<0.05$. After24 hours, animals were sacrificed, serum samples were collected and stored at -80ºC.ALT: Alanine aminotransferase.

AST Acute Study

Legend:
- Control
- Con A
- DFP(5) + ConA
- DFP(10) + ConA
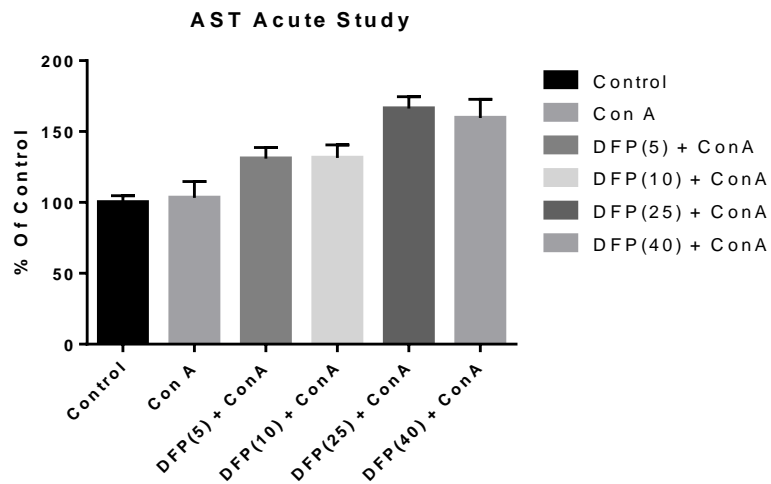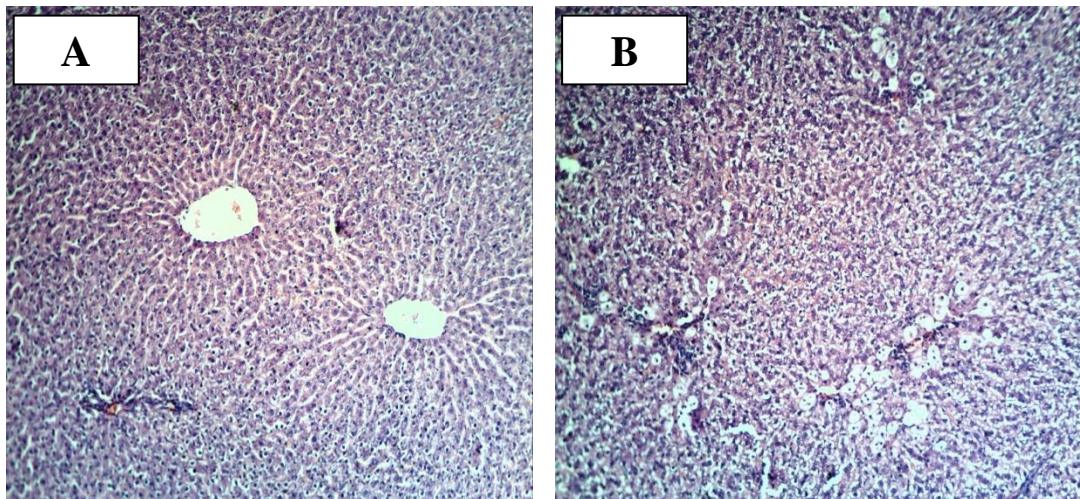- DFP(25) + ConA
- DFP(40) + ConA

Figure (2): Effects of pre-treatment with different doses of DFP (5, 10, 25, 40 mg/kg, oral) on serum levels of AST in rats subjected to acute Con A (20 mg/kg, i.v. 1 hour after DFP pre-treatment) hepatotoxicity. Values are percentage of control group (n=6). Comparisons between groups performed using one-way ANOVA followed by Tukey as post-hoc test. a,b,c,d: statistical significant difference from control, Con A, DFP(5) & DFP(10) groups respectively at $P < 0.05$. After 24 hours, animals were sacrificed; serum samples were collected and stored at -80°C. AST: Aspartate aminotransferase.
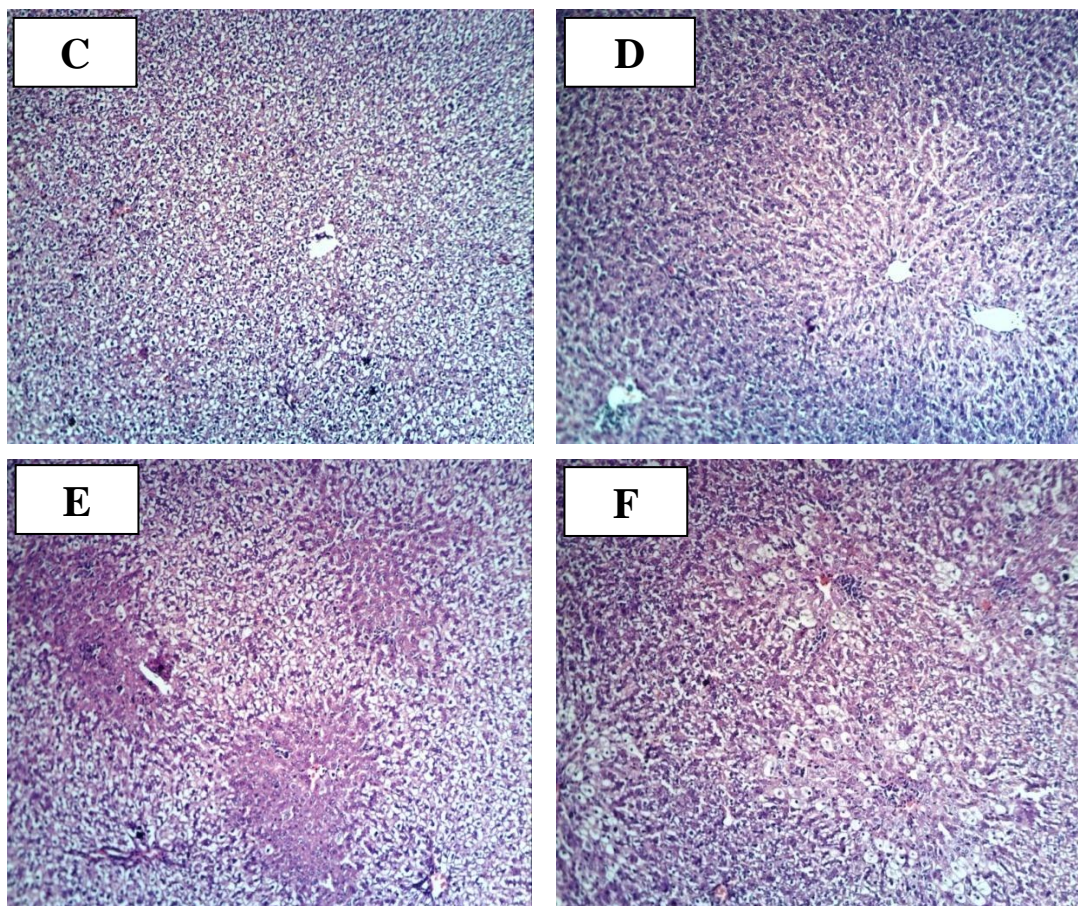
Figure (3): Representative photomicrographs of of liver sections stained by H&E (×100).

Figure legends:

A: Sections taken from liver of control rat showed normal histological structure of hepatocytes.

B: section taken from liver treated with con A showing severe inflammatory cells infiltration with fibrosis and central vein congestion.

C: section taken from liver pre-treated with DFP (5 mg/kg) showing normal architecture of hepatocytes.

D: section taken from liver pre-treated with DFP (10 mg/kg) showing mild changes in architecture of hepatocytes.

E: section taken from liver pre-treated with DFP (25 mg/kg) showing severe inflammatory cells infiltration.

F: section taken from liver pre-treated with DFP (40 mg/kg) showing severe histopathological changes summarized as cytoplasmic vacuolization of hepatocytes and massive inflammatory cells infiltration.
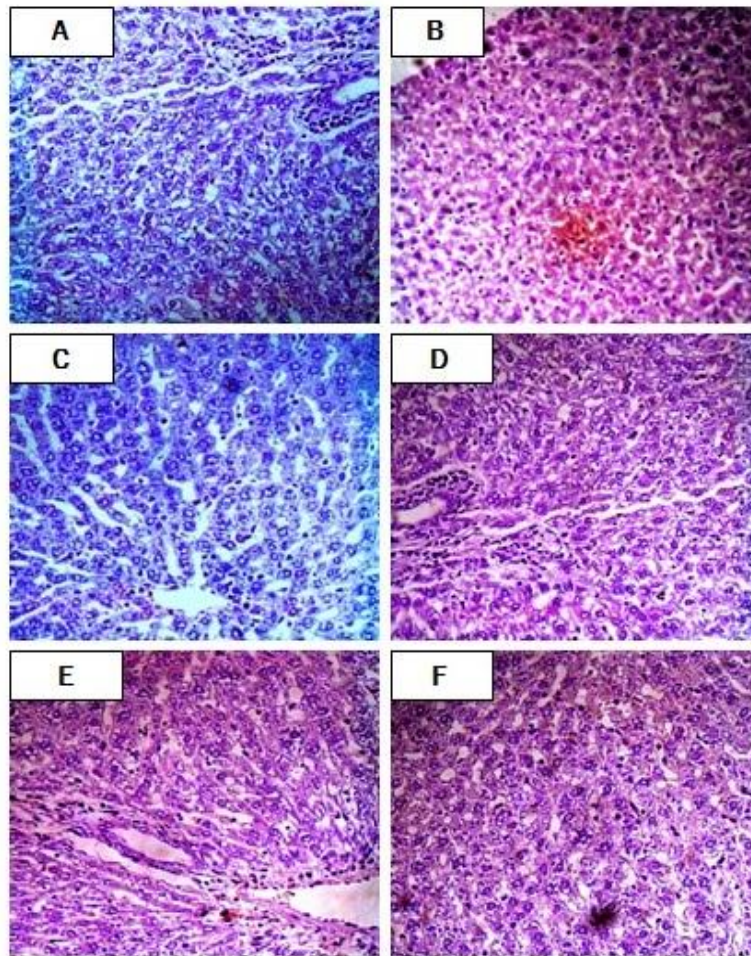
Figure (4): Immunohistochemical analysis of TNF-α in liver sections (×200).

Figure legends:

A: Transverse section of liver in control rat showing normal architecture with minimal degree of TNF-α (brown color) expression.

B: Transverse section of liver in con A group showing extensive TNF-α (brown color) expression in tissue.

C: Transverse section of rat liver pre-treated with DFP (5 mg/kg) showing normal architecture with minimal degree of TNF-α (brown color) expression.

D: Transverse section of rat liver pre-treated with DFP (5 mg/kg) showing mild TNF-α (brown color) expression in tissue.

E: Transverse section of rat liver pre-treated with DFP (5 mg/kg) showing moderate TNF-α (brown color) expression in tissue.

F: Transverse section of rat liver pre-treated with DFP (40 mg/kg) showing extensive TNF-α (brown color) expression in tissue.

Discussion:

Deferiprone has been investigated for its iron chelating action in iron overload cases. Iron induces oxidative stress and inflammation **(Poggiali et al., 2012).** In addition, iron stimulates collagen formation and extracellular matrix deposition through activation of hepatic stellate cells **(Brittenham, 2003)**. Moreover, phlebotomy preserved hepatocytes architecture in chronic hepatitis C males with mild iron overload **(Sartori et al., 2010**; **Armitage et al., 2014).).**

The con A model is a reproducible well established immunologic model that mimics hepatitis induced by hepatitis C virus in humans. A single con A injection can induce acute hepatotoxicity including hepatitis and liver fibrosis **(Hayashi and Sakai, 2011)**. Accordingly the present work was carried to screen the potential hepatoprotective dose of deferiprone against con A-induced acute hepatotoxicity.

It was found that a single con A injection induced a significant elevation of liver enzymes both AST and ALT as compared to the control group. These results were in context with other results, which confirmed that a bolus injection of 20 mg/kg of con A could induce acute hepatotoxicity in rats **(Nakano et al., 2010).** In the current study screening different doses of DFP showed that, pretreatment with DFP decreased liver enzymes levels as compared to con A group but the most hepatoprotective dose of DFP was 5 mg/kg evidenced by the significant improvement in liver enzymes levels.

The next step was the histological examination. The present study revealed that con A induced inflammation and excessive deposition of collagen fibers in liver tissues upon histopathological . these histological changes are in agreement with **(Murayama et al., 2008).** The pre-treatment with DFP (5 mg/kg) preserved hepatocytes normal architecture from destruction. These results supported the hepatoprotective effect of the chosen dose of DFP. This proves the protective effect of DFP which most probably is due to its iron chelating action.

Another pathway involved in hepatotoxicity is the inflammatory process **(de Souza-Cruz et al., 2016)**. In this regard, the inflammatory reaction following Con A injection was elaborated by the significant elevation in TNF-α indicating amplified inflammation. The present results coincided with previous studies, which indicated that hepatitis induced by Con A in a murine model of liver injury was driven by the up-regulation of inflammatory cytokines including TNFα **(Tiegs, 2007)**.

Conclusion:

In conclusion, the present work provides evidences for the potent hepatoprotective effects of DFP against Con A-induced liver toxicity. Therefore, the incorporation of DFP in therapy of hepatitis C virus (HCV) could enhance the therapeutic efficiency.

Acknowledgments:

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البحث عن جرعة الديفيريبرون المحافظة على الكبد في نموذج تسمم الكبد المحدث بواسطة الكونكانافالين أ

للسادة

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الحديد الزائد هو أحد الآليات التي يستخدمها فيروس الإتهاب الكبدي سي لإحداث إجهاد تأكسدي والذي قد يسهم في حدوث تليف وسرطان في الكبد. يعد فيروس الإلتهاب الكبدي سي هو السبب الرئيسي لتليف وتشمع وسرطان الكبد. يرتفع معدل الوفيات مع تليف الكبد بعد التشخيص والعلاج الناجح محدود. وقد تم تصميم الدراسة الحالية لفحص الجرعة المحتملة المحافظة على الكبد من الديفيريبرون ومدى إمكانية هذه الجرعة في تقليل التسمم الكبدي المحدث بواسطة الكونكانافالين أ في الجرذان. تم تقسيم ذكور الجرذان إلى ٦ مجموعات وتم إعطاءها كونكانافالين أ (٢٠ ملغ / كغ، مرة واحدة، بالوريد) مع/أو ديفيريبرون (٥، ١٠، ٢٥ أو ٤٠ ملغ / كغ / يوم، قبل الكونكانافالين أ بساعة واحدة، عن طريق الفم). تم قياس مستوى إنزيمات الكبد بالإضافة إلى فحص أنسجة الكبد. أن العلاج بالجرعة (٥ ملغ / كلغ) من الديفيريبرون أعادت إنزيمات الكبد إلى القيم الطبيعية. وعلاوة على ذلك، أكد فحص الأنسجة التأثير الوقائي لهذه الجرعة.

الخلاصة و الاستنتاج:

في الختام، يقدم هذا العمل الأدلة على فاعلية الديفيريبرون في حماية الكبد من التسمم المحدث بواسطة الكونكانافالين أ. ولذلك فإن دمج الديفيريبرون في علاج فيروس التهاب الكبدي سي يعزز الكفاءة العلاجية.