

EVALUATION OF OXIDATIVE STRESS IN EXPERIMENTAL ACETAMINOPHEN INDUCED HEPATOTOXICITY: EFFECTS OF DARBEPOETIN ADMINISTRATION

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ABSTRACT

• **Objective:** Effects of darbepoetin alfa on acetaminophen induced hepatotoxicity have been investigated by evaluating some oxidant/antioxidant parameters and liver histology.

• **Material and Method:** Fifty adult, male, Sprague-Dawley rats were randomly divided into five equal groups. Sham group (S); Acetaminophen (800 mg/kg, single dose, intraperitoneally) injected group (A); Darbepoetin alfa (10µg/kg, single dose, subcutaneously) administered at the same time with acetaminophen injection group (ASD); before acetaminophen injection darbepoetin administered group (DA); Darbepoetin administered after acetaminophen injection group (AD).

• **Results:** Plasma AST and ALT levels of the A group, the ASD group, the DA group and the AD group were significantly

higher than the S group ($p<0.001$). Plasma MDA levels of the ASD group, the DA group and the AD group were significantly higher than the A group ($p<0.001$). Plasma Cu-Zn SOD levels of the ASD ($p<0.01$), DA ($p<0.01$) and AD ($p<0.001$) groups was significantly lower than the A group.

Tissue Cu-Zn SOD levels of the ASD ($p<0.01$) group, the DA ($p<0.001$) group, and the AD ($p<0.001$) groups were significantly lower than the A group.

• **Conclusion:** Darbepoetin alfa administration could not support the antioxidant parameters in acetaminophen induced hepatotoxicity. Timing of darbepoetin alfa administration for liver toxicity could be an important factor for the efficacy of the treatment.

• **Key Words:** Acetaminophen; hepatotoxicity; darbepoetin alfa; oxidative stress. *Nobel Med* 2010; 6(3): 56-61

DENEYSSEL ASETAMİNOFEN İLE OLUŞTURULAN KARACİĞER TOKSİSİTESİNDE OKSİDATİF STRESİN DEĞERLENDİRİLMESİ: DARBEPOETİN UYGULAMASININ ETKİLERİ

ÖZET

• **Amaç:** Darbepoetin alfanın asetaminofen ile oluşturulan karaciğer toksisitesi üzerine etkileri karaciğer histolojisi ve bazı oksidan/antioksidan parametreler değerlendirilerek incelendi.

• **Materyal ve Metod:** Elli adet yetişkin, erkek Sprague-Dawley sıçan rastlantısal olarak beş eşit gruba ayrıldı. Sham grubu (S); Asetaminofen (800 mg/kg, tek doz, intraperitoneal) enjekte edilen grup (A); Darbepoetin alfa'nın (10µg/kg, tek doz, subkutan) asetaminofen enjeksiyonu ile birlikte verildiği grup (ASD); Darbepoetin alfa'nın asetaminofen enjeksiyonundan önce verildiği grup (DA); Darbepoetin alfa'nın asetaminofen enjeksiyonundan sonra verildiği grup (AD).

• **Bulgular:** A grubu, ASD grubu, DA grubu ve AD grubu plazma AST ve ALT değeri, S grubundan anlamlı derecede yüksekti ($p<0,001$). ASD grubu, DA grubu ve AD grubu plazma MDA değeri A grubundan anlamlı derecede yüksekti ($p<0,001$). ASD grubu ($p<0,01$), DA grubu ($p<0,01$) ve AD grubu ($p<0,001$) plazma Cu-Zn SOD değeri A grubundan anlamlı derecede düşüktü.

ASD grubu ($p<0,01$), DA grubu ($p<0,001$) ve AD grubu ($p<0,001$) grubu doku Cu-Zn SOD değeri A grubundan anlamlı derecede düşüktü.

• **Sonuç:** Darbepoetin alfa asetaminofen ile oluşturulan karaciğer toksisitesinde antioksidan özellik göstermemiştir. Karaciğer toksisitesinde darbepoetin alfa'nın kullanımında zamanlama tedavinin etkinliği açısından önemli bir faktör olabilir.

• **Anahtar Kelimeler:** Asetaminofen, hepatotoksisite, darbepoetin alfa, oksidatif stres. **Nobel Med 2010;** 6(3): 56-61

INTRODUCTION

Overdoses of acetaminophen which is an analgesic and antipyretic drug is one of the most common pharmaceutical product poisonings.^{1,2} Acetaminophen undergoes sulfation and glucuronidation to form conjugates that undergo renal elimination at therapeutic doses.³ The liver expresses many cytochrome P450 isoforms, which generates reactive oxygen species, activates many toxicologically important substrates, and causes oxidative stress. The cytochromes that oxidize acetaminophen to the reactive metabolite are 2E1, 1A2, 3A4, and 2A6.^{2,4-7} The reactive metabolite was found to be N-acetyl-p-benzoquinoneimine (NAPQI) which is formed by a direct two-electron oxidation. NAPQI is a cytochrome P450-mediated oxidation product of acetaminophen. Kupffer cell activation and neutrophil infiltration potentializes toxic injury by releasing reactive oxygen species, cytokines, and chemokines which induce neutrophil extravasation and activation.^{8,9} Glutathione (GSH) takes place in the detoxification process of NAPQI by forming an acetaminophen-GSH conjugate. Total hepatic GSH is depleted by as much as 90% when a toxic dose of acetaminophen was administered.⁷ The reactive metabolite covalently binds to a substantial number of cytosolic and mitochondrial proteins after depletion of the cellular glutathione stores.¹⁰ Lipid peroxidation resulting from oxidative stress contributes to the initiation and progress of liver damage.³ As a result of early event, oxidative stress markers are increased in acetaminophen induced hepatotoxicity.¹¹

Erythropoietin (EPO) is a renal glycoprotein hormone and which promotes the survival, proliferation, and differentiation of erythrocytic progenitors in hematopoietic tissues. EPO has also been recognized as an anti-apoptotic and tissue-protective pleiotropic cytokine.^{12,13} Sedopes et al. demonstrated the protective effects of erythropoietin in liver ischemia-reperfusion (I/R) injury in rats.¹⁴ The hyperglycosylated rHuEPO (recombinant human erythropoietin) analogue darbepoetin alfa has more sialic acid content compared to rHuEPO, resulting in longer (3-4 times) half life and a higher in vivo activity.^{13,15}

Although darbepoetin alfa is commonly used for correction of anemia in patients with chronic renal disease. It is postulated that darbepoetin alfa has neuro protective effect like that of rHuEPO because it binds same receptor with rHuEPO.¹⁶ It has been shown that treatment with darbepoetin in acute liver failure of mice also significantly abrogated necrotic cell death and liver enzyme release.¹² In the study, our aim was to investigate the effects of darbepoetin alfa administration in acetaminophen induced hepatotoxicity by evaluating various oxidative stress markers and liver histopathology.

MATERIAL and METHOD

This study was conducted according to the guidelines of the animal care review board of Istanbul University, Cerrahpasa Medical Faculty, in accordance with National legislation and The Council Directive of the European Communities on the Protection of Animals Used for

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Table 1: The results of biochemical parameters

Biochemical Parameters ----- Experimental Groups	Plasma MDA (nmol/L)	Plasma GSH (mg/g Hb)	Plasma Cu-Zn SOD (U/mL)	Plasma ALT (U/L)	Plasma AST (U/L)	Tissue MDA (nmol/g wet tissue)	Tissue Cu-Zn SOD (U/g wet tissue)
S (n=10)	2.10±0.18	3.70±0.14	21.74±1.95	27.30±2.82	37.27±3.37	1.11±1.07	291.38±16.86
A (n=10)	3.04±0.40 a**	1.83±0.53	23.96±2.45	79.39±11.28 a***	91.66±8.72 a***	45.78±42.01a**	1945.50±193.26 a***
ASD (n=10)	6.10±0.79 a***,b***	2.38±2.66	19.31±2.63 b**	80.04±2.23 a***	92.37±5.96 a***	23.66±23.14	1554.00±223.51 a***,b**
DA (n=10)	5.15±0.33 a***,b***,c**	2.70±0.26	18.97±2.35 b**	77.81±5.98 a***	90.10±7.23 a***	25.00±5.36	1458.00±237.15a***,b***
AD (n=10)	5.48±0.44 a***,b***	2.38±1.35	17.33±2.64 a*,b***	76.80±5.17 a***	94.21±5.63 a***	25.89±5.23	1385.00±211.59a***,b***

Significant differences defined with (*): p<0.05, (**): p<0.01, (***): p<0.001; Significance between the S group and the other groups defined with a; Significance between the A group and the other groups defined with b; Significance between the ASD and the other groups defined with c.

Experimental and Other Scientific Purposes (L358/1, November 24, 1986). Fifty adult male Sprague-Dawley rats weighing between 200-250 g were obtained from the Cerrahpasa Medical Faculty Experimental Animal Production and Research Laboratory. The rats were kept in standard colony cages (15x25x40cm) (3 or 4 rats per cage) under controlled conditions including temperature of 28 °C, light (10 h light to 14 h darkness), humidity 50% to 55%. The animals fed with standard rat chow and tap water ad libitum.

Experimental design

The rats were randomly divided into five equal experimental groups (n= 10): an acetaminophen-only (A) group, a (ASD) group that received darbepoetin at the same time with acetaminophen injection, a (DA) group that received darbepoetin 12 hour before acetaminophen injection, a (AD) group that received darbepoetin 12 hour later acetaminophen injection, a (S) sham group that received an equivalent volume of saline. The doses of acetaminophen and darbepoetin alfa were 800 mg/kg (single dose, intraperitoneally) and 10 µg/kg (single dose, subcutaneously) respectively for the experimental groups. The animals were killed by decapitation 24 hour after acetaminophen toxicity induction. Blood samples and liver specimens were collected for biochemical and histopathological analyses.

Liver was excised immediately and immersed in physiological saline. The plasma and the liver tissues were stored at - 80 °C until analysis. The blood samples were centrifuged for 5 min. at 1000 xg at 4 °C. All haemolytic blood samples were discarded. All reagents were analytical grade and purchased from Sigma Chemical Co.(St.Louis, MO, USA) and Merck (Darmstadt, Germany). Malondialdehyde (MDA) and Cu-Zn super oxide dysmutase (SOD) levels were measured in the plasma and the liver tissue. Glutathione

(GSH), aspartate aminotransferase (AST) and alanin aminotransferase (ALT) were assessed in plasma. Liver biopsies were fixed in 10% formaldehyde solution for histopathological analyses.

Biochemical Procedure

Preparation of tissue samples

About 190-200 mg of liver sample was weighed and diluted 20% w/v in 20 mM ice-cold tris-HCl, pH:7.4, and homogenized with a Bosch Scintilla SA, Switzerland. The homogenate was centrifuged at 5000g for 10 min, and various analyte determinations were performed in the supernatant fraction.

Assay of Plasma Aspartate Aminotransferas and, Alanin Aminotransferase

AST and ALT were measured by enzymatic methods using commercial kits (Olympus, Hamburg, Germany) on Olympus AU800 analyzer.

Assay of Cu-Zn SOD activity

Plasma and tissue Cu-Zn SOD activity was determined by the method of Sun et al.¹⁷ The assay involves inhibition of nitroblue tetrazolium (NBT) (Sigma chemical Co., St. Louis, USA) and reduction with xanthine-xanthine oxidase (Sigma chemical Co., St. Louise, USA) that was used as a superoxide generator. One unit of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. Intra and interassay coefficient of variation for GSH were 3.8% (n=10) and 3.9% (n=10), respectively.

Assay of malondialdehyde

Lipid peroxidation levels in plasma and tissue were→

Table 2: The results of histopathological parameters		
Groups	n	Histological scores
S	10	0 ± 0
A	10	2,62 ± 0,94*
ASD	10	0,96 ± 0,32
DA	10	1,42 ± 1,51
AD	10	2,28 ± 1,68*
*P<0,05		

measured with the thiobarbituric acid (TBA) reaction to according to the method of Buege and Aust.¹⁸ This method was used to obtain a spectrophotometric measurement of the color produced during the reaction to TBA with MDA at 535 nm. The coefficients of intra- and inter-assay variations for MDA assay were 3.3% (n= 10) and 5.2% (n= 10), respectively.

Assay of Glutathione

After centrifugation at 2500 g for 5 min, the plasma was removed and erythrocytes were washed three times in 5 mL of 9 g/L NaCl solution, hemolyzed by diluting fourfold with water and stored at -80 °C until GSH analyses. Erythrocytes GSH concentrations were measured by Beutler and Ellman method, respectively.^{19,20} One milliliter of erythrocyte preparation and tissue homogenate were deproteinized and then centrifuged at 600g for 20 min.

After addition of dithiobis-nitrobenzoate and phosphate buffer (pH 8.0) into clear supernatants of samples, the color developed was read at 412 nm. GSH concentration in samples was calculated by using 1, 36x10⁴ M- cm- as the molar absorption coefficient. Intra and interassay coefficient of variation for GSH were 3.4 % (n=10) and 3.5% (n=10), respectively.

Histopathological Procedure

The liver biopsies cut into longitudinal sections 2-4 mm in thickness. Liver tissue slices were then fixed in 10% buffered formalin and embedded in paraffin. Each section in 4µm thickness was stained with hematoxylin and eosin for light microscopic assessment. A certified pathologist scored samples in a blinded fashion.

An arbitrary scope was given to each microscopic field at a magnification of 20x, 40x, 100x. 10 representative areas from each section consisting of periportal and perivenous zones were examined and scored to obtain the mean value. Extents of necrosis and degeneration of hepatocytes around central vein were graded as

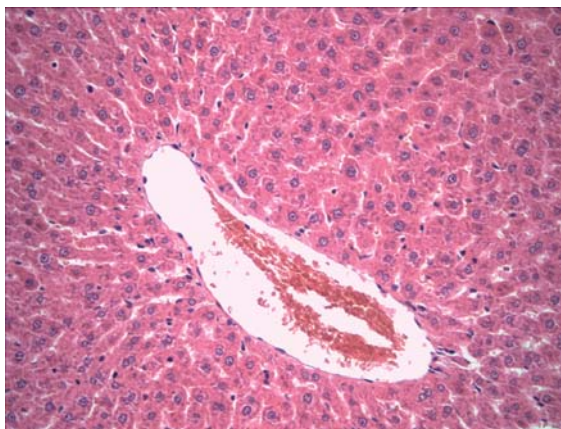


Figure 1. Normal liver histology

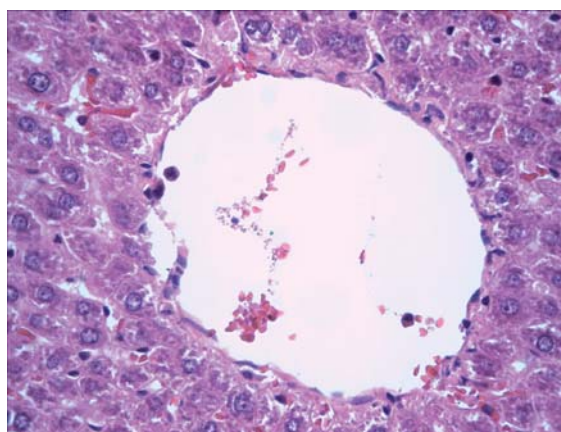


Figure 2. Pathologic changes in half of cells around the central vein.

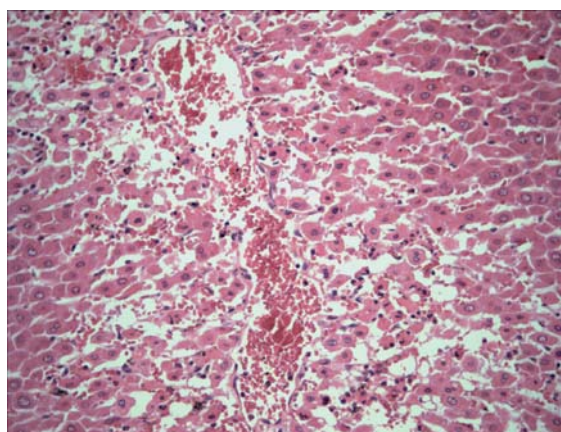


Figure 3. Marked pathologic changes around the central vein and in zone.

0= negative findings (Figure 1); 1= evidence of pathological changes in several hepatocytes; 2= pathologic changes in less than half of cells around central vein (Figure 2); 3= pathologic changes in more than half of cells around central vein; 4= marked pathologic changes around central vein and in zone 3 (Figure 3).

Statistical analysis

All the data are expressed as means and standard →

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deviations (means \pm SD) and 95% confidence intervals. The data was compared between groups using one-way ANOVA and post hoc Scheffe's test was used for statistical analysis. SPSS 12.0 (SPSS: Statistical Package for Social Sciences) was used for assessing the significance of differences between groups. $p < 0.05$ was considered significant.

RESULTS

The results of the biochemical parameters are summarized in the Table 1 with significances. Plasma MDA levels of the A ($p < 0.01$) group, the ASD ($p < 0.01$) group, the DA ($p < 0.001$) group and the AD ($p < 0.001$) group were significantly higher than the S group.

Plasma MDA levels of the ASD group, the DA group and the AD group were significantly higher than the A group ($p < 0.001$). Plasma MDA levels of the DA group was significantly lower than the ASD group ($p < 0.01$). There were no significances between the groups for plasma GSH levels. Plasma Cu-Zn SOD levels of the ASD group was significantly lower than the A group ($p < 0.01$). Plasma Cu-Zn SOD levels of the DA group was significantly lower than the A group ($p < 0.01$). Plasma Cu-Zn SOD levels of the AD group was significantly lower than the A group ($p < 0.001$). Plasma Cu-Zn SOD levels of the AD group was significantly lower than the S group ($p < 0.05$). Plasma AST and ALT levels of the A group, the ASD group, the DA group and the AD group were significantly higher than the S group ($p < 0.001$). Tissue MDA levels of the A group was significantly higher than the S group ($p < 0.01$). Tissue Cu-Zn SOD levels of the A group, the ASD group, the DA and the AD group were significantly higher than the S group ($p < 0.001$). Tissue Cu-Zn SOD levels of the ASD group ($p < 0.01$), the DA group ($p < 0.001$), and the AD groups ($p < 0.001$) were significantly lower than the A group.

There were no shown any histological changes in the S group (Figure 1). Acetaminophen caused central necrosis in the liver with infiltration of inflammatory cells in the A and AD group (Figure 3). Necrotic changes occurred to hepatocytes predominantly around the central vein and in zone 3. The ASD and DA groups showed lower histopathological scores than A group (Figure 2), without showing any statistical significance (Table 2).

DISCUSSION

Acetaminophen toxicity is a common cause of acute liver failure.² The generation of reactive oxygen species and NO, lipid peroxidation, mitochondrial dysfunction, disruption of calcium homeostasis, and induction of

apoptosis are all mechanisms which thought to be causes of acetaminophen induced hepatotoxicity.^{21,22} MDA is one of the end products of lipid peroxidation and is assayed as an index of membrane oxidative damage.²³ In our study, the lipid peroxidation, the liver enzymes and Cu-Zn SOD activity were increased after acetaminophen exposure. Histopathologic findings had supported the acetaminophen induced damage in the liver tissue (Figure 3). SOD metabolizes O_2 to H_2O_2 . During oxidant injury, O_2 accumulation was much greater. Therefore, SOD activity increased in order to detoxify O_2 . In some animal studies, SOD activity decreased during oxidant injury.²⁴⁻²⁶ However, in other experimental studies on oxidant injury increased SOD activities were reported.²⁷⁻²⁹ Cu-Zn SOD may be constitutively present only at low levels but is highly inducible under oxidative stress.³⁰

CONCLUSION

Protective effects of darbepoetin alfa have been demonstrated in various tissues.^{12,15,31} Belayev et al. have suggested that darbepoetin alfa confer behavioral and histological neuroprotection after focal ischemia in rats.¹⁵ In another study, renoprotective effects of darbepoetin alfa in ischemic acute renal failure have been reported.³¹ Akcora et al. showed the potential protective effects of darbepoetin alfa for preventing testicular injury caused by testis torsion.³² Le Minh et al. reported that pre and post treatment with darbepoetin attenuates inflammation and apoptosis in acute liver failure induced with D-galactosamine and E. coli lipopolysaccharide.¹² We have observed that darbepoetin administration increased plasma levels of MDA, and decreased plasma and liver tissue Cu-Zn SOD activity significantly. Darbepoetin alfa administration could not support the antioxidant parameters that we have assessed in acetaminophen induced hepatotoxicity and acted as an oxidant.

However, darbepoetin alfa administration before and at the same time with the induction of acetaminophen toxicity interfered the significant histopathologic damage which was observed in the darbepoetin administration after the induction of acetaminophen toxicity. Oxidative stress and lipid peroxidation are early events related to radicals generated during the hepatic metabolism of acetaminophen.³³ Increased oxidative stress could diminish liver functions and cause further structural liver tissue damage. Timing of darbepoetin alfa administration for liver toxicity could be an important factor for the efficacy of the treatment.

This current study forms a sound basis for further studies which would be done with immunohistochemical analysis and other oxidant/antioxidant parameters.



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