Original Article

Protective effects of fish oil, allopurinol, and verapamil on hepatic ischemia-reperfusion injury in rats

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Abstract

Background: The major aim of this work was to study the protective effects of fish oil (FO), allopurinol, and verapamil on hepatic ischemia-reperfusion (IR)-induced injury in experimental rats. **Materials and Methods:** Sixty male Wistar albino rats were randomly assigned to six groups of 10 rats each. Group 1 served as a negative control. Group 2 served as hepatic IR control injury. Groups 3, 4, 5, and 6 received N-acetylcysteine (standard), FO, allopurinol, and verapamil, respectively, for 3 consecutive days prior to ischemia. All animals were fasted for 12 h, anesthetized and underwent midline laparotomy. The portal triads were clamped by mini-artery clamp for 30 min followed by reperfusion for 30 min. Blood samples were withdrawn for estimation of serum alanine transaminase (ALT) and aspartate transaminase (AST) activities as well as hepatic thiobarbituric acid reactive substances, reduced glutathione, myeloperoxidase, and total nitrate/nitrite levels, in addition to histopathological examination. **Results:** Fish oil, allopurinol, and verapamil reduced hepatic IR injury as evidenced by significant reduction in serum ALT and AST enzyme activities. FO and verapamil markedly reduced oxidative stress as compared to control IR injury. Levels of inflammatory biomarkers in liver were also reduced after treatment with FO, allopurinol, or verapamil. In accordance, a marked improvement of histopathological findings was observed with all of the three treatments. **Conclusion:** The findings of this study prove the benefits of FO, allopurinol, and verapamil on hepatic IR-induced liver injury and are promising for further clinical trials.

Key words: Allopurinol, fish oil, ischemia-reperfusion, verapamil

INTRODUCTION

Liver injury is a frequent and multivariate phenomenon which can have dangerous and even fatal consequences. Liver damage involves in most cases oxidative stress and is characterized by a progressive evolution from steatosis to chronic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma.[1]

Hepatic ischemia-reperfusion injury (HIRI) can occur in the liver in response to a wide variety of clinical and operative situations, including hemorrhagic shock and severe hepatic trauma. HIRI can lead to liver dysfunction or even loss of function and thus represents a major therapeutic challenge.[2]

The pathogenesis of HIRI is multifactorial, involving hepatocellular Ca^{2+} overload, release of excessive oxygen-derived free radicals, inflammatory cytokines, Kupffer cell activation, impairment of microvessels, apoptosis, and nuclear factor kappa B.[3,4]

Oxidative stress plays an important mechanistic role in the progression of HIRI. When cells are deprived of oxygen, there is a rapid and massive fall in intracellular adenosine triphosphate (ATP) caused by impairment of oxidative phosphorylation.[5] ATP depletion reduces hepatic-reduced glutathione (GSH) synthesis and hence, oxidative stress takes place. Consequently, hepatocellular necrosis becomes the logic outcome.

Access this article online	
Quick Response Code:	Website: www.jnsbm.org
	DOI: 10.4103/0976-9668.160003

It is strongly suggested now that calcium accumulation is not just a result of hepatotoxicity but has a mechanistic hepatotoxic role as well. Even more, it may be considered as the primary event leading to hepatocellular necrosis.[6] Calcium ions also stimulate apoptosis via the initiation of the apoptotic cascade.[5] Hepatocytes contain several types of calcium channels.[7] Therefore, drugs that modulate calcium homeostasis, like calcium channel blockers and calmodulin antagonists, are targets for study as hepatoprotective agents, especially in cases when calcium accumulation is massive as in ischemia-reperfusion (IR) injury.

Recently, fish oil (FO) was found to play significantly protective roles in the liver, cardiovascular system, and kidney.[8] As expected, FO has been suggested to reduce the extent of tissue damage in HIRI by decreasing the oxidative stress.

Allopurinol has a well-known action in the blockade of xanthine oxidase (XO) enzyme, and it has a cytoprotective potential against injury provoked by reoxygenation.[9] Because Kupffer cells contain XO, which is one potential source of free radicals, allopurinol has been suggested to ameliorate hepatic injury by inhibition of XO and reduction of oxidative stress.

Verapamil is a slow calcium channel entry blocker. Based on the well-known role of calcium in the progression of ischemic injury in general, calcium channel blockade is expected to have a good protective role against HIRI.[10]

Based on the abovementioned facts, the present study was performed to investigate the possible hepatoprotective potentials of FO, allopurinol, and verapamil on HIRI in rats.

To achieve this goal, several types of parameters are to be measured. These include serum alanine transaminase (ALT), and aspartate transaminase (AST) activities to elucidate hepatocellular damage; hepatic contents of thiobarbituric acid reactive substances (TBARS) and reduced GSH to estimate oxidative stress; hepatic myeloperoxidase (MPO) and total nitrate/nitrite (NOx) as a measure of inflammation in addition to a histopathological study to confirm the results of the biochemical findings.

MATERIALS AND METHODS

Animals and treatments

Adult male Wistar albino rats of body weights ranging from 220 to 250 g were used. They were housed in the animal room in Pharmacology Department, Faculty of Pharmacy under conventional laboratory conditions on 12/12 h light/dark cycle and constant temperature (22°C ± 1°C). Throughout the study, food and water were supplied *ad libitum*. Exactly 12 h before IR operation, animals were fasted.[11] During fasting, animals were allowed free access to water and individually kept in separate cages with stainless steel mesh to avoid coprophagy. All experimental procedures were conducted in accordance with ethical procedures and policies approved by the Ethics Committee of Faculty of Pharmacy, Beni-Sueif University.

Chemicals, reagent kits, and apparatus

All chemicals and reagents used are of analytical grade. Allopurinol, Ellman's reagent, FO, *o*-dianisidine, malondialdehyde, reduced GSH, thiobarbituric acid, trichloroacetic acid, vanadium chloride, and verapamil were obtained from Sigma, USA. ALT and AST kits were obtained from Randox, UK. Sodium hydroxide and sodium nitrite were obtained from BDH, UK. Dipotassium hydrogen phosphate, potassium dihydrogen phosphate, and n-Butanol were obtained from Prolabo, France. Cetyltrimethyammonium bromide was obtained from Merck, Germany. Hydrogen peroxide was obtained from Loba Chemie, India. N-acetylcysteine (NAC) was obtained as a gift from SEDICO, Egypt. Formalin solution was obtained from El-Nasr Chemicals Co., Egypt. Tissue homogenization was performed using Ultra-Turrax T25, IKA Labortechnik, Germany. Measurement of optical density was performed by ultraviolet-150-02 double-beam spectrophotometer, Shimadzu, Japan.

Experimental design

After acclimatization period of 1-week, rats were divided into 6 groups, each of 6-10 rats, as follows:

- Group 1: Normal control group; receiving only vehicle and subjected to sham operation.
- Group 2: Hepatic IR control injury; receiving only vehicles and subjected to hepatic IR operation. Groups 3 through 6 were treated with standard or test agents on a daily basis for 3 consecutive days with the last dose administered 1 h prior to ischemia in the indicated doses.
- Group 3: (NAC; 1500 mg/kg/day, p.o)[12]
- Group 4: Received FO (300 mg/kg/day, p.o)[13]
- Group 5: Received allopurinol (50 mg/kg/day, p.o)[14]
- Group 6: Received verapamil (10 mg/kg/day, p.o).[15]

Blood and liver samples were collected soon after reperfusion.

Methods

Induction of hepatic ischemia-reperfusion injury

The IR model was performed according to the method previously described[16] with a slight modification. Rats

were fasted for 12 h before the operation, anesthetized and underwent midline laparotomy. The portal vein, hepatic artery, bile duct, and caudate hepatic lobe were freed by blunt dissection with the hepatoduodenal ligament separated. The portal vein, hepatic artery, and bile duct (portal triad) were clamped by mini-artery clamp for 30 min followed by reperfusion for 30 min. Appropriate clamping was confirmed by visual inspection of the ischemic lobes. During the period of hepatic ischemia, the animal's abdomen was covered with plastic wrap to prevent dehydration.

Assessment of hepatic injury

- Serum activities of ALT and AST enzymes were measured using test reagent kits as previously described by Reitman and Frankel.[17]
- Hepatic TBARS and GSH content was assessed in the liver homogenate according to the methods described previously.[18]
- Hepatic MPO activity was estimated according to the method described by Harada *et al.*[19]
- Total NOx production was assessed by the method described by Miranda *et al.*[20]
- Liver slides for histopathological study were prepared from the median lobes and stained with routine hematoxylin and eosin staining according to the method described by Bancroft and Steven.[21]

Statistical analysis

Analysis of the data was performed by one-way ANOVA, and subsequent analysis was performed by Tukey-Kramer multiple comparisons test using Instat version 2 computer program (GraphPad Software, Inc., San Diego, USA). The *P* values smaller than 0.05 were selected to indicate statistical significance between groups.

RESULTS

Serum alanine transaminase and aspartate transaminase activities

Induction of HIRI significantly raised serum ALT and AST enzyme activities as compared to normal control group. FO, allopurinol, or verapamil significantly reduced serum ALT and AST enzyme activities as compared to HIRI control. Values of serum ALT and AST were not significantly higher than standard treatment NAC in case of FO and verapamil [Table 1].

Oxidative stress biomarkers

The effect of FO, allopurinol, and verapamil on oxidative stress biomarkers is summarized in Table 2. FO and verapamil markedly reduced hepatic TBARS and markedly increased hepatic GSH content as compared to control IR injury. Allopurinol did not significantly affect the oxidative stress biomarkers TBARS and GSH as compared to control IR injury.

Liver myeloperoxidase and nitrate/nitrite

Fish oil, allopurinol, and verapamil significantly reduced liver inflammatory mediators MPO and NOx as compared to HIRI group [Table 3].

Histopathological findings

Liver section of HIRI group showed that the structure of liver lobules was severely damaged. Dilated and congested blood sinusoids were observed in addition to diffused

Table 1: Effect of FO, ALLO, and VERAP, as compared to standard treatment NAC on serum ALT and AST enzyme activities in adult male albino rats subjected to HIRI

Parameters	Sham control	Hepatic IR injury				
		Control	NAC	FO	ALLO	VERAP
Serum ALT activity (U/L)	24.7±1.86	128.0±8.92*	47.6±2.96*,@	60.0±5.59*,@	96.7±8.37*,@,#	52.4±3.87*,@
Serum AST activity (U/L)	126.0±7.09	505.1±32.66*	234.8±17.16*,@	222.5±15.53*,@	441.2±26.60*,@,#	226.9±18.43*,@

Data was expressed as mean of 6-10 rats ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey–Kramer multiple comparisons test. *Significantly different from sham-operated control value at $P<0.05$, @Significantly different from HIRI control value at $P < 0.05$, #Significantly different from standard treatment (NAC) value at $P < 0.05$. FO: Fish oil, ALLO: Allopurinol; VERAP: Verapamil; NAC: N-acetyl cysteine; ALT: Alanine aminotransferase; AST: aspartate transaminase; HIRI: Hepatic ischemia-reperfusion injury; SEM: Standard error of the mean

Table 2: Effect of FO, ALLO, and VERAP, as compared to standard treatment NAC on liver TBARS and reduced GSH levels in adult male albino rats subjected to HIRI

Parameters	Sham control	Hepatic IR injury				
		Control	NAC	FO	ALLO	VERAP
Liver TBARS (nmol/g wet tissue)	162.0±19.94	428.6±26.50*	246.2±17.34@	202.0±14.90@	370.7±40.74#	263.6±23.46@
Liver GSH (µmol/g wet tissue)	4.87±0.293	1.58±0.236*	4.49±0.303@	3.50±0.315*,@,#	1.74±0.239*,#	1.74±0.239*#

Data was expressed as mean of 6-10 rats±SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey–Kramer multiple comparisons test. *Significantly different from sham-operated control value at $P < 0.05$, @Significantly different from HIRI control value at $P < 0.05$, #Significantly different from standard treatment (NAC) value at $P < 0.05$. FO: Fish Oil; ALLO: Allopurinol; VERAP: Verapamil; NAC: N-Acetyl Cysteine; TBARS: Thiobarbituric acid reactive substances; GSH: Glutathione; HIRI: Hepatic ischemia-reperfusion injury; SEM: Standard error of the mean

cytoplasmic lipid vacuolations of hepatocytes with signet ring appearance [Figure 1a].

Nevertheless, the structure of liver hepatocytes was greatly restored after administration of FO. Almost hepatocytes are normal except few showing cytoplasmic vacuolations [Figure 1b].

A marked improvement of histopathological findings was observed in allopurinol treated group. Congested central vein (CV) with dilatation and massive congestion of blood sinusoids was observed. Moreover, hepatocytes showed cytoplasmic lipid vacuolations and some of them showed signet ring appearance [Figure 1c].

The structure of hepatocytes was greatly improved after administration of verapamil. Despite congested CV and blood sinusoids, almost hepatocytes were normal except few with cytoplasmic vacuolations [Figure 1d]

DISCUSSION

Results of the present study revealed that the induction of hepatic IR injury was associated with increased serum transaminases, as well as liver oxidative and inflammatory biomarkers. These results were further supported by histopathological examination. These findings are quite consistent with that of previous authors.[22] Oxidative stress affects membrane lipids as well as mitochondrial proteins leading to membrane injury, the loss of energy production and cellular ion control.[23] Dysfunction in energy-dependent metabolic pathways and transport mechanisms due to loss of mitochondrial respiration and subsequent reduction in ATP production was shown to be the leading factor in HIRI.[24]

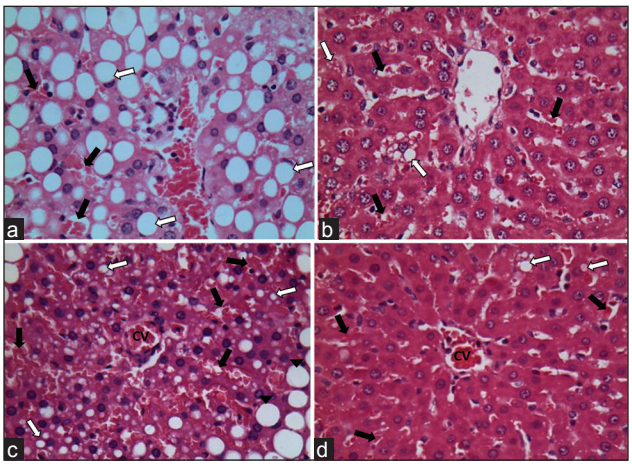

Figure 1: (a) A photomicrograph of liver section of hepatic ischemia-reperfusion injury control group. (b) A photomicrograph of liver section of fish oil-treated group. (c) A photomicrograph of liver section of allopurinol-treated group. (d) A photomicrograph of liver section of verapamil-treated group

Our data showed that supplying rats with FO for 3 consecutive days prior to HIRI significantly reduced hepatic IR injury as evidenced by reduced serum ALT and AST levels, reduced hepatic TBARS, MPO, and NOx levels, and increased hepatic GSH content. These findings are quite consistent with previous authors.[13] The hepatoprotective effects of FO could be attributed to the presence of omega-3 fatty acids which found to protect against ischemic injury in rats. Moreover, reduction of oxidative stress and severity of tissue damage due to modification of membrane fatty acids as well as modulation of both nitric oxide synthase activity and cyclooxygenase expression were suggested to be the mechanisms underlying this protective effect.[25]

In this study, allopurinol significantly improved HIRI as evidenced by significant reduction in serum ALT and AST enzyme activities. In addition, the inflammatory mediators MPO and NOx in liver were significantly reduced as compared to HIRI control group. There was also a marked improvement of histopathological findings. Allopurinol prevented liver injury by inhibition of free radical formation.[26] Another possible explanation for the beneficial effects of allopurinol is the preservation of hypoxanthine as a substrate to form ATP. Hypoxanthine is preserved through the blockade of XO and can form another possible explanation for the beneficial effects of allopurinol in the preservation of hypoxanthine as a substrate to form ATP.[27]

Verapamil administration improved serum ALT and AST levels and markedly corrected the oxidative stress

Table 3: Effect of FO, ALLO, and VERAP, as compared to standard treatment NAC on liver MPO and total NOx levels in adult male albino rats subjected to HIRI

Parameters	Sham control	Hepatic IR injury				
		Control	NAC	FO	ALLO	VERAP
Liver MPO (U/g wet tissue)	1.40±0.102	3.94±0.176*	2.27±0.155*,@	2.20±0.157*,@	1.87±0.211@,#	3.04±0.249*,@,#
Liver NOx (µmol/g wet tissue)	6.53±0.414	11.54±0.704*	7.88±0.652@	7.97±0.402@	9.84±0.890*,@,#	8.05±0.506@

Data was expressed as mean of 6-10 rats ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey-Kramer multiple comparisons test. *Significantly different from sham-operated control value at $P < 0.05$, @Significantly different from HIRI control value at $P < 0.05$, #Significantly different from standard treatment (NAC) value at $P < 0.05$. NAC: N-acetyl cysteine; FO: Fish oil; ALLO: Allopurinol; VERAP: Verapamil; HIRI: Hepatic ischemia-reperfusion injury; MPO: Myeloperoxidase; NOx: Nitrate/nitrite; SEM: Standard error of the mean

biomarkers TBARS and GSH in the liver as compared to control HIRI group. The inflammatory mediators MPO and NOx in liver were also significantly reduced. A marked improvement of histopathological findings was observed. The hepatoprotective effect of verapamil could be attributed to its calcium channel blocking activity. It blocks the calcium influx to hepatocytes through blockade of receptor-operated calcium channels present in Hepatocytes in addition to attenuation of chemo-attractant release by Kupffer cells after HIRI in the rat liver.[28] Some studies showed a correlation between the increase of intracellular calcium and oxidative stress injury.[29] Verapamil may also inhibit the overload of mitochondrial calcium, both in IR and normal conditions, as well as prevent the xanthine dehydrogenase to XO conversion in the cytoplasm.[30]

CONCLUSION

The present data support the beneficial effects of FO, allopurinol, and verapamil in management of HIRI as a liver injury model. The clinical significance of these results should be elucidated in further clinical studies.

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How to cite this article: Messiha BA, Abo-Youssef AM. Protective effects of fish oil, allopurinol, and verapamil on hepatic ischemia-reperfusion injury in rats. J Nat Sc Biol Med 2015;6:351-5.

Source of Support: Nil. **Conflict of Interest:** None declared.