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 Blood samples were withdrawn for estimation of serum alanine transaminase (ALT) and aspartate transaminase (AST) activities as well as hepatic thiobarbituric acid reactive substances,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 Ca2+ 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.
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.\[3\] 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 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.
- **Group 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 | Treatment |
|-------|-----------|
| 3     | (NAC; 1500 mg/kg/day, p.o)\[12\] |
| 4     | Received FO (300 mg/kg/day, p.o)\[13\] |
| 5     | Received allopurinol (50 mg/kg/day, p.o)\[14\] |
| 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\[14\] 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).

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 | Serum ALT activity (U/L) | Serum AST activity (U/L) |
|------------|-------------------------|-------------------------|
| Sham control | 24.7±1.86 | 120.0±8.92* |
| Control | 128.0±8.92* | 505.1±32.66* |
| NAC | 47.6±2.96** | 234.8±17.16** |
| FO | 60.0±5.59* | 222.5±15.53* |
| ALLO | 96.7±8.37*** | 441.2±26.60*** |
| VERAP | 52.4±3.87*** | 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                  | Liver TBARS (nmol/g wet tissue) | Liver GSH (μmol/g wet tissue) |
|-----------------------------|---------------------------------|-------------------------------|
| Sham control                | 162.0±19.94                     | 4.87±0.293                    |
| Control                     | 428.6±26.50*                    | 1.58±0.236*                   |
| NAC                         | 246.2±17.34**                   | 4.49±0.303*                   |
| FO                          | 202.0±14.90**                   | 3.50±0.315*                   |
| ALLO                        | 370.7±40.74**                   | 1.74±0.239*                   |
| VERAP                       | 263.6±23.46**                   | 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.

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
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]

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

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.
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.

REFERENCES

1. Kodavanti PR, Joshi UM, Young RA, Meydrech EF, Mehendale HM. Protection of hepatotoxic and lethal effects of CCl4 by partial hepatectomy. Toxicol Pathol 1989;17:499-505.
2. Pan LJ, Zhang ZC, Zhang ZY, Wang WJ, Xu Y, Zhang ZM. Effects and mechanisms of store-operated calcium channel blockade on hepatic ischemia-reperfusion injury in rats. World J Gastroenterol 2012;18:356-67.
3. Sato H, Takeo T, Liu Q, Nakano K, Osanai T, Suga S, et al. Hydrogen peroxide mobilizes Ca2+ through two distinct mechanisms in rat hepatocytes. Acta Pharmacol Sin 2009;30:78-89.
4. Huber N, Sakai N, Eisemann T, Shin T, Kuboki S, Blanchard J, et al. Age-related decrease in proteasome expression contributes to defective nuclear factor-kappaB activation during hepatic ischemia/ reperfusion. Hepatology 2009;49:1718-28.
5. Cooper BJ. Disease at the cellular level. In: Kitt T, editor. Text Book of Comparative General Pathology. ST. Louis: Mosby Company; 2002. p. 16-75.
6. Bernardi P, Rasola A. Calcium and cell death: The mitochondrial connection. Subcell Biochem 2007;45:481-506.
7. Gould RJ, Murphy KM, Snyder SH. Tissue heterogeneity of calcium channel antagonist binding sites labeled by [3H]nitrendipine. Mol Pharmacol 1984;25:235-41.
8. Fassett RG, Gobe GC, Peake JM, Coombs JS. Omega-3 polyunsaturated fatty acids in the treatment of kidney disease. Am J Kidney Dis 2010;56:728-42.
9. Andrade Jr DR, Andrade DR, Santos SA. Study of rat hepatocytes in primary culture submitted to hypoxia and reoxygenation: Action of the cytoprotectors prostaglandin E1, superoxide dismutase, allopurinol and verapamil. Arch Gastroenterol 2009;46:333-40.
10. Nauta RJ, Tsimoyiannis E, Uribe M, Walsh DB, Miller D, Butterfield JA. The role of calcium ions and calcium channel entry blockers in experimental ischemia-reperfusion-induced liver injury. Ann Surg 1991;213:137-42.
11. Xing HC, Li LJ, Xu KJ, Shen T, Chen YB, Sheng JF, et al. Intestinal microflora in rats with ischemia/reperfusion liver injury. J Zhejiang Univ Sci B 2005;6:14-21.
12. Wang T, Qiao S, Lei S, Liu Y, Ng KF, Xu A, et al. N-acetylcysteine and allopurinol synergistically enhance cardiac adiponectin content and reduce myocardial reperfusion injury in diabetic rats. PLoS One 2011;6:e23967.
13. Mardones M, Valenzuela R, Romanque P, Covarrubias N, Anghileri F, Fernández V, et al. Prevention of liver ischemia reperfusion injury by a combined thyroid hormone and fish oil protocol. J Nutr Biochem 2012;23:113-20.
14. Lee WY, Koh EJ, Lee SM. A combination of ischemic preconditioning and allopurinol protects against ischemic injury through a nitric oxide-dependent mechanism. Nitric Oxide 2012;26:1-8.
15. Jan M, Faqir F, Hamida, Mughal MA. Comparison of effects of extract of Myristica fragrans and verapamil on the volume and acidity of carbachol induced gastric secretion in fasting rabbits. J Ayub Med Coll Abbottabad 2005;17:69-71.
16. Colletti LM, Remick DG, Burch GD, Kunkel SL, Strieter RM, Campbell DA Jr. Role of tumor necrosis factor-alpha in the pathophysiological alterations after hepatic ischemia/reperfusion injury in the rat. J Clin Invest 1990;85:1936-43.
17. Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. Am J Clin Pathol 1957;28:56-63.
18. Mihara M, Uchiyama M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem 1978;86:271-8.
19. Harada N, Okajima K, Kushimoto S, Iseho B, Tanaka K. Antithrombin reduces ischemia/reperfusion injury of rat liver by increasing the hepatic level of prostacyclin. Blood 1999;93:157-64.
20. Miranda KM, Espey MG, Wink DA. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 2001;5:62-71.
21. Bancroft GD, Steven A. Theory and Practice of Histological Techniques. London: Churchill Livingstone Company; 2007.
22. Çekin AH, Gür G, Türkoglu S, Aldemir D, Yilmaz U, Gürsoy M, et al. The protective effect of L-carnitine on hepatic ischemia-reperfusion injury in rats. Turk J Gastroenterol 2013;24:51-6.
23. Masabuchi Y, Suda C, Horie T. Involvement of mitochondrial permeability transition in acetaminophen-induced liver injury in mice. J Hepatol 2005;42:110-6.
24. Yapur K, Kart A, Karapelahvan M, Atakosi O, Tunca R, Erginsoy S, et al. Hepatoprotective effect of L-carnitine against acute acetaminophen toxicity in mice. Exp Toxicol Pathol 2007;59:121-8.
25. Radosinska J, Bacova B, Bernatova I, Navarova J, Zhukovska V, Shysh A, et al. Myocardial NOS activity and connexin-43 expression in untreated and omega-3 fatty acids-treated spontaneously hypertensive and hereditary hypertiglyceridemic rats. Mol Cell Biochem 2011;347:163-73.
26. Marotto ME, Thurman RG, Lemasters JJ. Early mitozonal cell death during low-flow hypoxia in the isolated, perfused rat liver: Protection by allopurinol. Hepatology 1988;8:585-90.
27. McDord JM. Oxygen-derived free radicals in posts ischemic tissue injury. N Engl J Med 1985;312:159-63.
28. Ray SD, Kamendulis LM, Gurule MW, Yorkin RD, Corcoran GB. Ca2+ antagonists inhibit DNA fragmentation and toxic cell death induced by acetaminophen. FASEB J 1993;7:453-63.
29. Murata M, Monden M, Umeshiba K, Nakano H, Kanaï T, Gotoh M, et al. Role of intracellular calcium in superoxide-induced hepatoocyte injury. Hepatology 1994;19:1223-8.
30. Ishii K, Saita S, Sumimoto H. Effect of verapamil on conversion of xanthine dehydrogenase to oxidase in ischemic rat liver. Res Exp Med (Berl) 1990;190:389-99.

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