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Prevention of Ischemia-Reperfusion-Induced Oxidative Injury in Liver by Allopurinol and Pentoxifylline

Background: We investigated the effect of pentoxifylline (PTX) and allopurinol (AP) on ischemia-reperfusion (I/R) injury of rat liver. Rats were divided into four groups. I/R group: 30 minutes ischemia and then 20 minutes reperfusion was applied to the rat liver tissue. PTX group: Pentoxifylline (50 mg/kg) was injected i.p 10 minutes before reperfusion. PTX+AP group: PTX (50 mg/kg) and allopurinol (50 mg/kg) were given i.p 10 minutes before reperfusion. Control group: I/R was not done. This group was accepted as control group.

Results: Malondialdehyde (MDA) levels (nmol/g tissue) in the I/R group (18.29 ± 0.84) were found to be significantly higher than the control group (15.30 ± 0.66) ($p < 0.05$). MDA in the PTX group (15.92 ± 0.73) and the PTX+AP group (15.18 ± 0.71) were significantly decreased as compared to I/R group ($p < 0.05$). Reduced glutathione (GSH) levels (nmol/mg prot) in the I/R group (26.82 ± 0.76) were found to be significantly lower than in the control group (39.35 ± 2.0) ($p < 0.001$). GSH were found to be significantly increased in the PTX group (35.31 ± 3.15) and PTX+AP group (41.57 ± 1.54) as compared to I/R group ($p < 0.05$ in PTX group, $p < 0.001$ in PTX+AP group). Catalase activity (U/mg prot.) was found to be significantly low in the I/R group (39.11 ± 3.59) as compared to control group (74.65 ± 2.85) ($p < 0.001$). Catalase in the PTX group (68.48 ± 2.97) and PTX+AP group (76.91 ± 2.34) were found to be significantly increased as compared to I/R group ($p < 0.001$). In the PTX+AP group, GSH and catalase were higher than PTX group ($p < 0.05$, respectively).

Conclusions: Our findings have shown that PTX and PTX plus AP combined therapy affected enzymatic and non-enzymatic defence mechanisms in I/R of rat liver. Furthermore, PTX and combined therapy decreased peroxidative injury. Combined therapy was more effective than the PTX administration.

Key words:

ischemia/reperfusion, allopurinol, pentoxifylline, oxidative stress

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Allopurinol und Pentoxifyllin zum Schutz der Leber vor Ischämie-Reperfusion-induzierter oxidativer Schädigung

Hintergrund: Es sollte die Wirkung von Pentoxifyllin (PTX) und Allopurinol (AP) auf den Ischämie-Reperfusionsschaden (I/R-Schaden) in der Rattenleber untersucht werden. Die Ratten wurden in vier Gruppen eingeteilt. I/R-Gruppe: Das Gewebe der Rattenleber wurde einer 30-minütigen Ischämie ausgesetzt, gefolgt von 20

Minuten Reperfusion. PTX-Gruppe: Pentoxifyllin (50 mg/kg) wurde i.p. 10 Minuten vor Reperfusion injiziert. PTX+AP-Gruppe: PTX (50 mg/kg) und Allopurinol (50 mg/kg) wurden i.p. 10 Minuten vor Reperfusion verabreicht. Kontrollgruppe: Es wurde keine I/R durchgeführt. Diese Gruppe wurde als Kontrollgruppe akzeptiert.

Ergebnisse: Die Malondialdehyd (MDA)-Spiegel (nmol/g Gewebe) in der I/R-Gruppe ($18,29 \pm 0,84$) waren signifikant höher als in der Kontrollgruppe ($15,30 \pm 0,66$) ($p < 0,05$). MDA in der PTX-Gruppe ($15,92 \pm 0,73$) und in der PTX+AP-Gruppe ($15,18 \pm 0,71$) war im Vergleich zur I/R-Gruppe signifikant niedriger ($p < 0,05$). Die geringeren Glutathion (GSH)-Spiegel (nmol/mg prot.) in der I/R-Gruppe ($26,82 \pm 0,76$) waren signifikant niedriger als in der Kontrollgruppe ($39,35 \pm 2,0$) ($p < 0,001$). GSH war in der PTX-Gruppe ($35,31 \pm 3,15$) und in der PTX+AP-Gruppe ($41,57 \pm 1,54$) signifikant erhöht im Vergleich zur I/R-Gruppe ($p < 0,05$ in der PTX-Gruppe, $p < 0,001$ in der PTX+AP-Gruppe). Für die Katalasenaktivität (U/mg prot.) ergab sich ein signifikant niedrigerer Wert in der I/R-Gruppe ($39,11 \pm 3,59$) im Vergleich zur Kontrollgruppe ($74,65 \pm 2,85$) ($p < 0,001$). Katalase in der PTX-Gruppe ($68,48 \pm 2,97$) und PTX+AP-Gruppe ($76,91 \pm 2,34$) war signifikant höher im Vergleich zur I/R-Gruppe ($p < 0,001$). Für die PTX+AP-Gruppe ergaben sich bei GSH und Katalase höhere Spiegel als in der PTX-Gruppe ($p < 0,05$).

Schlussfolgerungen: Unsere Ergebnisse haben gezeigt, dass eine PTX-Therapie und eine Kombinationsbehandlung mit PTX plus AP Einfluss auf die enzymatischen und nicht-enzymatischen Verteidigungsmechanismen bei I/R in der Rattenleber ausüben. Darüber hinaus verringerten PTX- bzw. Kombinationstherapie die peroxidative Schädigung. Die kombinierte Therapie war dabei wirkungsvoller als die alleinige Gabe von PTX.

Schlüsselwörter:

Ischämie/Reperfusion, Allopurinol, Pentoxifyllin, oxidativer Stress

Introduction

Recent advances in liver transplantation present a serious challenge to the traditional treatment of chronic, ultimately fatal liver disease (1-2). Ischemia-reperfusion is an unavoidable process in liver transplantation and many cases of liver surgery (3-4). It has been reported that oxygen-derived free radicals play a crucial role in the pathogenesis of ischemic-reperfusion injury in many tissues, such as brain, heart, kidney, intestinal mucosa and liver (5-6). There are several reports already in the literature suggesting that xanthine oxidase is a major source of the reactive oxygen

species produced during reperfusion of ischemic liver (6-7). Reactive oxygen species such as superoxide anion (O_2^-), hydroxyl radical ($\bullet OH$) and hydrogen peroxide (H_2O_2) lead to peroxidation of lipids. Malondialdehyde (MDA) is the one of the end-products of lipid peroxidation. There are several enzymatic and non-enzymatic antioxidant systems such as catalase (E.C.1.11.1.6) and reduced glutathione (GSH) in the cell (8-10).

Protection of the liver against ischemia injury is a major concern in hepatic surgery, because it is often necessary to interrupt blood supply in the various surgical conditions, such as treatment of

liver tumors, vascular lesions or trauma to the liver (2). Various studies have demonstrated that different agents have beneficial effects on different organs after I/R injury, such as scavenger enzymes of superoxide radical, antioxidants and XO inhibitors (1). Allopurinol is a competitive inhibitor of XO. It is as effective as oxygen radical scavengers in the prevention of ischemic injury (6,11). Pentoxifylline is a methylxanthine derivative that has been successfully used in the treatment of chronic occlusive arterial disease. Pentoxifylline has also been used in other diseases related to decreased tissue oxygenation (12). Besides, pentoxifylline has recently been identified as an inhibitor of free radical generation (13). In the present study, using a rat model, the effect of PTX and PTX plus AP combined therapy on the antioxidant status of rat liver in I/R process was investigated. For this aim, we measured catalase, GSH and MDA levels in ischemic-reperfused rat livers.

Materials and Methods

Thirty-two female wistar albino rats (180-250 g body wt) were used in our study and were maintained on a basal diet and water. The animals were anesthetized with Urethan (800 mg/kg) by intraperitoneal injection (i.p.) and their body temperature was kept between 36.5 and 37.5 °C with a heating lamp. A carotid artery was cannulated. The arterial and portal venous blood supply to the left lateral and median lobes of the liver was then interrupted with a clamp for 30 minutes. After the period of ischemia, the clamp was removed and the liver was reperfused for 20 minutes. Rats were divided into four groups: 1) Control group (n=8): Ischemia-reperfusion was not done in this group of rats. 2) Ischemia-reperfusion group (n=8): All surgical procedures were done. However, a corresponding volume of saline solution was given to these animals. 3) Pentoxifylline group (n=8): PTX (50 mg/kg) was injected i.p. 10 minutes before reperfusion. 4) PTX + Allopurinol group (n=8). PTX (50 mg/kg) and allopurinol (50 mg/kg) were given i.p. 10 minutes before reperfusion into this group of rats.

At the end of the reperfusion period, the liver tissues were taken for the measurement of GSH, MDA and cata-

lase. The tissues were washed with physiological saline three times and they were stored in a deepfreeze at -25 °C until assay. The tissues were homogenized in phosphate buffer (1 M, pH=7) for catalase determination and in potassium chloride buffer (0.15 M) for MDA and GSH analysis with an ultraturax (T 25, Janke Kuntel, KA Labor technique). The homogenates were then sonicated with a Bandelin Sonopuls HD 70. After the homogenized materials had been centrifuged, the supernatant fractions were removed. The protein levels were analyzed by the colorimetric method of Lowry, using a commercial kit. Plasma alanine aminotransferase (ALT) levels were measured with Auto-analyser (BM-Hitachi 911) using colorimetric method. The levels of MDA in the liver were determined by a colorimetric reaction with the thobarbituric acid according to the method of Okhawa et al. (14). The catalase activity was determined according to the method of Beutler (15). The measurement of liver tissue GSH levels was measured according to the method of Ellman (16).

Statistical analysis was done by the student's t test and all data were expressed as mean ± SEM (standard error). Differences with p values of less than 0.05 were considered to be statistically significant.

Results

Plasma alanine aminotransferase levels are shown in table 1. ALT levels increased in the other three groups as compared to the control group (p < 0.001). There was no statistically significant difference between PTX group, PTX+AP group and I/R group.

MDA and GSH levels and Catalase activities in the liver tissue are shown in table 2. MDA levels were significantly increased in the I/R group as compared to control group (p < 0.05). MDA levels in the PTX and PTX+AP group were significantly lower than I/R group (p < 0.05). GSH levels and catalase activities were significantly decreased in the I/R group as compared to the control group (p < 0.001). On the contrary, GSH levels in the PTX and PTX+AP groups were significantly increased as compared to the I/R group (p < 0.05 and p < 0.001, respectively). In both treatment groups, catalase activities were

Tab. 1: The plasma ALT levels of control, I/R, PTX and PTX+AP groups (Mean ± SEM)

	ALT (U/L)
Control group (n = 8)	50.37 ± 4.69
Ischemia-reperfusion group (n = 8)	380.71 ± 75.24 ^a
Pentoxifylline treated group (n = 8)	310.22 ± 60.58 ^a
Pentoxifylline and allopurinol treated group (n = 8)	261.50 ± 51.56 ^a

a: p < 0.001 as compared to control group

Tab. 2: The liver tissue MDA, GSH levels and catalase activities of control, I/R, PTX and PTX+AP groups. (Mean ± SEM)

	MDA (nmol/gr tissue)	GSH(nmol/mg protein)	CATALASE (U/mg protein)
Control group (n = 8)	15.30 ± 0.66	39.35 ± 2.00	74.65 ± 2.85
Ischemia-reperfusion group (n = 8)	18.29 ± 0.84 ^a	26.82 ± 0.76 ^c	39.11 ± 3.59 ^c
Pentoxifylline treated group (n = 8)	15.92 ± 0.73 ^b	35.31 ± 3.15 ^b	68.48 ± 2.97 ^d
Pentoxifylline and allopurinol treated group (n = 8)	15.18 ± 0.71 ^b	41.57 ± 1.54 ^{d,e}	76.91 ± 2.34 ^{d,e}

a: p < 0.05 as compared to control group

b: p < 0.05 as compared to ischemia-reperfusion group

c: p < 0.001 as compared to control group

d: p < 0.001 as compared to ischemia-reperfusion group

e: p < 0.05 as compared to pentoxifylline and allopurinol group

significantly higher than I/R group (p < 0.001). In the PTX+AP group, GSH levels and catalase activities were significantly higher than PTX group (p < 0.05).

Discussion

It is proposed that xanthine dehydrogenase converts xanthine oxidase in ischemic tissues. XO in the presence of hypoxanthine or xanthine reduces molecular oxygen to O₂⁻ and H₂O₂. It is demonstrated that the enzyme can further reduce H₂O₂ to •OH. The liver, being rich in xanthine oxidase, would appear to have the potential for generation of high levels of oxygen species during post-ischemic reperfusion (7,17).

Peroxidation of lipids and denaturation of proteins caused by reactive oxygen species are involved in at least part of ischemic cellular damage (2). In our

study, plasma ALT levels as a marker of tissue damage in liver increased in I/R group as compared to the control group (p < 0.001). Moreover, MDA level in liver, which is indicator of peroxidative injury, was increased significantly in the I/R group as compared to the control group (p < 0.05). Okboy et al. have reported that MDA level in liver tissue was increased by reperfusion after 6 minutes (1).

GSH is a major intracellular thiol. It has an important role in the protection of the cell from the peroxidation injury which is caused by the antioxidant agents (6). The concentration of hepatic GSH decreases progressively during ischemia (5-6) with a corresponding increase in oxidized glutathione (GSSG). The ratio of GSSG/GSH increases significantly when the liver is exposed to ischemia (6). In our study, GSH levels of I/R group were lower than control group (p < 0.001).

Catalase catalyzes the reduction of H_2O_2 to water and molecular oxygen (18). In our study, catalase activities in the I/R group were decreased significantly as compared to the control group ($p < 0.001$). It has been suggested that during reperfusion, XO generates H_2O_2 (6,19). In our study, decreased liver catalase activities in I/R may depend on this phenomenon. Brown et al. have shown that erythrocytes decrease myocardial hydrogen peroxide levels and reperfusion injury (19,20). They suggested that red blood cells decreased reperfusion injury by GSH and catalase-dependent mechanisms. In our previous study, we indicated that erythrocyte catalase activities were decreased reperfusion following ischemia in the cardiopulmonary bypass (18).

In this study, MDA levels in the PTX and PTX+AP groups were decreased as compared to the I/R group ($p < 0.05$, respectively). There was no statistical difference between control and therapy groups. Low GSH levels in the I/R group of rat livers were increased in both therapy groups ($p < 0.05$ in the PTX group; $p < 0.001$ in the PTX+AP group). Besides, increase in the PTX+AP group was higher than in PTX group ($p < 0.05$). Catalase activities in the therapy groups were higher than I/R group ($p < 0.001$, respectively). Also as compared to the PTX treatment group, catalase activity in the combined therapy group was increased ($p < 0.05$).

Pentoxifylline has been recently identified as an inhibitor of free radical generation (12). In animal models and in vitro studies of human blood, high concentrations of pentoxifylline have significantly reduced superoxide anion production by leukocytes (13). On the other hand, PTX might decrease polymorphonuclear leukocyte adhesiveness to capillary endothelial cells. Decreased endothelial adherence of activated polymorphonuclear (PMN) leukocytes could decrease the severity of reperfusion injury, due to decreased superoxide radical release at the PMN leukocyte endothelial surface (12).

Several studies have reported that inhibitors of xanthine oxidase prevent reperfusion injury. In addition, it is reported that allopurinol induces antioxidant enzymes and scavenges reactive oxygen species directly (6,21-22). Parks et al. (6) have reported that allopurinol scavenges hydroxyl radical and hypochlorous acid (HOCl). Peterson et

al. have indicated that allopurinol acts as electron transfer agent and thereby reduces tissue injury through facilitation of electron transport during reperfusion (23). In our study, because pentoxifylline and allopurinol, particularly PTX+AP combined therapy, have a scavenger effect on reactive oxygen species, these agents could prevent catalase enzyme activity, GSH level and could reduce lipid peroxidation depending on reactive oxygen species scavenger effect, additional to the inhibitory effect of allopurinol on xanthine oxidase. Sahin et al. reported that allopurinol increased catalase activity and decreased MDA levels in I/R process (18). These results are similar to our findings.

In conclusion, our findings have shown that ischemia-reperfusion causes oxidant stress and peroxidative injury in liver tissue. Because allopurinol (50 mg/kg) and pentoxifylline (50 mg/kg) are effective on I/R mediated oxidant stress and peroxidative injury, pentoxifylline and pentoxifylline plus allopurinol combined therapy have a beneficial effect in the prevention of post-ischemic reperfusion injury. Combined therapy was more effective than the PTX therapy. We think that combination of these drugs must be examined in hepatic surgery.

Abbreviations

ALT	Alanine aminotransferase
AP	Allopurinol
H_2O_2	Hydrogen peroxide
•OH	Hydroxyl radical
I/R	Ischemia/Reperfusion
MDA	Malondialdehyde
PTX	Pentoxifylline
PMN	Polymorphonuclear
GSH	Reduced glutathione
$O_2^{\cdot-}$	Superoxide dismutase
XO	Xanthine oxidase

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