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Effects of alpha tocopherol and verapamil on liver and small bowel following mesenteric ischemia-reperfusion

Alfa tokoferol ve Verapamilin, mezenterik iskemi reperfüzyonuda karaciğer ve ince barsak üzerine etkileri

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Background/aims: *We have previously shown that alpha tocopherol is a potent antioxidant which prevents reperfusion injury in a kidney and small bowel autotransplant model. In this study, the effect of systemic alpha tocopherol and verapamil on small bowel and hepatic functions following mesenteric ischemi-reperfusion was evaluated.* ***Methods:*** *Fourty male Wistar Albino rats (weight, 250-300 g) all subjected to an ischemia-reperfusion experiment were divided into four groups of 10 as follows: Group 1: (SHAM), Group 2, given prophylactic and systemic alpha tocopherol, Group 3: given verapamil and Group 4: given (both verapamil and alpha tocopherol).* ***Results:*** *Glutaminase activities 120 minutes after reperfusion were found to be significant in liver tissues (p=0.004). The highest to the lowest glutaminase activities in liver tissue at 120 minutes after reperfusion were in Group 1, Group 3, Group 2 and Group 4 respectively. Significant differences in MDA levels were found in the small bowel at 30 minutes and 120 minutes time points (p<0.05). There were statistically significant higher glutaminase levels at 30 minutes and 120 minutes of reperfusion in the small bowel, especially in Group 4 (p=0.005).* ***Conclusion:*** *Both small bowel and liver injuries reperfusion, can be decreased by prophylactic use of alpha tocopherol and verapamil. Glutaminase activity in liver tissue can also be affected by small bowel ischemia-reperfusion.*

Keywords: Alpha tocopherol, verapamil, antoxidants, ischemi/reperfusion.

Amaç: *Alfa tokoferolün böbrek ve ince barsak dokusundaki reperfüzyon zedelenmesini önleyici etkisi, köpeklerde yapılan deneysel ototransplant modelinde gösterilmiştir. Bu çalışmada sistemik alfa tokoferol ve verapamilin mezenterik iskemi reperfdüzyon sonrasında ince barsak ve karaciğer fonksiyonları üzerine etkisi araştırılmıştır.* ***Yöntem:*** *Çalışmada ağırlıkları 250-300 mg arasında değişen Wistar Albino cinsi ratlar kullanılmıştır. Deneysel çalışma her biri 10 denekten oluşan ve tümüne mezenterik iskemi uygulanan 4 grupta planlandı (Grup 1:kontrol grubu, Grup 2: profilaktik ve sistemik alfa tokoferol, Grup 3:verapamil, Grup 4:verapamil+alfa tokoferol).* ***Bulgular:*** *Reperfüzyon sonrasında 120.dk' da alınan karaciğer dokusundaki glutaminaz aktiviteleri Grup 4'te anlamlı olarak yüksek bulundu (p=0.004). Ayrıca 30 ve 120.dk' larda ince barsak dokusundaki MDA düzeyleri,Grup 4' te anlamlı olarak düşük bulundu (p<0.05). Ayrıca 30.dk'da ince barsak dokusundaki glutaminaz aktiviteleri Grup 4'te anlamlı olarak daha yüksekti.* ***Sonuç:*** *Mezenterik iskemi-reperfüzyona bağlı olarak gelişince barsak ve karaciğer fonksiyon bozukluklarında, profilaktik alfa tokoferol ve verapamil koruyucu olabilmektedir. Ayrıca karaciğer dokusundaki glutaminaz aktivitesi ince barsak iskemi-reperfüzyonundan etkilenebilmektedir.*

Anahtar Sözcükler: Alfa tokoferol, verapamil, antioksidanlar, iskemi/reperfüzyon.

INTRODUCTION

Mesenteric ischemia/reperfusion (IR) is one of the most important causes of acute hepatic cellular injury (1). Reduction of hepatic blood flow due to circulating or paracrine vasoconstrictors, is considered to be responsible for this injury (2).

Hepatic blood flow is reduced by systemic hemodynamic changes or paracrine vasoconstrictor agents such as angiotensin II, after intestinal ischemia-reperfusion (3, 4). Multiple organ failure remains an important cause of death after trauma (5) and there are many studies which emphasise that the gut is the central organ in the pathogenesis of multiple organ failure. It is considered that oxidative reactions due to free oxygen radicals are responsible for both cellular and tissue injuries (6, 7). Both reactive oxygen metabolites and lipid peroxides are known to be the most important causes of multiple organ failure in shock. Impairment of redox balance in tissues accomparies a decrease of

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the amounts in both alpha tocopherol and glutathion and many investigations emphasise that the use of different antioxidants prolongs survival in sepsis and multiple organ failure (8-10).

We have previously shown that alpha tocopherol, as a potent antioxidant, prevents reperfusion injury in a kidney autotransplant model (11). The addition of alpha tocopherol to standard Euro Collins solution is also reported provide more effective protection against reperfusion injury in a canine small bowel autotransplantation model (12). Verapamil, another antioxidant agent is also known to protect against free radical damage, if present, during the ischemic period , especially in calcium rich tissues (13,14). One of the different mechanisms attributed to verapamil is its inhibitory effect on the conversion of xanthine dehydrogenase to xanthine oxidase (13). The protective effects of alpha tocopherol and verapamil on small bowel and hepatic function after mesenteric ischemi-reperfusion, however, has not been well investigated and this study was therefore undertaken to evaluate the effects of those agents.

MATERIAL AND METHODS

Fourty male Wistar Albino rats weighing 250 g to 300 g were given no food for 12 hours before the experiments, but allowed to drink water freely. There were then divided into four groups of 10: Group 1 (SHAM), Group 2 (prophylactic systemic alpha tocopherol), Group 3 (verapamil) and Group 4 (both verapamil and alpha tocopherol). The rats were anaesthetised with ketamine and midline laparotomy was performed. The superior mesenteric artery (SMA) was carefully dissected microscopically and a non-crushing vascular clamp was used to provide mesenteric ischemia for 120 min. Prior to ischemia and at 30 min and 120 min after reperfusion, tissue samples were obtained from the small bowel (distal ileum and midjejeunal segments) and liver (different part of left lobe) to study glutaminase activity. The amount of lipid peroxidation in small bowel tissue was measured at 30 min and 120 min time points, serum AST, ALT, LDH and bilirubin levels were assessed by taking blood from the tail veins of the animals 30 min and 120 min after reperfusion as was bile flow via a catheter (PE 50) inserted into the common bile duct. At the end of 120 min the animals were killed. Ten animals which died during the operation were excluded from the study. The average temperature of the operating room was kept at 21°C.

Administration of α-Tocopherol (α-T) and verapamil

10 mg/kg a-T (Sigma, natural alpha tocopherol crystalline, 1210 I.U/g) and 2.5 mg/kg verapamil (Knoll, 5 mg/2 ml) in 0.5 ml saline was administered intraperitoneally before the operation (13,14).

Measurement of amount of lipid peroxidation

The amount of lipid peroxidation was calculated with the use of Uchiyama's Thiobarbituric Acid method (TBA) (15). Weighed tissue in 25 mM Tris Hcl (pH 7.0) buffer was homogenated in an ice bath then 0.5 ml of homogenates (10% concentration) were mixed with 3 ml of 1 H_3PO_4% and 1 ml 6% TBA and stored in boiling water for 45 min. After cooling and adding 4 ml n- butanol to the tubes, they were shaken and centrifuged. Colour in n - butanol phase was read at 532 nm in spectrophotometry (Schimadzu UV 120-2 spectrophotometry). The amount of lipid peroxide was calculated with the use of molar absorbtivity malonylaldehide colour. Lipid peroxide levels were expressed as nanomoles per gram of tissue.

Homogenate preparations and glutaminase assays

For the assessment of mucosal glutaminase, maltase and lactase activity, frozen tissues were weighed and the mucosa was stripped from a 0.5 cm long segment of the graft transferred into 1ml phosphate buffer (containing 1 mM EDTA, pH 7.8) and stored on ice until homogenization. Homogenization was performed with polytron homogeniser at 4000 rpm for one min on ice. Homogenate was then centrifuged at 1500 g at 4° C for 15 min. The method defined by Windmueller (16) was utilized for the determination of glutaminase. Glutaminase activity was expressed as the amount of glutamate (µmol) produced in the assay during one hr/mg protein.

Measurement of bile flow

Bile flow has been shown to be a sensitive marker of acute hepatic injury (17). Following laparotomy and just prior to mesenteric non-crushing vascular clamp application the common bile duct was cannulated at the level of the portal hilus by micro-dissection. Bile was collected in preweighed vials and measured gravimetrically for volume. After a 30 min equilibration period, bile flow rates were assessed by measuring the volume of bile collected during a 15 min period. Results were reported as the volume of bile collected in ml per 15 min per 100 g.

Table 1. Glutaminase activities (mmol/h/mg protein) and MDA levels (MDA/g tissue) in liver tissue 120 min after reperfusion were found to be significant in liver tissues

	Glutaminase activities (µmol/h/mg protein)
	Mean±SD(min-max)*
Group 1 (n=10)	2.09±0.37 (1.59-2.70)
Group 2 (n=10)	1.03±0.15 (0.80-1.18)
Group 3 (n=10)	1.66±0.26 (1.40-2.10)
Group 4 (n=10)	0.98±0.16 (0.80-1.20)**
	MDA levels (MDA/g tissue)
	Mean±SD(min-max)*
Group 1 (n=10)	124±23 (99-147)
Group 2 (n=10)	101±12 (86-113)
Group 3 (n=10)	106±22 (89-131)
Group 4 (n=10)	89±16 (80-1.20)†

*Standard deviation
**p=0.004, Kruskal Wallis one way ANOVA
† p=0.004, Kruskal Wallis one way ANOVA

Statistical analysis

To analyze the results, Kruskall Wallis one way ANOVA and Mann Whitney U tests were used and values above 0.05 were reported as significant.

RESULTS

In all groups, with regard to both MDA levels and glutaminase activities, no significant difference was found in either liver or small bowel tissues taken just prior to ischemia. Although MDA levels in liver were not statistically different at 30 min, those at 120 min were found to be the highest in Group 1and the lowest in Group 4 (p=0.004) (Table 1). Also, MDA levels in small bowel tissues obtained prior to ischemia did not show any significant difference (p>0.05). No significant differences in glutaminase activities in liver tissues were found 30 min after reperfusion. However, glutaminase activities in liver tissues 120 min after reperfusion were found to be significant (p=0.004) and the highest to the lowest activity level was found in Group 1, Group 3, Group 2 and Group 4 respectively (Table 1).

Although no differences in MDA levels in the small bowel were found prior to ischemia, significant differences were seen at 30 min and 120 min time points (p<0.05) (Table 2).

Table 2. A. Amounts of lipid peroxidation in small bowel tissues (MDA /g tissue)

	Normal	*30 min*	*120 min*
Group 1 (n=10)	51 ±1.2	55.5 ±5.6	64.1 ±3.36
Group 2 (n=10)	50 ±2.2	56.2 ±5.9	66.3± 9.6
Group 3 (n=10)	49±0.89	55.5± 3.4	64.12 ±3.5
Group 4 (n=10)	48 ±3.4	50.12 ± 2.5	53.8± 8.2**

B. Glutaminase activities showing significant differences at 30 and 120min, in small bowel tissues (µmol/hour/mg protein)

	30 minute†	*120 minute‡*
Group 1 (n=10)	2.73 ± 0.29	2.08 ±0.58
Group 2 (n=10)	3.05± 0.08	3.45± 0.39
Group 3 (n=10)	2.94 ±0.70	3.71± 0.66
Group 4 (n=10)	3.73± 0.21	3.30 ±0.13

*Standard deviation
**p<0.05, Kruskal Wallis one way ANOVA
†p=0.03, ‡p=0.04, Kruskal Wallis one way ANOVA

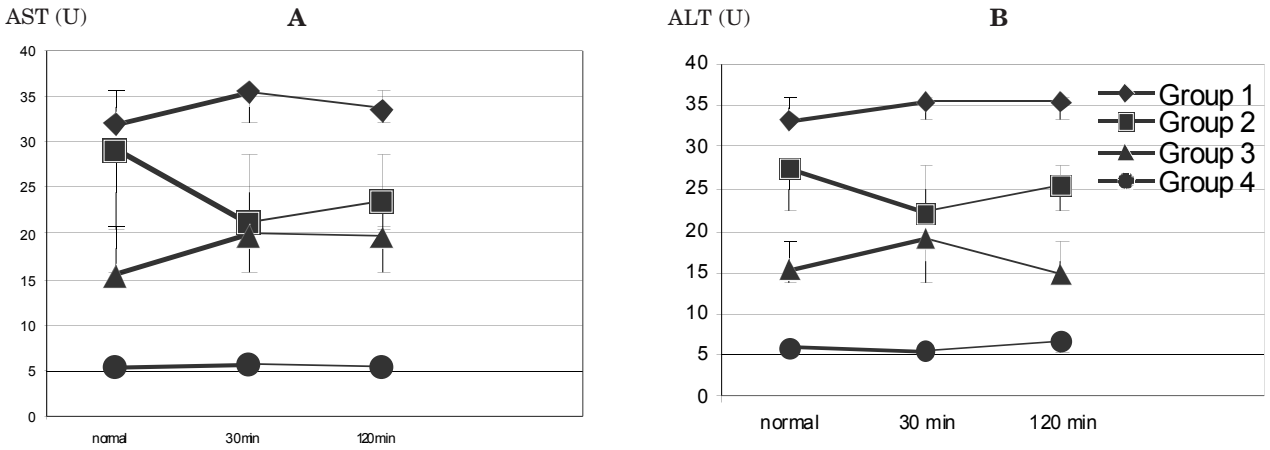

Figure 1. Serum AST (A) and ALT (B) level changes with time (U)

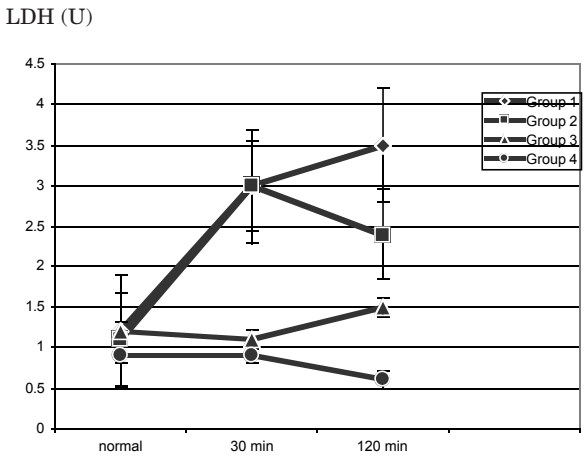

Figure 2. Serum LDH changes with time in groups (U)

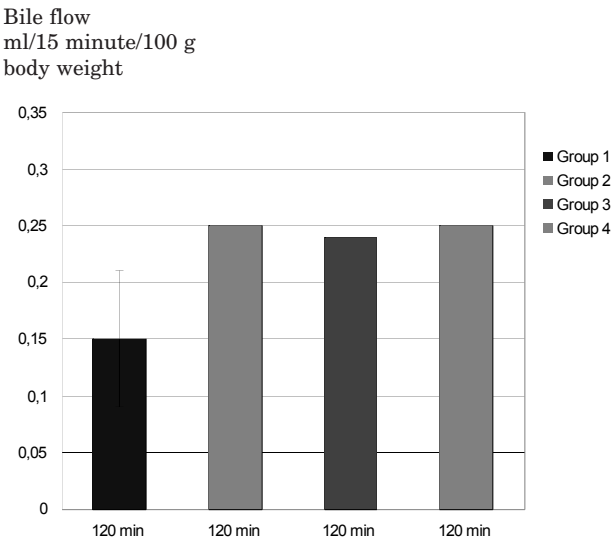

Figure 3. Values of bile flow rates after 120 min (ml/15 minute/100 g body weight)

There was a significant difference in the glutaminase level at 30 min of reperfusion in small bowel (p=0.005), with a higher level of glutaminase activity in Group 4 than in Group 2, Group 3 and Group 1 respectively. At 120 min the level of glutaminase activity was found to be greater in Group 3 than in Group 2, 4, and 1 respectively (p=0.003) (Table 2). In all groups, both serum AST and ALT levels showed significant differences before ischemia and also at 30 min and 120 min after reperfusion (p=0.001). At all time points, serum AST and ALT levels were found to be highest, especially in Group 1, and lowest in Group 4. Serum LDH levels prior to ischemia were not significant (p>0.05), but at 30 min and 120 min showed a progressive decrease (p=0.0004). Similar serum total bilirubin levels were found prior to ischemia. After 30 min and 120 min of reperfusion, serum total bilirubin levels with the greatest increase were found in Group 1, Group 2, Group 3 and Group 4 respectively (p=0.002) (Figure 1,2). There was no significant change bile flow at up to 30 min of reperfusion in all groups while at 120 min of reperfusion, bile flow rates in Group 1, 2, 3 and 4 were 0.15±0.05, 0.25±0.06, 0.24±0.05 and 0.25±0.08 respectively as ml/15 min/100 g body weight (p=0.04) (Figure 3).

DISCUSSION

It is well known that mesenteric ischemia-reperfusion causes acute hepatic cellular injury and this type of injury liver is an important of multiple organ failure (1). Recently, the gastrointestinal system has been thought to be the central organ of multiple organ failure (18, 19). Potent circulating vasoactive substance such as angiotensin II have been shown to affect splanchnic blood flow which is thought to be an important causative factor of splanchnic vasoconstriction and mesenteric hypoperfusion. The subsequent consequent reperfusion injury yields arachidonic acid metabolites which affect many organ systems (20). Experimentally, Pogetti et al (21) showed that hepatic dysfunction was caused by small bowel ischemia-reperfusion. It established that hepatic blood flow is decreased by both systemic hemodynamic changes and paracrine vasoconstrictor agents released after mesenteric ischemia-reperfusion (3, 4). However, not only complement activation but also tumor necrosis factor has been shown to be responsible for reduced hepatic blood flow. Also, it has been shown experimentally that increased intraabdominal pressure profoundly reduce hepatic blood flow (22- 24). On the basis of these previous findings in the literature, hepatic blood flow monitoring with the use of doppler ultrasonography was not considered appropriate in this study. The aim of our experimental model, was to induce pathological changes due to mesenteric ischemia-reperfusion both on the small bowel and the liver tissues. It is known that experimental mesenteric ischemia for more than 45 min causes irreversible mucosal epithelial damage and a animal mortality of almost 100% (21). Turnage et al induced a 120 min ischemia period on the small bowel in an acute cellular hepatic injury model due to mesenteric ischemia-reperfusion (25). On the basis of this model, a microvascular clip was placed across the proximal superior mesenteric artery for 120 min to investigate the effects of different antioxidants on small bowel and liver tissues after mesenteric ischemia-reperfusion.(26).

Several kinds of antioxidants have been used to prevent ischemia-reperfusion injury. Superoxide dismutase and allopurinol have been shown to be increase liver regeneration after ischemia-reperfusion produced by directly cross-clamping the hepatic pedicle (27). In transplantation surgery in particular, it is considered this type of injury is decreased with the addition of different antioxidant agents to preservation solutions (superoxide dismutase, allopurinol etc.)(28,29). We have previously shown that the addition of alpha tocopherol to standard Euro Collins solutions decreased reperfusion injury in the canine small bowel autotransplantation model (12). The preventive effects of alpha-tocopherol and glutathion peroxidase in kidney, liver and myocardial tissues has been demonstrated in different experimental models (11, 30, 31). The preventive effects of calcium channel antagonists on reperfusion injury in small bowel and liver tissues has also been proven (32).

Hepatocellular injury due to intestinal ischemia-reperfusion is characterized by both elevated serum transaminase levels and decreased bile flow (4, 30). In this study, tissue glutaminase levels in liver were also evaluated. Glutaminase found in Disses space in the liver is thought to be valuable in determining liver function. This enzyme is important not only for glutamine metabolism and ammonium formation, but also the ornitine – urea cycle. In sepsis, the amount of glutamine uptake from the systemic circulation is increased by liver (33). The present authors therefore considered that glutaminase activity in liver tissue might be a parameter for the assessment of hepatocellullar injury due to small bowel ischemia-reperfusion. Although decreases of serum AST, ALT, LDH and total bilirubin levels at 120 min were attributed to the protective effects of verapamil and alpha tocopherol in all groups, changes in glutaminase levels in the liver were not in keeping with this trend.

Bile flow rates were found to be similar in Groups 2, 3 and 4, while the significantly lower bile flow rates found in Group 1 may have been related to the antioxidant effects of alpha tocopherol and verapamil. Thus it can be concluded that the use of alpha tocopherol and verapamil together does not effect bile flow rate positively.

There are different studies in which mucosal glutaminase activity was used as an indicator in the assessment of small bowel ischemia-reperfusion injury (12, 34). Glutaminase activity reflects enterocyte loss and concomitant mucosal absorptive functions and decreased glutaminase activity is related to the degree of small bowel ischemia-reperfusion injury (34, 35). It was on this basis that glutaminase activity as an indicator of small bowel ischemia-reperfusion injury was used in the present study.

The absence of any differences between tissue samples obtained from liver and small bowel prior to ischemia may have been due to the use of homogeneous rats. According to our results, glutaminase activity in liver tissue was only affected by small bowel ischemia-reperfusion, after 120 min. When used either alone or together alpha tocopherol and verapamil may have negative effects on the prevention of reperfusion injury in liver tissue that are not yet to be defined. In the present study, the lowest MDA levels at 120 min in Group 4 can be speculated as a synergistic interaction between alpha-tocopherol and verapamil.

According to our results, , the highest mucosal glutaminase activity at 30 min in Group 4 may be due to a maximum protective effect of alpha tocopherol at this time point. However, owing to the maximum protective effect of verapamil at 120 min, we found the highest mucosal glutaminase activity at the same time point. In small bowel, there were no difference between the protective effect of alpha tocopherol at 30 min and that of verapamil at 120 min. Explanations for this may include the later protective effect of verapamil in small bowel. The decrease in glutaminase activity with combination therapy was interesting. It was shown that verapamil and alpha tocopherol may not act synergistically thorough RNA polymerase in isolated rat liver nuclei (36). The amount of lipid peroxidation with the greatest decrease in Group 4 may due to synergistic effects of both alpha tocopherol and verapamil on small bowel tissue.

In conclusion, both small bowel and liver injuries, caused by mesenteric ischemia-reperfusion can be decreased by the prophylactic use of alpha tocopherol and verapamil. Glutaminase activity in liver tissue can also be affected by small bowel ischemia-reperfusion, but further investigations areneeded to show whether this enzyme may be used as an indicator of hepatocellular injury due to reperfusion. Alpha tocopherol and verapamil may act synergistically in the prevention of small bowel reperfusion injury but further studies are required to confirm this. But this requires further confirmations.

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