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The effects of a - tocopherol and verapamil on mucosal functions after gut ischemia / reperfusion*

intestinal iskemi/reperfüzyon sonrasında a - tokoferol ve verapamilin mukozal fonksiyonlar üzerine etkisi

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Background/Aims: *We have previously shown that in the canine small bowel autotransplantation model, adding a - tocopherol to Euro Collins solution for perfusion enhanced the integrity of the small bowel mucosa for up to 12 hours in the posttransplantation period. The purpose of this study was to investigate the effects of verapamil and a tocopherol on reperfusion injury in the canine small bowel autotransplantation model.* ***Methods:*** *The study consisted offour groups of six animals each. In Group 1 (control group) grafts were perfused with Euro Collins solution while those of Group 2 were perfused with Euro Collins and a- tocopherol. Group 3 grafts were perfused with Euro Collins + verapamil and Group 4 with Euro Collins, a- tocopherol and verapamil. Autotransplantation model was used to avoid immunological injury. Graft function and mucosal integrity were assessed by the analysis of enzymatic activities (mucosal glutaminase, maltase and lactase) and histopathological examination. Malonyldialdehyde (per gram tissue) was used as an indicator of lipid peroxidation.* ***Results:*** *Glutaminase activity of Group 4 was found to be higher than that of the other groups at all time points ($p<0.05$). In the verapamil group (Group 3), the amount of lipid peroxidation showed the greatest decrease in comparison to other groups at the 12 hour time point ($p<0.05$). Maltase activity in Group 3 was significantly different at all time points ($p<0.05$). Lactase activity showed no significant difference between groups at each time point. The microscopic appearance of tissue injury shown by histopathological examination of tissue samples obtained at different time points was related to decrease in mucosal enzymatic activities.* ***Conclusions:*** *a- tocopherol is a promising agent for the prevention of tissue injury caused by free oxygen radicals during organ transplantation. Verapamil, as an antioxidant, also has preventive effects in organ preservation. Using verapamil together with a- tocopherol in the same preservation solution did not increase the preventive effect of a— tocopherol.*

Key words: Antioxidant, Alpha-tocopherol, Verapamil, Reperfusion, Autotransplantation

Amaç: *Daha önce yaptığımız çalışmamızda, a - Tocopherol (a T)' ün Euro Collins (EC) solüsyonuna perfüzyon için eklenmesinin ince barsak mukozasının bütünlüğünün korunmasında post-transplant dönemde 12. saate kadar etkili olduğu gösterilmiştir. Köpeklerde yapılan bu deneysel çalışmada, verapamil ve a T 'ün reperfüzyon zedelenmesine etkisi, ince barsak ototransplant modelinde incelenmiştir.* ***Yöntem:*** *Çalışma her biri 6 denekten oluşan toplam 4 grupta planlandı. Grup l'de (kontrol grubu) greftler EC solüsyonu ile perfüze edilirken, Grup 2'de EC+a T ile perfüze edildi. Grup 3'te perfüzyon için EC+verapamil kullanılırken, Grup 4'te EC+a T+verapamil kulanıldı. immünolojik zedelenmeyi ekarte etmek için ototransplantasyon modeli kullanıldı. Greft fonksiyonları ve mukoza bütünlüğünün değerlendirilmesi, enzimatik aktivitelere (mukozal glutaminaz, maltaz ve laktaz) ve histopatolojik incelemelere dayanılarak yapıldı. Lipit peroksidasyonunun değerlendirilmesi için gram doku başına malonildialdehit düzeyine bakıldı.* ***Bulgular:*** *Örneklerin alındığı tüm zamanlarda Grup 4'te glutaminaz aktivitesi diğer gruplardan yüksekti ($p<0.05$). Grup 3'te lipit peroksidasyonu miktarı 12.saatte en az düzeye inmiştir ($p<0.05$). Grup 3'te maltaz aktivitesi bütün zamanlarda farklılık gösterdi ($p<0.05$). Bütün gruplarda laktaz aktivitesi tüm zamanlarda farklılık göstermedi. Enzim aktivitelerini saptamak için aynı zaman periyotlarında ince barsaklardan alınan örneklerin histopatolojik incelemesi de mukozal enzimatik aktivitelerdeki azalmayı teyid eder özellikteydi.* **Sonuç:** *a T serbest oksijen radikallerine bağlı doku zedelenmesinin önlenmesinde etkili bir ajandır. Verapamil de bir antioksidan olarak organ prezervasyonund etkilidir. Verapamilin a T ile birlikte EC solüsyonu içinde kullanılması a Tün koruyucu etkinliğini artırmamaktadır.*

Anahtar kelimeler: Antioksidan, alfa-tokeferol, veramapil, repervizyon, ototransplantasyon.

INTRODUCTION

Free oxygen radicals generated during reperfusion cause serious tissue injury after revascularization in transplantation (1-2). Injury due to reperfusion is more severe than the ischemic type and is known to have a negative effect on preservation time in transplantation surgery (3). Several agents have been used to eliminate oxygen radicals during organ transplantation (4,5). Previous

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experimental studies have shown that successful preservation of the small bowel is possible up to six hours with Euro Collins (EC) solution (6). Experimentally, oc-tocopherol (a T), an antioxidant, has been shown to decrease cellular injury in liver ischemia/reperfusion (7). We have previously shown that a T has a protective effect against free oxygen radicals generated during reperfusion on small bowel tissue in the canine autotransplantation model (8). Verapamil, a calcium channel antagonist, has been demonstrated as having an antioxidant effect in small bowel reperfusion injury when used together with EC (9). However, there is also controversy as to whether a T and verapamil act synergisticlly in preventing reperfusion injury (10,11). In this study, we investigated the efficacy and interaction of these agents on reperfusion injury in the canine small bowel autotransplantation model. Also we considered whether both a T and verapamil, when used together, may increase the six hour's cold preservation time, which is one the most important measures of small bowel transplantation.

METERIALS AND METHODS

Experimental technique

The canine small bowel autotransplant model was used in this study. Male mongrel dogs weighing 15 kg and age 2-3 years were fasted for a minimum of eight hours before surgery. General anesthesia was induced with ketamine (5 mg/kg) and xylazine (2.5 mg/kg intramuscularly) and maintained with repeated doses of ketamine (15 mg/kg). Intravenous access was established with a venous catheter placed in a branch of the femoral vein. After a midline laparotomy was performed, the superior mesenteric artery and vein were dissected. Careful attention was given to not damage the inferior pancreaticoduodenal and inferior mesenteric branches of the superior mesenteric vein. The common colic and inferior pancreaticoduodenal branches of the superior mesenteric artery were also protected. A graft including the entire small bowel was removed from the abdomen, with warm ischemia time never exceeding two minutes. At the end of six hours of perfusion and preservation, a second laparotomy was performed, and the superior mesenteric vein and artery were anastomosed, respectively. Vein anastomosis was performed by 7.0 polypyline suture, also 7.0 polypyline suture materal was used for arterial anastomosis. Proximal continuity of the autotransplanted graft was achieved through a jejunojejunostomy by means of 5.0 silk suture, while the distal end of the graft was anastomosed to the skin, creating an ileostomy.

Experimental groups

The study included for four groups of six animals each. In all groups the small bowel was taken out of the abdomen by transection of the superior mesenteric artery and vein. Group 1 (control group) grafts were perfused with EC solution (1000 ml) which contained: potassium dihydrogen phosphate (2.05 g), potassium monohydrogen phosphate (7.40 g), potassium chloride (1.12 g), sodium bicarbonate (0.84 g), glucose monohydrate for injections (0.84 g) and 35.00 g anhydrous glucose for injections (38.50 g) (Fresenius Ag Bad Hamburg). Group 2, Group 3 and Group 4 were perfused respectively with EC + a T (30 mmol/L) , EC + verapamil (25 mg/L), and EC + a T (30 mmol/L) + verapamil (25 mg/L). All grafts were preserved for six hours in 200 ml of these solutions at 4°°C, followed by autotransplantation.

Perfusion and preservation

Intravascular perfusion via the superior mesenteric arterial catheter was carried out with the clear perfusion solution entering from the superior mesenteric vein. Intraluminal perfusion was performed following intravascular perfusion. During the six - hour cold preservation period, animals were awakened from anesthesia. After preservation and following repeat intraluminal and intravascular perfusion, the graft was autotransplanted into the animals after they were reanesthetized. The intravascular perfusion pressure was maintained between 25-30 mm Hg.

Parameters

Intestinal biopsies were taken immediately before the warm ischemia period (normal tissue activity) and at O minutes, 30 minutes, 1 hour, and 12 hour's posttransplantation. For the evaluation of mucosal integrity, mucosal enzymatic activities (glutaminase, maltase, lactase) were measured. Also, malonyldialdehyde (MDA) levels were measured for lipid peroxidation, and a histopathological examination was performed for each time point. Samples at 12 hours of reperfusion were obtained from the edge of the ileostomy. Histopathological examination was made using

the grading system described by Scholten and Sonnino (12,13).

Homogenate preparations

For the assessment of mucosal glutaminase, maltase and lactase activity, after weighing of frozen tissues, the mucosa was stripped from a 0.5 cm segment of the graft transferred into ImI phosphate buffer (containing 1 mM EDTA, pH 7.8) stored on ice until homogenization. Homogenization was performed with polytron homogenizer at 4000 rpm for 1 min on ice. Homogenate was then centrifuged at 1500 g at 4°C for 15 minutes.

Mucosal enzyme assay

The method defined by Windmueller was utilized for the determination of glutaminase (14). Glutaminase activity was expressed as the amount of glutamate (mmol) produced in the assay during 1 hr/mg protein. Mucosal maltase and lactase activity were determined by the method described by Dahlquist and activity was calculated from the amount of glucose produced per hour and expressed in U/g protein (15).

Lipid peroxidation

The amount of lipid peroxidation was calculated using Uchiyama's thiobarbituric acid method (TBA) (16). Weighed tissue in 25 mM Tris HcI (pH 7.0) buffer was homogenated in ice bath. 0.5 ml of homogenates (10% concentration) were mixed with 3 ml % 1 H3PO4 and 1 ml 6% TBA and stored in boiling water for 45 minutes. After cooling and adding 4 ml n- butanol to the tubes, they were shaken and then centrifuged. Color 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 the molar absorptivity malonydilaldehyde color. Lipid peroxide levels were expressed as nanomoles per gram tissue.

Histopathological examination

Full thickness small bowel biopsies were fixed in 10% buffered formalin and stained with hemotoxylin-eosin for microscopic examination. The degree of reperfusion injury was classified as follows: O, normal mucosa with only mild edema; 1 (mild), apical sloughing of the villi, variable congestion or edema of the lamina propria and otherwise intact mucosa; 2 (moderate), epithelial damage involving at least upper half of the villi, variable edema, congestion and hemorrhage of the lamina propria and sparing the crypt area; 3 (severe), epithelial damage with total villus sloughing and loss of crypt. The histopathological examination was made in a blinded fashion by the pathologist.

Statistical analysis

Kruskall Wallis one-way ANOVA test was used to analyze data obtained at designated sampling times. Differences between repeated measurements of enzymatic activity and lipid peroxidation were assessed by repeated measurements of ANOVA.

RESULTS

The data for enzyme activity and lipid peroxidation are shown in Table 1. There was no statistical difference among the four groups regarding enzymatic activity and lipid peroxidation in biopsies taken just prior to warm ischemia, except in lactase activity. Glutaminase activity of the a T +verapamil group was found to be higher than that of other groups at O and 30 minutes and 1 and 12 hour time points. The amount of lipid peroxidation of the verapamil group was the lowest at the 12 hour time point ($P<0.05$). Maltase activity showed the highest level at O minutes and 12 hours in Group 3, while the greatest decrease was found in Group 1 at 12 hours ($P<0.05$). Lactase activity of the groups was the same at all time points.

Histopathological examination of samples taken at different times (normal, O, 30 minutes and 1 and 12 hours) revealed that there were eight grade 3 in Group 1 and three grade 3 in Group 2 and four grade 3 in Groups 3 and 4. The distributions of histopathological grades of groups did not differ significantly at any time point ($P>0.05$) (Table 1 and 2).

DISCUSSION

Small bowel dysfunction always accompanies both motility and absorption disorders due to malnutrition and electrolyte imbalance. The commonest causes are massive resections with loss of much of the small intestine resulting in short bowel syndrome. Small bowel transplantation is an alternative treatment in this situation (6,17,18). It is a difficult to protect small bowel tissue, which is

Table 1. Glutaminase (mmol/hour/mg protein), maltase (mmol/hour/mg protein), the amount of lipid peroxidation malonyldialdehyde level/gram tissue), and lactase (unit/gram tissue) activities in groups

	Normal	O min	30 min	1 hour	12 hour
Glutaminase					
Group 1	3.1 ±1.0	2.9± 1.07	2.62 ±0.49	2.21± 1.07	1.38± 0.49
Group 2	3.0±0.9	3.56±0.69	3.55±0.62	2.98±0.43	2.33±0.86
Group 3	3. 14 ±1.1	3.0± 1.0	3.0 ±0.9	2.9 ±0.38	2.7 ±0.5
Group 4	3.0+1.2	3.8+0.14*	3.7±0.1*	3.3±1.14*	3.0±0.1*
Lipid peroxidation					
Group 1	52.0± 8.2	55.5 ±5.9	52.8± 2.29	64.1± 3.28	75.7 ±5.6
Group 2	52.0±7.9	56.2±5.9	61.8±2.0	66.0±8.9	75.7±4.5
Group 3	53.8±2.7	50.5+2.0	60.3±1.8	64.0±3.5	63.7±2.8*
Group 4	52.0±8.2	52.5±13.9	56.5±14.1	66.4±10.8	69.1+11.1
Maltase					
Group 1	48.0± 7.0	47.2± 8.0	41.6 ±3.8	42.8± 7.1	21.3± 9.6*
Group 2	49.0± 10.1	51.2± 7.3	44.2± 9.6	46.9 ±7.7	33.7± 10
Group 3	73.2 ±8.6	73.2 ±9.1 *	64.9± 10	64.2 ±11.3	73 ±4.2*
Group 4	52± 8.2	59.8± 4.1	59.2 ±6.0	57.7 ±13.7	43.4± 3.1
Lactase					
Group 1	7.91± 2.3	9.0± 1.35	7.75±0.6	8.07+ 0.65	4.4± 0.81
Group 2	8.0± 2.4	9.24 ±1.99	7.53± 1.1	7.59±1.9	4.5 ±2.6
Group 3	6.2 ±5.4	7.1±0.32	6.78± 0.92	5.88± 0.14	4.5± 0.6
Group 4	7.5± 1.41	8.4 ±0.85	9.0 ±1.5	7.45± 1.1	6.89 ±0.9

Mean ±SD **

*statistically significant, **standard deviation

rich in lymphoid cells and proteases, from the injuries related to either ischemia or reperfusion.The amount of lipid peroxidation following reperfusion is closely related to the duration of cold preservation time (19). Several agents have been used to eliminate the injurious effects of free oxygen radicals. We used the canine small bowel autotransplant model as defined by Lillehei to avoid confounding issues of immunologic injury (20). Oishi and Sarr showed that sufficient length of transplanted graft prevents diarrhea due to symphathetic denervation. At least an 80 cm graft is necessary (21). In our study autotransplanted grafts were 100 cm in length. Hamamoto et al showed that success ful preservation time of up to six hours was possible using EC solution (22). In clinical practice EC, University of Wisconsin (UW) and Ringer's lactate have been used for graft preservation. Although some investigators have used UW solution, there are others who prefer either UW or EC (23-28). It has been shown that UW alone, does not prevent reperfusion injury unless it is used with superoxide dismutase (29). Takara et al showed better results with EC experimentally, after simple hypothermic cold storage for 24 hours (30), thus it was our preference for preservation. In the same study, it was shown that addition of verapamil to preservation solution prolonged the cold ischemia period. Verapamil also has beneficial effects on hepatic ischemia /reperfusion injury in rats (31). Recent studies demonstrated it was shown that addition of T to commercial EC solution improved graft function and decreased lipid peroxidation in the canine small bowel and kidney autotransplantation models (8,32). In our study addition of verapamil to standard EC solution decreased the amount of lipid peroxidation significantly, but use of verapamil with a T in EC caused no decrease.

Post-transplantation graft dysfunction is due to lymphatic disruption, denervation and immunological and reperfusion injury. De Bruin et al showed that there was no nutritional deficit except decrease in serum triglyeride level after

Table 2. The distributions of histopathological grades of groups were not significantly different at any time point (P>0.05).

	Grades				
	Normal	O min	30 min	1 hour	12hour
Group 1	0	1	1	1	2
	0	0	2	2	3
	0	1	2	2	3
	1	2	3	3	3
	1	2	2	3	3
	1	2	2	2	3
Group 2	0	1	2	2	3
	0	2	2	2	2
	0	1	1	2	2
	1	1	1	2	3
	1	1	2	2	2
	0	2	2	2	3
Group 3	1	1	1	2	3
	1	2	1	1	3
	0	1	1	2	2
	0	1	1	2	3
	1	1	2	2	2
	1	2	2	2	2
Group 4	0	1	1	2	3
	0	2	1	1	2
	0	1	1	2	2
	1	1	1	2	3
	0	1	2	2	3
	1	2	2	2	3

transplantation; thus growth retardation was an important indicator of graft dysfunction (33). Schier et al demonstrated lymphatic regeneration occurring eight days posttransplantation so lymphatic anostomosis was not necessary (34). Since these are related to long-term graft functions, we did not assess lymphatic regeneration or serum triglyeride levels in this study. However, we monitored glutaminase, maltase and lactase activities as indicators of mucosal integrity. Mucosal glutaminase and maltase activities are reported to be reliable methods of determining reperfusion injury in the small bowel (35). In this study we also measured lactase activity as a possible indicator of reperfusion injury. Glutaminase enzyme activities were found to be superior. As seen in Table 1, maltase activity in Group 3 was already significantly higher before the start of the experiments. We also found statistically different maltase activity levels in normal tissue samples. This can be attributed to some properties of maltase being related to genetic and environmental factors. Thus, maltase activity cannot be considered reliable for the assessment of mucosal integrity after ischemia-reperfusion injury. In Group 1, the greatest decrease in enzyme activities was seen at the 12th hour, which may be evidence of maximum lipid peroxidation. In Group 4, glutaminase and lactase activities showed a similar pattern, reaching the lowest level at the 12 th hour. Nevertheless, and in view of no significent differences in lactase activities at any time point, glutaminase activity can be considered a better indicator than maltase and lactase activity when the measurements are checked against the amount of lipid peroxidation and histopathological findings. Higher glutaminase activity levels than normal at the end of the cold ischemia periods in the a T + verapamil group may indicate the synergistic action of these two agents during the six hour cold preservation. This may be attributed to verapamil's inhibitor effect on the conversion of xanthine dehydrogenase to xanthine oxidase as reported previously in the literature (9). Verapamil, as an antioxidant agent, protects against free radical damage, if present, during the

ischemic period by blocking calcium channels, especially in calcium rich tissues (9,31). Verapamail has also been shown to inhibit in-vitro T-cell motility and lymphocytic transmigration (36). On the basis of these findings, we prefer verapamil during the cold ischemic period in preservation solutions, but not systemically. However, a T was used in the same manner as in our previous study (8).

The higher number of histopathological appearance grade 3's found in Group 1 can be attributed to the use of no antioxidant agent in that group. This may reflect the protective effects of a T and verapamil.

It therefore can be concluded that a T may be a promising agent for the prevention of tissue injury caused by free oxygen radicals during the cold ischemia period in organ transplantation and that addition of verapamil to the EC solution also effectively prevents this injury. Verapamil, used with a T in the same preservation solution did not increase the preventive effect of a T, although further investigation is needed.

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