The Effects of Amlodipine on the Biochemical and Histopathological Changes in the Rabbit Ileum Subjected to Ischemia-Reperfusion

Tavşanlarda Amlodipin'in Ileal Iskemi-Reperfüzyonun Histopatolojik ve Biyokimyasal Değişiklikleri Üzerine Etkisi

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Abstract

Objective: The aim of this study was to determine the potential, protective effects of amlodipine in an experimental, ischemia-reperfusion (I/R) model in the rabbit small intestine.

Materials and Methods: The rabbits were divided into four groups: sham-operated, amlodipine (10 mg/kg) + sham-operated, I/R, and I/R + amlodipine (10 mg/kg) groups. An intestinal I/R model was applied to the rabbits. The superior mesenteric artery was occluded for 1 h with an atraumatic vascular clamp and then was reperfused for 2 h. Animals in the amlodipine and I/R + amlodipine groups received the amlodipine by oral gavage. At the end of the 2-h-reperfusion period, the animals were sacrificed.

Results: Pretreatment with amlodipine significantly increased SOD activity and GSH levels to values close to those found in the serum from the I/R group. Rabbits in the I/R group showed high levels of serum MDA. Amlodipine pretreatment significantly reduced the serum MDA levels compared to the I/R group, although the MDA levels in the I/R + amlodipine group were still higher than in the sham-operated group. The I/R damage was ameliorated by amlodipine pretreatment, as evidenced by histopathological analysis.

Conclusion: The present study is the first to report an attenuation of I/R-induced intestinal injury by the systemic administration of amlodipine.

Key Words: Amlodipine, Ischemia-Reperfusion, Glutathione, Superoxide Dismutase, Malonyldialdehyde, Rabbit

Özet

Amaç: Biz bu çalışmada, tavşan ince barsak iskemi-reperfüzyon (I/R) deneysel modeli üzerine amlodipin'in koruyucu etkisini inceledik.

Gereç ve Yöntem: Tavşanlar dört guruba ayrıldı: şam operasyon gurubu, 10mg/kg amlodipin+şam operasyon gurubu, I/R gurubu, I/R+ 10 mg/kg amlodipin gurubu. İntestinal iskemi/reperfüzyon modeli tavşanlara uygulanıldı. Superior mezenterik arter 1 saat sure ile atravmatik vasküler klemp ile klempe edildi sonrasında 2 saat reperfüzyon uygulanıldı. Amlodipin ve amlodipin+I/R guruplarına oral gavaj ile amlodipin verildi. İki saatlik reperfüzyon süresinin sonunda hayvanlar sakrifiye edildi. I/R hasarının amlodipin uygulaması ile düzeldiği histopatolojik analizler ile ispatlandı.

Bulgular: Amlodipin uygulaması I/R 'da serumda bulunan SOD aktivitesi ve GSH seviyesini anlamlı şekilde artırdı. Tavşanlarda intestinal I/R'ında yüksek seviyede MDA gözlendi. Amlodipin uygulaması intestinal I/R bulunan tavşan serumunda MDA aktivite seviyesini anlamlı şekilde düşürürken bu düşüş I/R+amlodipin gurubu control gurubu ile mukayese edildiğinde çok daha fazlaydı.

Sonuç: Mevcut çalışma I/R ile oluşan intestinal hasar üzerine amlodipin'in sistemik uygulamasının iyleştirici etkisini gösteren ilk çalışmadır.

Anahtar Kelimeler: Amlpdipin, İskemi-Reperfüzyon, Glutatyon, Süperoksit Dismutaz, Malonyldialdehid, Tavşan

Introduction

Ischemia-reperfusion injury (IRI) in the intestine is associated with high morbidity and mortality in both surgical and trauma patients [1]. The intestine, an area that is highly sensitive to IRI, is composed of labile cells that are easily injured by episodes of ischemia, and reperfusion results in further damage to the mucosa [2]. IRI occurs in situations of blood flow disruption to the gut, such as in abdominal aortic-aneurysm surgery, cardiopulmonary bypass, strangulated hernias, neonatal necrotizing enterocolitis, mesenteric insufficiency, and intestinal transplantation [3, 4]. IRI in the intestine also occurs in septic and hypovolemic shock [5, 6].

Interruption of the blood supply results in ischemic injury, which leads to metabolically active tissue damage. Paradoxically, re-establishing blood flow to the ischemic tissue initiates a succession of events that may lead to additional cell injury, known as reperfusion injury. This reperfusion damage frequently exceeds the original ischemic insult [7]. The mechanisms underlying IRI damage are not clear, but over the past decades, free radicals are considered as the primary injury mediators that are initiated upon reperfusion.

Essentially, any reduction in the blood supply results in Reactive Oxygen Species (ROS) damage to the intestinal mucosa, and subsequent reperfusion of the intestine results in further ROS damage to the mucosa [8]. For these reasons, there is increasing interest in studying potential new drugs based on antioxidants. A number of promising candidates have been used to ameliorate IRI in the intestines of animal models [9]. For example, dihydropyridine calcium-channel blockers are commonly used in the medical field and are now being used to treat several disorders, such as hypertension, arrhythmia, angina pectoris, left ventricular diastolic dysfunction, myocardial infarction, Raynaud's phenomenon, and progressive systemic sclerosis. [10, 11]. In addition, experimental studies have shown that the use of these drugs can be extended to many additional indications.

In previous studies, we demonstrated that amlodipine exerts anti-inflammatory, anti-osteoporotic, and protective effects against ovarian ischemia-reperfusion (I/R) damage [12, 13]. Amlodipine has also been clinically and experimentally shown to have antiatherogenic effects because it decreases and/or blocks inflammation from forming in the atheroma plaques on the vascular endothelium by inhibiting nitric oxide synthase activity [14]. Its anti-atherogenic mechanism is not only related to inhibiting nitric oxide production, but it also involves modulating gene expression, remodeling vascular smooth-muscle cells, generating antioxidants, and inhibiting vascular smooth-muscle cell proliferation and migration [15]. The potent antioxidant activity of amlodipine results in lower organ failure following vascular ischemia episodes [15], and the anti-inflammatory effects of this drug are now well recognized [16]. Amlodipine counteracts the roles of calcium in inflammatory pathogenesis, which results in a pronounced anti-inflammatory effect. In a stroke model of hypertensive rats treated with amlodipine, brain tissue damage was low, and this effect was suggested to be associated with the increasing effect of amlodipine on superoxide dismutase (SOD) activity [17]. Amlodipine treatment in rats has also decreased cardiac damage associated with coronary artery occlusion [18].

At the present time, there is still insufficient data to conclude that amlodipine has a protective effect on the intestinal injury associated with I/R. However, amlodipine administration prior to the initiation of the reperfusion damage may reduce intestinal reperfusion injury. The aim of the present study was to determine the potential, protective effect of amlodipine in an experimental, I/R model of the rabbit small intestine by examining its histopathological and oxidant/antioxidant effects.

Materials and Methods

Animals

A total of 24 rabbits (3.5 to 3.8 kg) were used for the experiments. Rabbits were obtained from the Gulhane Military Medical University's Experimental Animal Laboratory of Medicinal and Experimental Application and Research Center, Ankara, Turkey. Animal experiments and procedures were performed in accordance with the national guidelines for the use and care of laboratory animals and were approved by Gulhane University's local animal care committee. The rabbits were housed in standard plastic cages on sawdust bedding in an air-conditioned room at 22±1°C under lighting controls (14 h light/10 h dark cycle). Standard rabbit chow and tap water were given ad libitum.

Chemicals

All chemicals were purchased from Sigma Chemical Co. (Germany). Amlodipine (Norvasc, 10 mg tablet) and ketamine (Ketalar, 500 mg vial) were obtained from Pfizer (Istanbul-Turkey). Xylazine (Rompun, 50 ml vial**)** was obtained from Roche (Istanbul-Turkey).

Experimental Design

The rabbits were divided into four groups, each composed of six animals: 1) the sham-operated control group (control group); 2) the 10 mg/kg amlodipine-treated, sham-operated group (amlodipine group); 3) the ischemia and reperfusion control group (I/R group); and 4) the ischemia and reperfusion, 10 mg/kg amlodipine-treated group (I/R + amlodipine group). The groups were housed separately. Animals in the amlodipine and I/R+amlodipine groups received amlodipine by oral gavage suspended in saline to a final concentration of 2 mL before operation. Animals in the control and the I/R groups received the same amount of normal saline by oral gavage.

Intestinal ischemia-reperfusion model

An intestinal I/R model was used in this study. The rabbits were fasted overnight but were allowed to drink water ad libitum. They were anesthetized by subcutaneous administration of ketamine (30 mg/kg) and xylazine (5 mg/kg) with atropine sulfate (0.15 mg/kg). An intravenous cannula was placed in the dorsal auricular vein of the rabbits, and NaCl (0.9%) was administered at a rate of the 10 mL/kg/h until the end of the experiment to prevent dehydration. If necessary, the rabbits received additional ketamine intravenously (5 to 10 mg/kg). After the abdomen was shaved, a longitudinal incision was performed in the midline area of the abdomen. After a median laparotomy, the animals were laid on their right side so that the intestinal mesentery removed from the abdomen was on the level of the aorta. The superior mesenteric artery (SMA) was separated from the aorta, and then it was occluded for 1 h with an atraumatic vascular clamp and reperfused for 2 h (Figure 1). Between surgical interventions, the midline incision was sutured and covered with plastic wrap to minimize fluid loss. To maintain an adequate, anesthetic plane, ketamine was administered as needed. The rabbits were placed on heating pads at 37°C throughout the experiment. The wound was bathed in 1% lidocaine solution to ensure analgesia. The sham-operated groups received laparotomy, and the groups' intestines were manipulated. At the end of the 2-h reperfusion period, the animals were sacrificed. Cardiac blood samples were collected immediately and transferred to the laboratory for biochemical analysis. For all groups, the intestines were surgically removed to a 10% formaldehyde solution for histological examination.

Biochemical analyses

Blood was collected in two different tubes, one with anticoagulant (EDTA) for whole blood and another without anticoagulant for serum. The serum was separated and centrifuged using a refrigerated centrifuge at 5°C, 4000G, 10 minutes. Serum total (Cu-Zn and Mn) Superoxide dismutase (SOD) activity (EC 1.15.1.1) was determined according to the method of Sun et al (19). Serum malonyldialdehyde (MDA) and glutathione (GSH) levels were determined with some modification by the methods of Ohkawa et al. [20] and Sedlak et al. [21], respectively.

Histopathological examinations

Intestinal segments from the experimental groups were initially placed in 10% formalin, paraffin-fixed, cut into 5-μm thick sections, and stained with hematoxylin and eosin (H&E). One section from each rabbit was graded blindly, and semi-quantitative, histological evaluations were graded from 0 to 5 by a single observer, according to the index of Park et al. [22]:
Grade 0: normal morphology
Grade 1: subepithelial edema and partial separation of apical cells

Grade 2: moderate lifting of enterocytes from the tips of the villi
Grade 3: lifting of enterocytes from both the tips and the sides of the villi (including superficial crypts)
Grade 4: partial mucosal necrosis of the lamina propria
Grade 5: total mucosal necrosis

Statistical analyses

The SPSS computer program (version 13.0 Chicago-USA) was used for statistical analyses. All data were expressed as mean±standard deviation (SD). The differences in the serum biochemical parameters between groups were evaluated using a one-way analysis of variance (ANOVA) with Tukey's tests for post-hoc comparisons. $p<0.05$ was considered statistically significant.

Results

Histopathological examinations

Histological scores are summarized in Table 1. Intestinal epithelium and architecture were normal (Grade 0) in the control and amlodipine groups (Figure 2 and Figure 3, respectively). Villi (Grade 2) or villi and crypt (Grade 3) ischemic damage was observed in the I/R and I/R + amlodipine groups (Figures 4 and 5). However, the number of crypts with villi damage (Grade 3) in the I/R group was greater than in the I/R + amlodipine group (Grade 1). In the I/R + amlodipine group, all cases showed villi damage (Grade 2) only, except for one case with both villi and crypt damage (grade 3; Figure 5).

Oxidant and antioxidant levels in rabbit blood
Serum MDA levels after intestinal I/R

We examined the levels of MDA in rabbit serum. MDA levels in the amlodipine group were higher than those in the control group, but no significant difference was observed between these two groups (Table 2). In the rabbits with I/R, high levels of MDA were observed compared to the control animals; however, amlodipine pretreatment significantly reduced MDA activity in these animals. The MDA levels in the I/R + amlodipine group were still higher than the control group (Table 2).

Serum SOD activity and GSH levels after intestinal I/R

Serum SOD activity was higher in the amlodipine group than in the control group (Table 2). Following intestinal I/R, SOD activity significantly decreased compared to the control rabbits. However, pretreatment with amlodipine significantly increased SOD activity to values close to those found in controls (Table 2). Serum GSH levels from the control and amlodipine groups averaged 335±35 and 348±29 nmol/ml, respectively, and no significant difference occurred between these two groups. Following intestinal I/R, GSH levels significantly decreased compared to the control rabbits; however,

amlodipine pretreatment significantly increased the serums GSH levels in the rabbits with intestinal I/R, which were comparable to those of the controls (Table 2).

Discussion

This experiment indicated that amlodipine administered to rabbits before ischemia protected the intestinal tissue from I/R injury. In our study, the SMA was clamped for 1 h and reperfused for 2 h. The amlodipine-mediated protective effects on both oxidative status and histopathological

Figure 1. *Schematic of the vascular clip application to the SMA in rabbits (ischemia).*

change are due, in part, to the suppression of oxidative stress. The present study is the first to demonstrate the effects of amlodipine in preventing I/R-induced intestinal injury. The rabbit intestinal mucosa, like other intestinal tissues previously reported [2], was clearly injured by I/R in the present study.

Recent studies have shown that pharmacological preconditioning with amlodipine protects the heart, kidney, and neuronal cells from ischemic injury [23-25]. As oxidative stress plays an important role in intestinal I/R injury, free radical scavengers have been demonstrated to reduce intestinal I/R damage [26]. Several factors have been associated with I/R injury, including free radicals and neutrophils [27]. When the intestinal tissue is subjected to I/R, activated neutrophils induce tissue injury through the production and release of ROS and cytotoxic proteins, such as proteases, MPO, and lactoferrin, into the extracellular fluid. This initiates inflammatory cascades that trigger the radical-induced I/R injury [28, 29].

I/R stimulates the generation of oxygen free radicals (OFRs), which appear to be responsible for the increase in activated neutrophils. Moreover, OFRs lead to lipid peroxidation within the cell membranes by reacting directly with polyunsaturated fatty acids. Various studies have demonstrated that OFRs cause tissue damage in the intestinal tissue subjected to I/R [30]. In the present study, the intestinal content of MDA, a product of lipid peroxidation, was significantly increased in the I/R group compared to the control. Pretreatment with amlodipine markedly reduced the MDA content as compared to the I/R group, suggesting that amlodipine inhibits lipid peroxidation and protects the tissue against free radical damage. These findings are in agreement with previously published studies concluding that amlodipine reduces oxidative stress and protects against
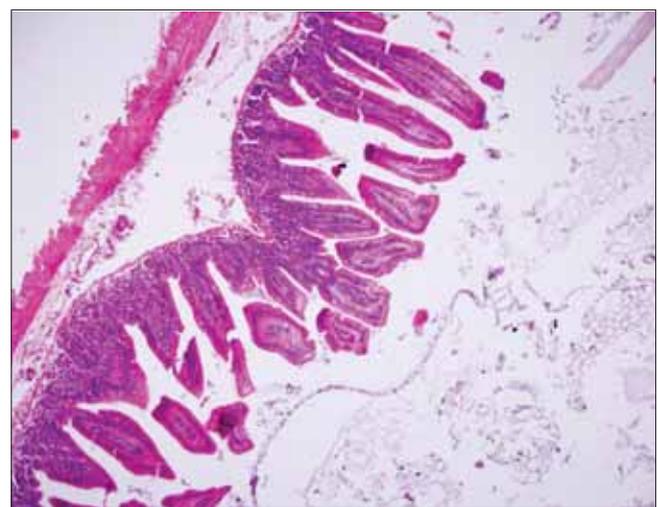

Figure 2. *Light microscopy of an intestine from the sham-operated control group. Normal intestinal tissue (grade 0; HEx100).*

Figure 3. *Light microscopy of an intestine from the sham-operated group treated with 10 mg/kg amlodipine. Normal intestinal tissue (grade 0; HEx100).*

Table 1. Grades of ischemic damage for the control and three study groups

Case number	Sham	Sham+ Amlopidine 10 mg/kg	Ischemia/ reperfusion (I/R)	I/R+10 mg/kg Amlopidine
1	0	0	3	2
2	0	0	2	2
3	0	0	3	3
4	0	0	3	2
5	0	0	2	2
6	0	0	2	2

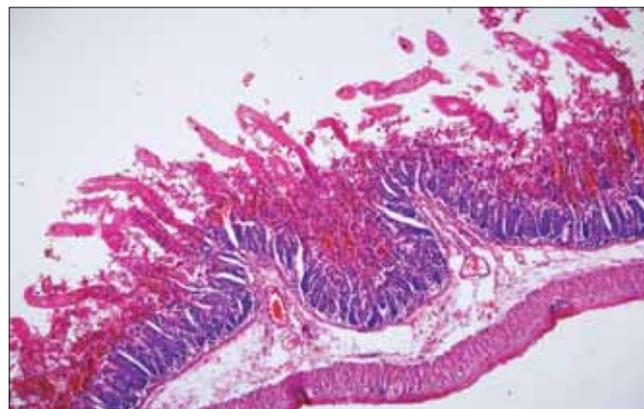**Table 2. Effects of amlodipine treatment on serum MDA (nmol/ml) and GSH (nmol/ml) levels and SOD activity (µU/l)**

GROUPS	N	MDA (nmol/ml)	GSH (nmol/ml)	SOD (µU/l)
1. Sham Control	6	3.12±0.35	335±35	87±8.1
2. Sham+Amlodipine	6	3.24±0.19	348±29	92±7.3
3. I/R	6	4.55±0.42*	236±55*	61±8.4*
4. Amlodipine+IR	6	3.58±0.33**	345±32**	84±12**

*Significant differences between the sham and I/R groups.
**Significant differences between the I/R and amlodipine-treated group. $p < 0.05$
MDA: Malonyldialdehyde, GSH: Glutathione, SOD: Superoxide dismutase

Figure 4. *Light microscopy of an intestine in the ischemia-reperfusion (I/R) group. The image shows ischemic villous necrosis without crypt damage (grade 2; HEx100).*
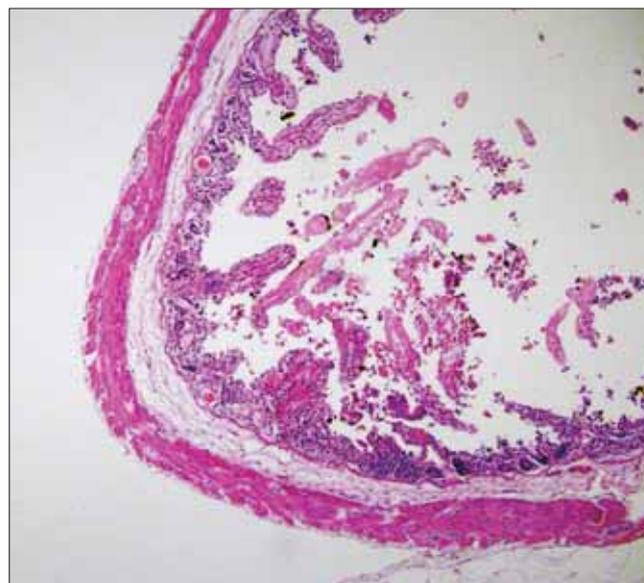
Figure 5. *Light microscopy of an intestine from the ischemia/reperfusion (I/R)+amlodipine (10 mg/kg) group. The image shows ischemic villous necrosis with crypt damage (grade 3; HEx100).*

lipid peroxidation in cell membranes, a likely contributor to the cell death observed after I/R [13].

GSH plays a principal role in the cellular, protective system against toxic, free radical-induced tissue injury [31], and depletion of GSH increases the chance of free radical-induced damage [32]. Therefore, GSH levels signify the antioxidative capacity of reperfused tissue [33]. Improvement in the GSH levels after amlodipine administration may have prevented an exacerbation of the tissue damage caused by I/R. Under physiological conditions the damaging effects of ROS are prevented by endogenous antioxidant enzymes, such as SOD, which rapidly reduce superoxide radicals [34]. According to many other reports, the oxidant/antioxidant capacity may change during intestinal reperfusion. In the present study, SOD was markedly decreased in the I/R group, which indirectly indicates the presence of excessive amounts of superoxide.

Amlodipine treatment resulted in enhanced SOD activity, suggesting that the attenuation of the intestinal I/R injury was due to detoxifying the oxygen free radicals. The mechanism of this antioxidative effect of amlodipine is not clear, although previous reports show antioxidative effects of amlodipine. In one study, Umemoto et al. showed that amlodipine suppressed ROS generation by upregulating Cu/Zn super-

oxide dismutase [17]. Recently, Li et al. demonstrated that amlodipine decreased the production of MDA and increased antioxidants, such as SOD and GSH, in gentamicin-induced renal-tubular toxicity in rats. [35].

In conclusion, the present study is the first to report the attenuation of I/R-induced intestinal injury by systemic administration of amlodipine. It also shows a more complete structural regeneration in rabbits with critical, partial intestinal ischemia. Amlodipine acts as a pharmacological preconditioning agent against injury caused by I/R in several tissues, which now includes the intestinal mucosa. Although amlodipine's mechanism of action should be further investigated, it is most

likely through reducing oxidative stress, which is supported by amlodipine inhibiting the production of MDA and increasing GSH levels and SOD activity under I/R conditions. Therefore, we believe that amlodipine should be added to the list of pharmacological preconditioning agents because it introduces a novel, protective strategy in intestinal ischemia.

Conflict of interest statement: The authors declare that they have no conflict of interest to the publication of this article.

This research has 26 February 2010 date and 10/6-K numbered Gulhane Military Academy Ethic Committee's approval.

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