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Local and systemic effects of adrenomedullin after intestinal ischemia reperfusion

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Adrenomedullin (AM) is a peptide proven to increase cellular tolerance to hypoxia and oxidative stresss and contribute angiogenesis. Despite its known therapeutic effects on myocardial, renal or spinal ischemic reperfusion injuries, its local and systemic effects on intestinal ischemic reperfusion injury still remain unknown. This study aims to demonstrate the local and systemic effects of AM on Intestinal Ischemic Reperfusion Injury (I-IRI) demonstrated in rats. Thirty male rats were randomly allocated to five groups: Control, Adrenomedullin (AM), Intestinal Ischemic Reperfusion Injury (I-IRI), Adrenomedullin and Intestinal Ischemic Reperfusion Injury (AM+I-IRI), and Intestinal Ischemic Reperfusion Injury and Adrenomedullin (I-IRI+AM). Blood and tissue samples were obtained for biochemical and histopathological evaluation. The results were expressed as mean±SEM and, P <0.05 was considered statistically significant. The levels of inflammatory cytokines were found to be elevated in I-IRI group and depleted in I-IRI+AM group. The biochemical and histopathological markers of injuries at the intestine and remote organs were found to be recuperated when the AM applied before the reperfusion phase. Results of this study demonstrated that the therapeutic drug adrenomedullin (AM) could reverse the intestinal and remote organ injuries related to intestinal ischemic reperfusion injury (I-IRI). These effects might be related to the antioxidant, anti-inflammatory, and anti-apoptotic activities of AM.

Keywords: Inflammation, Intestinal ischemic reperfusion injury, Oxidative stress

Ischemic reperfusion injury (IRI) is a dual problem leading to either reversible or irreversible damage that occurs due to insufficient blood flow at the ischemic phase[1]. During the reperfusion phase, despite the expectation of clearance of toxic metabolites and regeneration with reflow, the reflow of blood to the ischemic area paradoxically leads much more serious damages than ischemia alone[2]. Many mechanisms including formation of free oxygen radical derivatives from molcular oxygen, are blamed for these damages[3]. IRI is a clinically important process involving inflammation, oxidative stress, apoptosis, calcium over load and leukocyte adhesion[4,5].

Intestinal IRI (I-IRI) is an important problem seen during numerous pathologies like acute mesenteric ischemia, abdominal aortic aneurysm surgery, volvulus, trauma, and even severe burns[1]. The damage caused by I-IRI is not only limited to the site, moreover it may result remote organ injury, and may even cause multiple organ failure[6]. The remote organ injury is related with free oxygen radicals and mediators such as cytokines, complement proteins, or arachidonic acid products that are released into the systemic circulation due to the injury of the intestinal mucosal barrier[5,7].

Adrenomedullin (AM) is a peptide containing of 52 amino acids. It was discovered in 1993 by Kitamura *et al*., and was isolated from human pheochromocytoma cells. The peptide can be produced by many tissues such as adrenal medulla, endothelial cells, smooth muscle cells, myocardium, and central nervous system. Vasodilation and natriuresis are the most known and important physiological effects of the peptide via secondary messengers like cAMP and cGMP induced by nitric oxide[8-11]. This peptide has different effects on tissues such as cell proliferation, contraction, migration, and interaction with other neurohormonal factors. Also, AM has specific effects on gastrointestinal system like modulating gastrointestinal functions, improving endothelial barrier function and contributing mucosal host defense[12,13].

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In the literature, AM has been used to reduce the effects of IRI in tissues and was shown to be beneficial[8,9]. This healing effect is related to not only its potent vasodilator effect, but also its antioxidant activity. The antioxidant effect of AM on IRI was first studied by Oyar *et al*.[8] in 2008. In this study, we tried to evaluate the recuparative effect of adrenomedullin (AM) on the site and at the remote organs following I-IRI in experimental rats.

Materials and Methods

Experimental protocol

This study was approved by Gazi University Ethical Committee for Experimental Research on Animals (Project no: B.30.2.GÜN.0.05.06.00/158-18762) and supported by Gazi University Research Fund. Twenty four male adult Wistar albino rats weighing of 242 g (270±28 g) were included and fed with standard chow and tap water *ad libitum*, lived at 14 h light and 10 h dark cycle. Rats were housed under standard temperature and humidity. All procedures have been performed according to Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals of National Health Institutes (NHS; Bethesda, USA). All animals were anesthetized using xylazine hydrochloride (5 mg/kg, Alphazyne, Ege Vet, Izmir, Turkey) and ketamine hydrochloride (40 mg/kg, Ketalar, Eczacibasi, Istanbul, Turkey), and all surgical procedures were performed under sterile conditions. Rats were randomly divided into four groupsviz. Group Control (Gr C, n=6): The rats underwent laparotomy and dissecting of the superior mesenteric atery (SMA). No further intervention was performed; Group Adrenomedullin (Gr AM, n=6): The tail vein was catheterized and AM (12 µg/kg) was applied intravenously to the rats in this group without surgical intervention; Group Intestinal Ischemia Reperfusion Injury (Gr IRI, n=6): The ischemia model was performed by clamping (Fig. 1) the SMA for 90 min. After 90 min, the vascular clamp was removed and the abdomen was closed; Group Adrenomedullin+ Intestinal Ischemia ReperfusionInjury (Gr AM+I-IRI, n=6): Before the ischemic period, AM (12 µg/kg) infusion was performed intravenously through the cannulated tail vein. Intestinal ischemia was performed for 90 min by clamping SMA. After 90 min of ischemia period, clamp was removed; and Group Intestinal Ischemia Reperfusion Injury + Adrenomedullin (Gr I-IRI+AM, n=6): Intestinal ischemia was performed for 90 minutes by clamping SMA. After 90 minutes ischemia period, clamp was removed. With the onset of reperfusion, AM (12 µg/kg) infusion was performed intravenously through the cannulated tail vein.

The rats were sacrificed with intracardiac blood aspiration under anesthesia 4 h after the procedure. Lung, liver, heart, intestine, and kidney were resected for pathological, immunohistochemical, and biochemical evaluations. Blood samples were collected in Ethylenediaminetetraacetic acid (EDTA) tubes and centrifuged as soon as possible at 3.000×*g* for 10 min at 4ºC. All plasma and tissue samples were stored at –80°C.

Biochemical analysis

Plasma tumor necrosis factor alpha and interleukin 6 measurements

Solid-phases and wich Enzyme Linked-Immuno Sorbent Assay (ELISA) was used in order to evaluate the plasma tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) levels.

Determination of tissue catalase activity

The activity was measured by degradation of hydrogen[14]. Cayman catalase (CAT) assay kits at 540 nm was used to determine tissue CAT activities. The values were presented as nmol/min/mg protein.

Determination of tissue superoxide dismutase activity

The activity was measured by degradation of tissue superoxide by superoxide dismutase (SOD) enzyme and the production of O_2^- with xanthine oxidase. Spectrophotometric determination of the end product was performed after the reaction with nitroblue tetrazolium[15]. Cayman SOD assay kits at 440 nm was used to determine tissue SOD activities. The values were presented as U/mg protein.

Determination of tissue glutathione levels

Aliphatic thiol groups of the sample was determined using Ellman's reagent 5,5'-Dithiobis-(2-Nitrobenzoic Acid or DTNB). Spectrophotometric

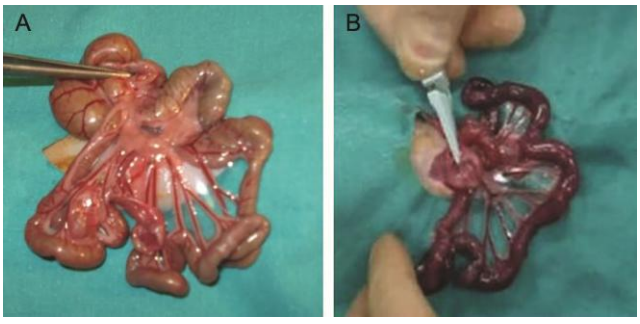

Fig. 1 — Intestinal ischemia model: (A) preischemia; and (B) postischemia

determination of tissues that are reacted with the Ellman reagent and *p*-nitrophenol anion per every thiol group were performed the at the mild alkali pH[16]. Cayman glutathione (GSH) assay kits at 410 nm was used to determine tissue GSH levels. The values were presented as nmol/mg protein.

Determination of tissue total nitrate/nitrite levels

The assay is dependent to the following reactions. In the presence of nitrate reductase, nitrate is conversed to nitrite by using nicotinamide adenine dinucleotide phosphate (NADPH) and the nitrite reacts with N-(1-naphthyl) ethylenediamine and sulphonamide in order to reveal an end product. Spectrophotometrical determination of the end product was performed[17]. Cayman total nitrate/nitrite (NOx) assay kits at 540 nm was used to determine NOx. The values were presented as µM.

Determination of tissue malondialdehyde levels

Malondialdehyde (MDA) levels were determined using the reaction between lipid peroxides and thiobarbituric acid (TBA). Spectrophotometrical determination of the end product was performed[18]. Cayman MDA assay kits at 530 nm was used to determine tissue MDA level. The values were presented as nmol/g tissue.

Determination of tissue protein levels

Tissue protein levels were determined using Bradford method[19]. Bovine serum albumin (BSA) was used as the standard.

Histopathological evaluation

The intestinal samples from groups were fixed in 10% formaline for 3 days before they were embedded into paraffin blocks. The fixated tissues were sectioned axially at 5 µm thickness by microtome (RM 2245, Leica, Wetzlar, Germany). After sectioning, Hematoxylin and Eosin (H&E) staining procedure was performed. The slides were assesed under a light microscope (DMI 4000 B; Leica, Wetzlar, Germany). Spesicial scoring system which is described by Chiu *et al*.[20] was applied for evaluation of ischemic damage at the mucosal level.

Immunohistochemical evaluation

Intestinal samples were exposed to 37°C overnight and then incubated at 60°C for an hour. Afterwards, xylol application was performed to the samples twice for 15 min. Subsequently samples were treated with different alcohol series (96 and 80% ethanol) for 10 min, and in dipped into distilled water twice for 5 min.

For determination of caspase-3 immunoreactivity; samples were placed in 10% citrate buffer and incubated in the oven. After incubation, sections were kept at room temperature for 20 min. Hydrophobic pen (Super PAP PEN IM3580, Immunotech) was used to mark surroundings of the sections. 0.3% hydrogen peroxide (Fisher Scientific, Melrose Park, IL) was used for 10 min to block endogenous peroxidase activity. After washing with PBS, caspase-3 primary antibody (Labvision/NeoMarkers Corp.) was applied for 1 h. Subsequently, biotinylated goat antibody with streptavidin peroxidase activity was used and sections were placed in 3-amino, 9-ethyl carbazole (TA-125-HA, Thermo Fisher Scientific) chromogen for 10 min after rewashing with PBS. Two minute Mayer's hematoxylin exposure was used for counter staining.

Examination was done under light microscopy (DMI 4000 B, Leica) by a single histopathologist who is blinded to the experimental groups. Quantitative examination of relative immunoreactive densities of caspase-3 staining was conducted according to the criteria described by McCarty *et al*.[21] Histological score (H-score) was formulated as $\sum Pi\ (I + 1)$, as "I" represented the intensity of staining (0: none; 1: mild; 2: moderate; and 3: intense) and "Pi" represented the fraction of stained cells for each intensity.

Statistical analysis

All statistical analyses were performed by using the statistical package SPSS for Windows, version 17.0 (SPSS, Chicago, Illinois, USA). The descriptive statistics were represented as mean±SEM. Statistical analyses were carried out using Kruskal-Wallis test and Mann-Whitney *U*-tests. The value of $P < 0.05$ was considered statistically significant.

Results

Biochemical evaluations

Plasma TNF-α and IL-6 level

Table 1 represents the plasma TNF-α and IL-6 levels. The plasma levels of TNF-α and IL-6 were found higher in Gr I-IRI when compared to Gr C and were found lower in Gr I-IRI+AM when compared to Gr I-IRI ($P < 0.05$).

Tissue CAT and SOD activities, GSH, NOx, and MDA levels

Intestinal levels

Intestinal SOD activity and GSH level were lower in Gr I-IRI when compared to the Gr C and higher in Gr I-IRI+AM when compared to Gr IRI. Moreover the GSH level was also found higher in Gr AM+I-IRI

Table 1 — Plasma TNF-α and IL-6 levels (mean±SEM)

Parameters\Groups	Gr C (n=6)	Gr I-IRI (n=6)	Gr AM (n=6)	Gr I-IRI+AM (n=6)	Gr AM+I-IRI (n=6)
TNF-α (pg/mL)	124.22±20.70	388.26±81.70*	169.16±40.12	242.55±54.59†	326.26±84.93
IL-6 (pg/mL)	16.29±1.81	280.24±89.95*	34.33±5.69	53.11±15.84†,‡	248.22±71.67

[Biochemical analysis of plasma, results presented as mean±Standart error of mean (SEM). *P <0.05 compared to control group; †P <0.05 compared to IRI group; ‡P <0.05 compared to AM+IRI group; AM, adrenomedullin; TNF-α, tumor necrosis factor α; IL-6, interleukin 6]

Table 2 — Biochemical analysis of intestinal segments, and renal, hepatic, cardiac and lung tissues (mean±SEM)

	Gr C (n=6)	Gr I-IRI (n=6)	Gr AM (n=6)	Gr I-IRI+AM (n=6)	Gr AM+I-IRI (n=6)
Intestinal segments					
CAT (nmol/min/mg protein)	46.67±8.03	31.23±18.11	46.12±9.42	45.32±16.59	37.05±31.11
SOD (U/mg protein)	54.27±9.21	32.23±3.42*	37.06±17.51	60.44±37.64†	38.02±14.05
GSH (nmol/mg protein)	11.41±5.08	5.77±2.01	11.44±2.72	13.71±3.77†	11.06±3.06†
NOx (µM)	3.12±3.00	9.74±3.80*	2.52±1.36	8.75±6.10	10.09±4.82
MDA (nmol/g tissue)	2.92±1.64	19.29±6.66*	5.65±2.01	8.87±4.58†	12.79±4.45
Renal tissue					
CAT (nmol/min/mg protein)	36.17±9.88	25.00±8.23*	34.95±17.03	46,69±15,7 †,‡	27.03±7.67
SOD (U/mg protein)	13.84±5.23	8.57±3.89	16.64±4.68	16,03±7,82†,‡	8.61±2.04
GSH (nmol/mg protein)	10.14±3.26	5.79±2.31	9.28±5.52	8.58±2.61	8.71±4.12
NOx (µM)	13.24±5.19	33.92±14.61*	16.39±7.98	24.89±2.81	29.18±11.54
MDA (nmol/g tissue)	9.60±3.75	22.35±4.07*	14.94±3.12	10.78±3.18†	15.58±2.36†
Hepatic tissue					
CAT (nmol/min/mg protein)	30.78±6.47	24.27±5.78	27.78±5.69	39.45±9.33†	29.74±11.23
SOD (U/mg protein)	21.45±8.07	12.23±2.16	19.67±3.89	22.43±9.58	12.58±7.09
GSH (nmol/mg protein)	14.89±2.37	10.74±4.25	13.64±4.70	14.65±1.06	11.23±2.45
NOx (µM)	23.10±6.28	25.73±5.81	25.83±5.15	21.58±6.22	18.16±9.44
MDA (nmol/g tissue)	7.28±2.06	14.58±2.67*	9.46±1.18	8.44±3.16†	11.05±5.46
Cardiac tissue					
CAT (nmol/min/mg protein)	87.11±33.28	51.56±8.66*	67.50±15.71	100.38±34,46†,‡	73.29±28.65
SOD (U/mg protein)	59.07±17.30	49.55±7.03	65.27±17.41	66.98±20.69	66.94±15.52
GSH (nmol/mg protein)	12.28±2.97	12.35±6.55	11.23±1.84	18.48±9.43	14.43±5.71
NOx (µM)	5.78±1.54	2.88±0.69	6.63±3.70	5.85±2.84	5.67±3.05
MDA (nmol/g tissue)	4.32±0.37	11.19±4.35*	7.10±2.06	4.64±2.02†	8.04±3.68
Lung tissue					
CAT (nmol/min/mg protein)	77.94±13.71	53.76±23.97	75.22±18.15	97.10±42.75	55.56±9.19
SOD (U/mg protein)	53.25±9.72	40.75±4.40*	55.20±6.27	57.24±36.21†	55.69±7.25
GSH (nmol/mg protein)	21.65±5.92	10.80±5.71	14.39±6.45	18.56±9.75	15.88±6.37
NOx (µM)	5.64±2.87	9.76±9.45	5.04±3.63	6.76±2.41	6.57±3.07
MDA (nmol/g tissue)	9.75±2.18	20.66±5.96*	15.40±4.06	9,91±5,08†,‡	17.11±2.96

[Biochemical analysis of lung tissue, results presented as mean±Standart error of mean (SEM); *P <0.05 compared to control group; †P <0.05 compared to IRI group; ‡P <0.05 compared to AM+IRI group; AM, adrenomedullin; CAT, catalase; SOD, superoxide dismutase; GSH, glutathione; Nox, total nitrate/nitrite; and MDA, malondialdehyde]

when compared to Gr IRI. Mean while the intestinal MDA level was higher in Gr I-IRI when compared to the Gr C and lower in Gr I-IRI+AM when compared to Gr IRI (P <0.05) (Table 2).

Renal levels

Renal CAT activity was lower in Gr I-IRI when compared to the Gr C and higher in Gr I-IRI+AM when compared to Gr IRI whereas renal MDA level was higher in Gr I-IRI when compared to the Gr C and lower in Gr I-IRI+AM and Gr Gr AM+I-IRI when compared to Gr IRI (P <0.05) (Table 2).

Hepatic levels

Hepatic MDA level was higher in Gr I-IRI when compared to the Gr C and lower in Gr I-IRI+AM when compared to Gr IRI (P <0.05) (Table 2).

Cardiac levels

Cardiac CAT activity was lower in Gr I-IRI when compared to the Gr C and higher in Gr I-IRI+AM when compared to Gr IRI whereas cardiac MDA level was higher in Gr I-IRI when compared to the Gr C and lower in Gr I-IRI+AM when compared to Gr IRI (P <0.05) (Table 2).

Pulmonary levels

Pulmonary SOD activity was lower in Gr I-IRI when compared to the Gr C and higher in Gr I-IRI+AM when compared to Gr IRI whereas pulmonary MDA level was higher in Gr I-IRI when compared to the Gr C and lower in Gr I-IRI+AM when compared to Gr IRI (P <0.05) (Table 2).

Histopathological evaluation

The intestinal ischemic injury score was statistically significantly higher in Gr I-IRI when compared to Gr C (P <0.05) and lower in Gr I-IRI+AM and Gr I-IRI+AM when compared to Gr I-IRI (P <0.05) (Table 3). There were no ischemic findings in the samples of Gr C and Gr AM, stained with Hematoxylen and Eosin (Fig. 2 A and C). The histopathologic findings of ischemic damage, such as wide spreaddes quamation and mucosal congestion were seen Gr I-IRI (Fig. 2B). Mild ischemic findings such as foca desquamation and mild mucosal congestion was detected in Gr I-IRI+AM (Fig. 2D).

Intestinal caspase-3 and MMP-9 activities

Intestinal caspase-3 and MMP-9 activities were higher in Gr I-IRI when compared to Gr C and lower in Gr IRI+AM when compared to Gr I-IRI (P <0.05) (Table 7).

Table 3 — Mean histopathological scoring, intestinal caspase-3 immunoreactivity, and MMP-9 levels for all group (mean±SEM)

	Gr C (n=6)	Gr I-IRI (n=6)	Gr AM (n=6)	Gr I-IRI+ AM (n=6)	Gr AM+ I-IRI (n=6)
Mean histopathologic scores (grade)	0.16± 0.40	2.66± 0.81*	0.33± 0.51	0.66± 0.51†,‡	1.50± 0.54†
Caspase-3	0.85±0.36	2.95±0.44*	1.01±0.45	0.96±0.33†	1.15±0.49†
MMP-9	1.78±0.35	3.06±0.20*	1.91±0.41	2,03±0,33†,‡	2.70±0.49†

[Intestinal mean histopathological scoring, intestinal caspase-3 immunoreactivity and MMP-9 levels, results presented as mean±Standart error of mean (SEM); *P <0.05 compared to control group; †P <0.05 compared to IRI group; ‡P <0.05 compared to AM+IRI group. AM, adrenomedullin; MMP-9, matrix metalloproteinase 9]

Discussion

In this study, we evaluated the effects of adrenomedullin (AM) in an experimental model of Ischemic Reperfusion Injury (I-IRI) and AM has been shown to have local and systemic recuparative effects especially applied at the beginning reperfusion process. The biochemical evaluation has confirmed the ischemic reperfusion injury at the intestinal level and AM application, at the beginning of reperfusion phase following I-IRI revealed beter outcomes with decreased peroxidation products and strengthened antioxidant defense. However the histopathological and immunhistochemical evaluations revealed beter results with either application time of AM. Even the mean histopathalogic score was beter with either application time of AM, the best results was obtained in the group of AM application at reperfusion period. Likewise the MMP-9 staining results revealed best outcome with the application of AM at reperfusion period. AM treatment, mostly the application before there perfusion period, was shown to have antioxidant and antiapoptotic effects. In a study by Okumura *et al*., the effects of AM applied at the reperfusion period following cardiac IRI were evaluated[22].

The authors have stated that the cardio protective effects of AM are mainly credited to its antipitotic

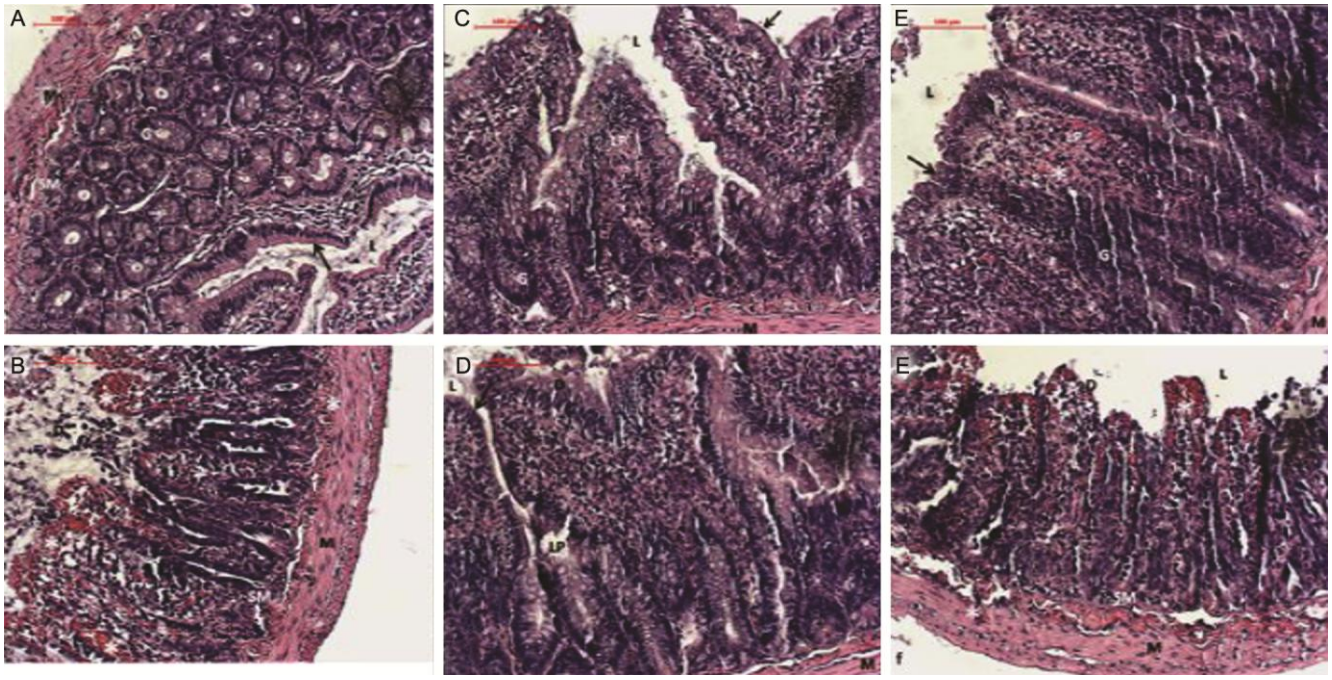

Fig. 2 — Histopathology of small intestine stained with Hematoxylin and eosin in all groups

effects via PI3K/Akt-dependent pathway. In another myocardial IRI study, Yin *et al*.[23], investigated the signaling mechanisms of AM. The investigators induced expression of recombinant AM in cardiac cells at the preischemic phase and demonstrated antiapoptotic activity of AM through the Akt-GSK-caspase signaling pathway after myocardial IRI[21]. Nishida *et al*.[24] studied the effects of AM applied different phases of IRI on isolated perfused hearts and found AM to be beneficial either way. They stated that AM before ischemia effects via PKA-mediated activation of mitoKCa channels and AM during reperfusion effected via P13-K-mediated pathway We also found AM to be effective either way and these effects could be attributed to different pathways induced by AM. But when the biochemical and histopathological findings were considered together, we think AM application during reperfusion to be more effective at the local site.

The damage related to IRI that occurred in the intestine was not local limited to the site. The consequences like loss of the active intestinal mucosal barrier, increased intestinal permeability and bacterial translocation, elevated cytokine release due to the systemic circulating macrophages and neutrophils that are activated secondary to inflammation could cause remote organ damage due to I-IRI[25].In this study, we also investigated the effects of I-IRI at he distant organs such as the lung, heart, liver, and kidney, as well as the effects of AM on this condition.

It was observed that all distant organs that we examined were damaged due to IRI and biochemical parameters improved with AM treatment at there perfusion period. It is possible that the significant decreases in systemic cytokines may have an effect on the healing seen secondary to the treatment at intestine and distant organs. In our study, the elevated TNF-α following ischemia probably induced apoptosis significantly and AM treatment reversed this effect.

This may be related either to the known direct anti-apoptotic effect of AM or TNF-α lowering effect of AM. Zhang *et al*.[26] investigated the efficacy of AM treatment, which they combined with AM binding protein-1 (AMBP-1), on intestinal damage in I-IRI, and achieved recovery rates similar to our results. Dwivedi *et al*.[27] reported that combined treatment with AMBP-1 is protective in lung damage caused by I-IRI. Besides, Carrizo *et al*.[28] evaluated an inflammatory response and distant organ damage after I-IRI with combined therapy. They have evaluated distant organ damage in a simpler way than our method, only by studying liver and kidney functional parameters in serum and stated the effectiveness of the treatment. They explained the necessity of combined therapy as follows: Ischemic damage decreases the level of AMBP-1 and therefore, the level of AM in the circulation decreases relatively due to ischemia[25]. In our study, we used AM alone and although we know that the effect of AM is potentiated by AMBP-1 we obtained an effective tissue response using the dose of AM used in the literature.

Conclusion

The above results suggest that the therapeutic drug adrenomedullin (AM) when applied during the reperfusion period of ıschemic reperfusion ınjury (IRI), can reverse the damage on the site and at the remote organs related to intestinal (I)-IRI. This effect may be due to the antioxidant, anti-inflammatory, and antiapoptotic activity of the drug AM and it seems promising for treatment in mesenteric ischemia reperfusion related pathologies.

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Conflicts of interest

Authors declare no competing interests.

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