

1 **Introduction**

2 Tissue ischemia is a key event in clinical conditions such as myocardial infarction and stroke
3 and may also occur as a complication following surgical procedures. Tissue damage due to ischemia is
4 paradoxically further increased after reperfusion. This negative effect of reperfusion especially
5 becomes more pronounced in cases of prolonged ischemia and is caused by a series of inflammatory
6 reactions, also known as ischemia-reperfusion (I/R) injury [1]. The pathophysiology of I/R injury is
7 extremely complex including vascular endothelial cells, leucocytes, oxygen radicals, inflammatory
8 mediators such as platelet-activating factor and tumor necrosis factor, and adhesion molecules [2].

9 Small intestine is probably the most sensitive visceral organ to I/R injury. Intestinal I/R injury is
10 associated with high morbidity and mortality rates in trauma and surgical patients [3]. Conditions
11 causing any kind of interruption and/or reduction of intestinal blood flow lead to I/R injury. These
12 conditions may include abdominal aortic aneurysm surgery, cardiopulmonary bypass, strangulated
13 hernias, neonatal necrotizing enterocolitis, intestinal transplantation, and septic and hypovolemic
14 shock. Interruption of blood supply rapidly results in ischemic injury in the metabolically active
15 intestinal tissue and as well as restoration of blood flow paradoxically leads to reperfusion injury [3],
16 which is even greater than the initial damage caused by ischemia itself, as a result of a series of events.
17 As intestinal mucosa is a critical site where several acute-phase proteins, hormones, and cytokines are
18 synthesized, intestinal damage is not limited to this region and results in impairment of functions and
19 integrity of distant organs, as well. Moreover, bacterial translocation due to intestinal damage may
20 lead to further deterioration of the clinical picture by causing sepsis, shock, and multiple organ failure
21 [3].

22 Considerable increase in surgical interventions and organ transplantations in recent years and
23 high morbidity and mortality rates associated with ischemia-related disorders have led to a rise in
24 interest regarding I/R injury. Currently no measures have been shown to be effective in prevention or
25 treatment of I/R injury, and there are ongoing studies investigating different potential agents. In the
26 present study, the effects of apelin 13 (AP) on intestinal I/R injury were investigated in a rat model.

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29 **Materials and Methods**

30 **Study Design**

31 This study was approved by the Animal Ethics Committee and conducted at the Laboratory
32 Animals Care Unit in accordance with the guidelines for the care and use of laboratory animals.
33 Twenty four male Sprague-Dawley rats aged 6 to 8 weeks and weighing 280 ± 20 g were used. Using a
34 computer generated table of random numbers, rats were randomly assigned to the following groups:
35 control group (group C, n=8), ischemia-reperfusion group (group I/R, n=8), and ischemia-
36 reperfusion+apelin group (group I/R+AP, n=8). The animal room was maintained at a temperature of
37 $22\pm 2^\circ\text{C}$ and a relative humidity of $55\pm 15\%$, with a 12-hour light-dark cycle. Tap water and chow were
38 freely available throughout the acclimatization and study periods.

39 **Chemicals and Reagents**

40 Apelin-13 (Apelin®, Phoenix Pharmaceutical, Belmont, CA, USA) was commercially
41 purchased. Apelin was administered intraperitoneally beginning three days prior to the surgical
42 procedure in group I/R+AP at a dose of $2\ \mu\text{g}/\text{kg}/\text{day}$ as previously described by Petrescu [4]. Same
43 amount of normal saline was administered intraperitoneally in groups I/R and C.

44 **Surgical Procedure**

45 After an overnight fasting period, each animal was anesthetized by intramuscular administration
46 of 5 mg/kg of xylazine (Rompun, Bayer Ilac Sanayi, Sisli, Istanbul, Turkey) and 30 mg/kg of ketamine
47 hydrochloride (Ketalar, Eczacibasi Ilac San, Istanbul, Turkey). Abdomen of each rat was then shaved
48 and cleansed by povidone iodine solution (Isosol, Merkez Laboratory, Ilac San, Istanbul, Turkey).
49 Using a sterile technique, all rats underwent laparotomy through a 3 cm midline incision. The aorta
50 and visceral arteries were exposed in the abdominal cavity, and the ligament of Treitz was also incised
51 to better expose the superior mesenteric artery (SMA). Group C underwent SMA mobilization only
52 without any clamping. In groups I/R and I/R+AP, an atraumatic microvascular bulldog clamp was
53 placed across the SMA at its point of origin from the aorta with special care to avoid occlusion of the
54 superior mesenteric vein. Mesenteric ischemia was confirmed by noting loss of mesenteric pulsations
55 and observing intestinal paleness. The bowel was then returned to the abdominal cavity, and the area
56 of incision was closed by interrupted atraumatic 4.0 silk sutures. After 60 minutes of ischemia,

57 relaparotomy was performed to remove the microvascular clamp on the SMA for 3 hours of
58 reperfusion. Mesenteric reperfusion was confirmed by noting the restoration of pulsations and
59 intestinal color. The bowel was again returned to the abdominal cavity, and the area of incision was
60 closed with 4.0 silk sutures. The bowel was left in the abdomen during reperfusion. At the end of 3
61 hours, tissue samples were obtained from an area of small intestine 5 cm proximal to the ileocecal
62 region. The bowel specimens from each animal were harvested for both biochemical and
63 histopathological analysis. The tissues were rinsed with cold saline solution and all of the tissue
64 specimens were fixed in 10% buffered formalin for histopathological analysis.

65 **Malondialdehyde and Glutathione assays**

66 The bowel tissue samples were washed with physiological saline and kept in a freezer until the
67 day of the experiment. These samples were homogenized with 150 mmol/L ice-cold KCl for the
68 measurement of malondialdehyde (MDA) and glutathione (GSH) levels. Homogenates were
69 centrifuged at 2600 x g for 10 min at 4 °C. The MDA concentrations in the renal tissue, an indicator of
70 lipid peroxidation, were assayed in the form of thiobarbituric acid-reacting substances [5]. Then, 0.2
71 mL of 8.1% sodium dodecyl sulphate, 1.5 mL of 20% acetic acid, 1.5 mL of 0.8% thiobarbituric acid,
72 and 0.6 mL of distilled water were added to supernatant. This mixture was heated to 95°C for 60 min.
73 After cooling with tap water, 1.0 mL of distilled water and 5.0 mL of a mixture of n-butanol: pyridine
74 (15:1, v/v) were added and the mixture was shaken vigorously and centrifuged at 2600 x g for 10 min
75 at 25°C. The absorbance of the organic layer was read at 532 nm. Malondialdehyde was quantified
76 using an extinction coefficient of 1.56x10⁵ L/mol per cm and expressed as nmol MDA/mg tissue. The
77 glutathione level was determined by Ellman method [6]. The concentration of GSH was monitored
78 spectrophotometrically at 412 nm, and the results were expressed as µmol/g tissue.

79 **Histopathological Examination**

80 Tissue specimens were fixed in 10% formalin for 24 hours, then embedded in paraffin and
81 sliced into 5 µm sections. Slides were stained with hematoxylin and eosin (H&E) and examined under
82 a light microscope. Each slide was evaluated by two expert investigators blinded to the experiment and
83 data. Intestinal injury was classified into a five-tiered scale defined by Chiu et al. [7] as grade 0: no
84 diagnostic change; grade 1: subepithelial layer lifting from the lamina propria, usually at the apex of

85 the villus; grade 2: moderate epithelial cell layer lifting from the lamina propria; grade 3: loss of a few
86 villi with massive epithelial lifting from the lamina propria with a few denuded villi; and grade 4:
87 disintegration of the lamina propria with ulceration and hemorrhage.

88 **Statistical Analysis**

89 Data were analyzed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA) for Windows.
90 Histopathological findings of the groups were compared using Kruskal-Wallis test. For group
91 comparisons, Mann Whitney U test was used. Bonferroni correction was used to adjust the p value due
92 to multiple comparisons and p value less than 0.017 was considered significant.

93 **Results**

94 Serum MDA and GSH levels of the study groups are presented in Table 1. There was a significant
95 difference between the groups in terms of MDA levels. Serum MDA level in group C was
96 significantly lower than those in group I/R and group I/R+AP (p=0.001 and p=0.008, respectively).
97 However, there was no significant difference between group I/R and group I/R+AP regarding MDA
98 level (p=0.294; Fig. 1). Moreover, no significant difference was found between the study groups in
99 terms of GSH level (Fig. 2).

100 No pathological changes were noted on histopathological evaluation in any of the subjects in the
101 control group. Grade 4 intestinal damage was observed in all of the subjects in I/R group. Whereas,
102 grade 0 damage was noted in 3, grade 2 damage was noted in 2, grade 3 damage was noted in 2, and
103 grade 4 damage was noted in one subject among 8 subjects in group I/R+AP (Fig. 3). According to
104 these histopathological results, median damage grade was 4 in I/R group and 2 in I/R+AP group and
105 the difference was significant (p=0.001). (Fig. 4).

106 **Discussion**

107 Mesenteric ischemia constitutes about 0.1% of all hospital admissions and 1-2% of admissions
108 due to abdominal pain. Despite the increase of knowledge on mesenteric ischemia, it is still associated
109 with high mortality rate [8]. Being a branch of the abdominal aorta, SMA supplies a large part of the
110 intestine, from duodenum to distal transverse colon. Pathological conditions causing an interruption or
111 reduction of blood flow in SMA may lead to mesenteric ischemia. Major goal of the treatment is the
112 restoration of blood flow as soon as possible. During the period of interrupted blood flow, ischemic

113 damage occurs in two stages. The first stage begins immediately after ischemia and continues for a
114 period of 2-3 hours. The second stage begins 12-24 hours after ischemia and lasts about 3-4 days.
115 Clinical presentation may vary depending on the duration of ischemia. Cytokines and acute phase
116 proteins released from the intestinal mucosa during reperfusion following ischemia causes
117 dissemination of the pathological process beyond the intestinal mucosa and affect distant organs.
118 Subsequently, cell death and organ failure occurs. Therefore, there has recently been a growing
119 amount of interest in this severe clinical condition resulting from I/R injury [8].

120 Several agents have been investigated for the prevention and/or treatment of mesenteric I/R
121 injury. Most of the information has been established from experimental studies using animal models.
122 Agents under investigation include antioxidants, several amino acids, phospholipids, hormones,
123 polyphenols and flavonoids, herbal extracts, pharmacological agents, carbon monoxide inhalation,
124 erythropoietin, statins, and hyperbaric oxygen [8, 9].

125 It has been demonstrated in an experimental study that intravenous immunoglobulin has a
126 protective effect against mesenteric I/R damage in rats [10]. In another study using a rat model, it has
127 been reported that hypocapnia reduces I/R injury despite having direct harmful effects on the liver
128 [11]. It has also been shown that alpha-melanocyte-stimulating hormone (MSH) significantly limits
129 postischemic injury in rat small intestine [12]. Furthermore, pyruvate infusion has been reported to
130 have a local protective effect against I/R injury in rat small intestine [13]. It has been shown in a rat
131 model that proanthocyanidin has a protective effect against mesenteric I/R injury both at intestinal and
132 distant organ level [14]. It has also been reported that application of pyrrolidine dithiocarbamate
133 prevents mesenteric I/R injury in rats [15].

134 Apelin has been shown to play significant role in the regulation of cardiovascular functions and
135 fluid homeostasis. Apelin receptor (APJ) mRNA can be detected in several human organs including
136 brain, spleen, thymus, prostate, testis, ovary, small and large intestines [16]. It has been demonstrated
137 that exogenous apelin treatment has a protective effect against myocardial I/R injury in animal models
138 [17-19]. In the study by Zeng et. al. [19], in which Langendorff model of myocardial I/R injury was
139 used in adult male rats, 20 min stabilization followed by 40 min global ischemia (achieved by total
140 perfusion arrest) and then 30 min reperfusion were performed on isolated hearts. They reported a

141 cardioprotective effect in the group in which they used a buffer containing AP. They also
142 demonstrated the protective effect of apelin in a cell culture model using primary neonatal rat
143 cardiomyocytes. We also investigated the effects of apelin on mesenteric I/R injury in a rat model. We
144 found that the median histopathological grade was significantly lower in I/R+AP group compared to
145 I/R group.

146 It is known that oxidative stress induced by ischemia leads to formation of GSH and MDA [20,
147 21]. Turnage et al. [21] evaluated hepatic tissue following experimentally induced intestinal I/R injury
148 for lipid peroxidation products, and oxidized and reduced glutathione. They demonstrated that
149 oxidized glutathione was increased significantly following 30 and 60 min of reperfusion; however,
150 they reported no increase in any of the products of lipid peroxidation. In the present study, we also
151 measured GSH and MDA levels to evaluate ischemia induced oxidative damage in tissues, but failed
152 to demonstrate this damage through these levels.

153 In conclusion, although we demonstrated positive effects of apelin on experimentally induced
154 I/R injury at histopathological level, we did not find any positive effects on oxidative injury. Even
155 though apelin appeared to have a positive effect on oxidative injury, this did not reach statistical
156 significance. It should be noted, however, that the role of this adipocytokine in the initial treatment of
157 intestinal ischemia needs further investigation, and associated findings need to be confirmed by future
158 large scale animal model studies before being tested in clinical conditions in humans. Furthermore,
159 clinical picture resulting from I/R injury is still associated with high morbidity and mortality rates.
160 Studies investigating effectiveness of novel pharmacological agents are needed in order to reduce
161 morbidity and mortality.

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169 **References**

- 170 1. Diepenhorst GM, van Gulik TM, Hack CE (2009) Complement-mediated ischemia-reperfusion
171 injury: lessons learned from animal and clinical studies. *Ann Surg* 249:889-899
- 172 2. Carden DL, Granger DN (2000) Pathophysiology of ischaemia-reperfusion injury. *J Pathol*
173 190:255-266
- 174 3. Mallick IH, Yang W, Winslet MC, et al (2004) Ischemia-reperfusion injury of the intestine and
175 protective strategies against injury. *Dig Dis Sci* 49:1359-1377
- 176 4. Petrescu BC, Gurzu B, Iancu RI, et al (2010) Apelin effects on lipopolysaccharide-increased
177 pulmonary permeability in rats. *Rev Med Chir Soc Med Nat Iasi* 114:163-169
- 178 5. Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric
179 acid reaction. *Anal Biochem* 95:351-358
- 180 6. Ellman GL (1959) Tissue sulfhydryl groups. *Arch Biochem Biophys* 82:70-77
- 181 7. Chiu CJ, McArdle AH, Brown R, et al (1970) Intestinal mucosal lesion in low-flow states. I. A
182 morphological, hemodynamic, and metabolic reappraisal. *Arch Surg* 101:478
- 183 8. Martinez JP, Hogan GJ (2004) Mesenteric ischemia. *Emerg Med Clin North Am* 22:909-928
- 184 9. Chatterjee PK (2007) Novel pharmacological approaches to the treatment of renal ischemia-
185 reperfusion injury: a comprehensive review. *Naunyn Schmiedebergs Arch Pharmacol* 376:1-43
- 186 10. Anderson J, Fleming SD, Rehrig S, et al (2005) Intravenous immunoglobulin attenuates
187 mesenteric ischemia-reperfusion injury. *Clin Immunol* 114:137-146
- 188 11. Duggan M, Engelberts D, Jankov RP, et al (2005) Hypocapnia attenuates mesenteric ischemia-
189 reperfusion injury in a rat model. *Can J Anaesth* 52:262-268
- 190 12. Hassoun HT, Zou L, Moore FA, et al (2002) Alpha-melanocyte-stimulating hormone protects
191 against mesenteric ischemia-reperfusion injury. *Am J Physiol Gastrointest Liver Physiol*
192 282:G1059-G1068
- 193 13. Petrat F, Rönn T, de Groot H (2011) Protection by pyruvate infusion in a rat model of severe
194 intestinal ischemia-reperfusion injury. *J Surg Res* 167:e93-e101
- 195 14. Sizlan A, Guven A, Uysal B, et al (2009) Proanthocyanidin protects intestine and remote organs
196 against mesenteric ischemia/reperfusion injury. *World J Surg* 33:1384-1391

- 197 15. Teke Z, Kabay B, Aytakin FO, et al (2007) Pyrrolidine dithiocarbamate prevents 60 minutes of
198 warm mesenteric ischemia/reperfusion injury in rats. *Am J Surg* 194:255-262
- 199 16. Kleinz MJ, Davenport AP (2005) Emerging roles of apelin in biology and medicine. *Pharmacol*
200 *Ther* 107:198-211
- 201 17. Kleinz MJ, Baxter GF (2008) Apelin reduces myocardial reperfusion injury independently of
202 PI3K/Akt and P70S6 kinase. *Regul Pept* 146:271-277
- 203 18. Simpkin JC, Yellon DM, Davidson SM, et al (2007) Apelin-13 and apelin-36 exhibit direct
204 cardioprotective activity against ischemia-reperfusion injury. *Basic Res Cardiol* 102:518-528
- 205 19. Zeng XJ, Zhang LK, Wang HX, et al (2009) Apelin protects heart against ischemia/reperfusion
206 injury in rat. *Peptides* 30:1144-1152
- 207 20. Janssen M, Koster JF, Bos E, et al (1993) Malondialdehyde and glutathione production in isolated
208 perfused human and rat hearts. *Circ Res* 73:681-688
- 209 21. Turnage RH, Bagnasco J, Berger J, et al (1991) Hepatocellular oxidant stress following intestinal
210 ischemia-reperfusion injury. *J Surg Res* 51:467-471
- 211