

BASIC RESEARCH

# Pretreatment with adenosine and adenosine A1 receptor agonist protects against intestinal ischemia-reperfusion injury in rat

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## Abstract

**AIM:** To examine the effects of adenosine and A1 receptor activation on reperfusion-induced small intestinal injury.

**METHODS:** Rats were randomized into groups with sham operation, ischemia and reperfusion, and systemic treatments with either adenosine or 2-chloro-N<sup>6</sup>-cyclopentyladenosine, A1 receptor agonist or 8-cyclopentyl-1,3-dipropylxanthine, A1 receptor antagonist, plus adenosine before ischemia. Following reperfusion, contractions of ileum segments in response to KCl, carbachol and substance P were recorded. Tissue myeloperoxidase, malondialdehyde, and reduced glutathione levels were measured.

**RESULTS:** Ischemia significantly decreased both contraction and reduced glutathione level which were ameliorated by adenosine and agonist administration. Treatment also decreased neutrophil infiltration and membrane lipid peroxidation. Beneficial effects of adenosine were abolished by pretreatment with A1 receptor antagonist.

**CONCLUSION:** The data suggest that adenosine and A1 receptor stimulation attenuate ischemic intestinal injury via decreasing oxidative stress, lowering neutrophil infiltration, and increasing reduced glutathione content.

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**Key words:** Adenosine; Adenosine A1 receptor; Intestinal ischemia; Pharmacological preconditioning

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## INTRODUCTION

Ischemia-reperfusion (I/R) injury of the intestine is a significant problem in a numerous situations such as abdominal aortic aneurysm surgery, small bowel transplantation, cardiopulmonary bypass, strangulated hernias, and neonatal necrotizing enterocolitis<sup>[1]</sup>. Decreased contractile activity, increased microvascular permeability, and dysfunction of mucosal barrier are all associated with intestinal I/R<sup>[2,3]</sup>. I/R injury of the intestine is an intricate and multifactorial pathophysiological process that involves the formation and action of oxygen free radicals (OFRs)<sup>[3-9]</sup>, inflammatory cytokines, the complement system<sup>[1,3]</sup> and neutrophil infiltration<sup>[1-3,6,7,10]</sup> at the site of damage.

The purine nucleoside adenosine is one of the major local regulators of normal tissue function, acting in both an autocrine and paracrine fashion. Its regulatory function becomes pronounced especially when energy supply ceases abruptly as in the case of ischemia, and fails to meet cellular energy demand. Adenosine exerts its effects by interacting with its receptors, four of which have been cloned and characterized as adenosine A<sub>1</sub>, A<sub>2a</sub>, A<sub>2b</sub>, and A<sub>3</sub><sup>[11-13]</sup>. The physiological role of adenosine in the gastrointestinal tract is still poorly understood, particularly with regard to colonic and ileal motor functions. It has been reported that A<sub>1</sub> adenosine receptor (A<sub>1</sub>AR) antagonists increase defecation in rats<sup>[13]</sup> and that A<sub>1</sub>AR agonists can inhibit intestinal fluid secretion and peristalsis *via* adenosine A<sub>2B</sub> and A<sub>1</sub> receptors, respectively<sup>[14]</sup>.

One of the cellular events observed during ischemia is the increased consumption of ATP, leading to accumulation of adenosine with thereby elevating extracellular adenosine. The accumulation of adenosine is believed to contribute to cytoprotection in the ischemic tissue<sup>[11,12]</sup>. Furthermore, adenosine which is released during short periods of ischemia followed by reperfusion, provides cytoprotection against a subsequent sustained ischemia in heart, resulting in reduced infarct size<sup>[15-20]</sup>. This is known as the preconditioning effect of adenosine, which is mediated mostly through the activation of cardiac A<sub>1</sub>ARs before ischemia<sup>[11,12,18]</sup>. It is well documented that the early<sup>[19,20]</sup> and late<sup>[11,15,18]</sup> phases of ischemic tolerance are mediated by adenosine in myocardium. That adenosine exerts anti-ischemic actions is indicated by

a number of studies using adenosine receptor agonists and antagonists<sup>[15-18]</sup> as well as animals overexpressing or lacking A<sub>1</sub>AR<sup>[21,22]</sup>. Administration of adenosine either prior to ischemia or during reperfusion has been shown to attenuate myocardial injury<sup>[23,24]</sup>. Treatment with adenosine A<sub>1</sub>AR agonist initiates preconditioning not only in heart<sup>[15-18,25]</sup> but also in tissues such as kidney<sup>[26,27]</sup> and brain<sup>[28,29]</sup>, resulting in attenuation of ischemic injury. One of the underlying mechanisms suggested for adenosine receptor-mediated preconditioning in the heart is through involvement of protein kinase C (PKC) in heart<sup>[11,12,30]</sup>. Activation of PKC induces opening of ATP-sensitive K<sup>+</sup> channels<sup>[11,12,31]</sup>. Among other effectors that most likely contribute to the cytoprotection by adenosine are mitogen activated protein (MAP) kinases<sup>[25,31-33]</sup>, heat shock proteins (HSPs)<sup>[11-15,34]</sup>, antioxidant enzymes<sup>[15,34]</sup> and inducible nitric oxide synthase (iNOS)<sup>[11,12]</sup>. Moreover, induction and activation of manganese superoxide dismutase (Mn-SOD) is also believed to be a significant factor in mediating myocardial adaptation in response to activation of A<sub>1</sub>AR<sup>[15]</sup>.

In the phenomenon of ischemic preconditioning (IPC), a short period of ischemia protects the organs (e.g. heart) against a subsequent more substantial ischemic injury<sup>[35]</sup>. In fact, IPC has been one of the most promising strategies against reperfusion injury during the last few years. It appears to elevate the tolerance of the intestine to I/R injury. A number of experimental studies have shown that reperfusion injury in small intestine is prevented by IPC<sup>[36-40]</sup>. IPC conducted in small intestine reduces postischemic leukocyte adhesion by maintaining the bioavailability of nitric oxide<sup>[41]</sup>. Moreover, it lowers the expression of P-selectin<sup>[38]</sup>, which is a downstream effector target of the adenosine-initiated, PKC dependent, signalling pathway in intestine. Although activation of PKC triggered by adenosine has been a crucial factor for initiating the beneficial actions of IPC in most tissues, the effector of the preconditioning phenomenon appears to vary among tissues. Activated-K<sup>+</sup> channels<sup>[42]</sup>, nitric oxide<sup>[39,41]</sup> and endogenous opioid peptides<sup>[43]</sup> have reported to be the other downstream effectors of IPC in intestine. Based primarily on animal experiments, the identification of the molecular mechanisms that are responsible for protection by IPC, has provided opportunities to consider several rational targets for pharmacological intervention. Consequently, a variety of drugs have been demonstrated to be able to mimic IPC when applied instead of ischemia. This is known as pharmacological preconditioning (PPC). Recently, various studies carried out in rat small intestine have demonstrated that establishing PPC by administration of either adenosine<sup>[37]</sup> or A<sub>1</sub>AR agonist<sup>[38]</sup> mimics the protective effects of IPC. Intensive investigation has been focused on explaining how adenosine accomplishes the beneficial effect of preconditioning. For instance, currently published studies suggest important anti-ischemic roles of the A<sub>1</sub><sup>[18,25,30]</sup>, A<sub>3</sub><sup>[17,21]</sup> or A<sub>2a</sub><sup>[44,45]</sup> adenosine receptors in heart. On the other hand, relatively little data are available on the role of the different adenosine receptors in mediating cytoprotection in intestinal tissue which is exposed to I/R. The majority of studies strongly suggest that adenosine can promote protection against I/R injury via activation of different adenosine receptors in various

tissues. A substantial number of studies report that IPC has been beneficial in human heart and the liver. However, both prospective controlled studies in human and experimental studies in animals are lacking<sup>[1]</sup>. Furthermore, research based on administration of drugs that can mimic the effects of IPC is required further to explore the cellular events during I/R injury of the intestine. To date, there is no direct evidence showing possible effects of adenosine and A<sub>1</sub>AR activation on reduced contractility of intestinal smooth muscle due to I/R injury. Therefore, the present study was constructed to explore the possible effects of adenosine and A<sub>1</sub>AR activation on reperfusion injury of small intestinal tissue by evaluating contractile response and levels of thiobarbituric acid-reactive substances (TBARS, a marker of lipid peroxidation), reduced glutathione (GSH, an endogenous antioxidant), and myeloperoxidase (MPO an index of neutrophil infiltration), in terminal ileum subjected to I/R.

## MATERIALS AND METHODS

### Animals

Following Ethical Committee approval, forty adult male Wistar rats, weighing 200-230 g, were obtained from the Experimental Research Section of Zonguldak Karaelmas University, where animals have been reared and maintained under standard conditions, such as stable room temperature (23 ± 2°C), a 12 h light: 12 h dark cycle, and feeding with commercial rat chow and tap water *ad libitum*. Experimental manipulations and surgical operations were approved by the Animal Ethical Committee of the University. Maximum care and a humane approach to use of animals was of primary consideration.

### Experimental groups and operative procedures

The surgical goal was to induce mesenteric ischemia in rats for 30 min followed by a 180 min reperfusion period. On the day before surgery, each animal was fasted overnight with unlimited access to water. Briefly, each animal was anesthetized by an intraperitoneal injection of 50 mg/kg sodium thiopental followed by a midline incision made into the peritoneal cavity. The small bowel was exteriorized gently to the left onto moist gauze, and then the superior mesenteric artery (SMA) was carefully exposed, isolated, and clamped using a microvascular clamp. Intestinal ischemia was confirmed by obvious lack of pulse in the SMA and paleness of the jejunum and ileum. The intestines were then meticulously placed back into the abdomen which was closed with two small clamps. Following 30 min of occlusion time, the clamping was gently released and the intestine inspected for proper reperfusion characterized by regular pulsation. Throughout the surgical procedure, each animal was placed under a heating lamp to maintain constant body temperature (e.g. 37°C). For the purpose of assessing the roles of adenosine and A<sub>1</sub>AR agonist, animals were randomly divided into five groups: (1) Sham-operated group, subjected to laparotomy without performing the occlusion of the SMA; (2) I/R group, subjected to the occlusion of SMA followed by reperfusion; (3) CPA-treated group (0.1 mg/kg, 5 min prior to ischemia) + I/R; (4) Adenosine-treated group (10 mg/kg, 5 min prior

to ischemia) + I/R; (5) DPCPX pretreatment (1 mg/kg, 15 min prior to adenosine administration) + adenosine treatment (10 mg/kg, 5 min prior to ischemia) + I/R. In the last group, confirming the possible effect of selective A<sub>1</sub>AR agonist CPA on reperfusion injury, the selective A<sub>1</sub>AR antagonist DPCPX was administered 15 min prior to adenosine treatment. The route and volume for drug administration were the tail vein and 200  $\mu$ L, respectively. To animals in both sham-control and I/R-control groups were given sterile serum physiological solution in the same volume instead. Choice of dose regimen for the drugs was based on published studies in the literature<sup>[22,37,38]</sup>.

### Preparation of terminal ileum

Upon completion of the I/R period, and whilst still unconscious, the animals were sacrificed by exsanguination of the abdominal aorta. Strips of terminal ileum of 10 mm length were immediately removed 10 cm oral to the ileocecal junction and transferred into a Petri dish containing Krebs solution (in mmol/L: NaCl 118, NaHCO<sub>3</sub> 24.88, KH<sub>2</sub>PO<sub>4</sub> 1.18, KCl 4.7, MgSO<sub>4</sub> 1.16, CaCl<sub>2</sub> 2.52 and glucose 11.1). Then, tissue was longitudinally suspended in a standard organ chamber, and continuously perfused with 20 mL of preoxygenated Krebs solution (pH 7.4), which was bubbled constantly with a mixture of 950 mL/L O<sub>2</sub> and 50 mL/L CO<sub>2</sub> gas and maintained at a temperature of 37°C. One end of the tissue strip was tied to a fixed post and the other attached to an isometric force transducer under a resting tension of 2 g. Isometric responses were monitored by external force displacement transducer (FDA-10A, Commat Iletisim Co., Ankara, Turkey) and recorded on the computer using MP 30 software (Biopac Systems Inc., Santa Barbara, CA, USA). In the organ bath, each strip was allowed to equilibrate for 1 h with intervening washes every 15 min before adding any compound. Tissue samples also obtained from small intestine approximately 10 cm proximal to the ileocecal area were frozen immediately and stored at -40°C for biochemical measurements.

### Concentration-response curves

At the beginning of each experiment to observe dose-contractile response relationship, KCl was added to the organ chamber to a final concentration of 30 mmol/L. For the preparation of high K<sup>+</sup> solutions, NaCl was exchanged for an equimolar amount of KCl to maintain the physiological osmolarity of the Krebs solution. The contraction recorded in response to KCl was considered as a reference response. Afterwards, the contractions in response to carbachol and substance P at various final concentrations ranging from 10<sup>-9</sup> mol/L to 10<sup>-2</sup> mol/L were recorded by pipetting these compounds into the organ bath in a cumulative fashion at equal intervals. At the end of the experiment, the response to 30 mmol/L KCl was measured again to confirm and evaluate the degree of tissue viability. The amplitude of all contractions was then normalized for each g of tissue and expressed as percentage of the initial KCl-reference response. The number of experiments, represented as “n”, indicates that each experiment was performed with a tissue sample taken from one animal.

All experiments were conducted in a paired way. For the purpose of evaluating the effects of ligand, agonist, and antagonist, the maximum response ( $E_{max}$ ) and pD<sub>2</sub> values (e.g. the negative logarithm of the concentration for the half-maximal response, ED<sub>50</sub>) were computed by using GraphPad Prism Software 3.02 (GraphPad Prism Inc., San Diego, CA, USA)<sup>[46]</sup>. The pD<sub>2</sub> values (apparent agonist affinity constants) were calculated from each agonist concentration–response curve by linear regression of the linear median part of the sigmoid curve and taken as a measure of the sensitivity of the tissues to each agonist.

### Drugs

Adenosine, CPA, DPCPX, carbachol and substance P were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). They were dissolved in double distilled water, except for CPA and DPCPX which were initially prepared in dimethyl sulphoxide and then diluted in physiological saline. Adenosine, CPA, and DPCPX were prepared fresh just before usage. Carbachol and substance P were made up at different concentrations and kept frozen in aliquots. Compounds, which were used for preparing Krebs solution, were purchased from Merck (Merck KGaA, Darmstadt, Germany). All other reagents, including trichloroacetic acid (TCA), thiobarbituric acid (TBA), butylated hydroxy toluene (BHT), and dithiobisnitrobenzoate (DTNB) were obtained from Sigma.

### Determination of tissue TBARS and GSH

Tissue TBARS content was measured in order to estimate the extent of lipid peroxidation in the injured terminal ileum. Samples obtained from each group were stored at -40°C until assayed. Tissue samples were washed in ice-cold Krebs solution, blotted on absorbent paper and weighed. Afterwards, each sample was minced followed by homogenization with 10 mL of 100 g/L TCA per g of tissue, using a motor-driven homogenizer (Heidolph Dixa 900, Heidolph Elektro GmbH&Co.KG, Kelheim, Germany). Then, the tissue TBARS levels were measured spectrophotometrically based on a method described by Casini *et al*<sup>[47]</sup> and expressed as nmol/g of tissue weight. Briefly, following two consecutive centrifugations at 3000 g for 15 min, 750  $\mu$ L supernatant was added to equal volume of 6.7 g/L TBA and heated to 100°C for 15 min. The absorbance of the samples was then measured spectrophotometrically at 535 nm (Smart Spectro, LaMotte Co., Chestertown, MD, USA).

The GSH content of the samples were measured by applying a modified Ellman method<sup>[48]</sup>. In brief, 2 mL of 0.3 mol/L Na<sub>2</sub>HPO<sub>4</sub> solution was mixed with 0.5 mL of supernatant obtained by employing the homogenization procedure described above. Into the mixture, 0.2 mL of DTNB solution was added followed by reading absorbance at 412 nm. The tissue GSH levels were expressed as  $\mu$ mol/g of tissue weight.

### Measurement of tissue MPO activity

The degree of neutrophil accumulation in the intestinal tissue samples was measured by assaying MPO activity as described by Bradley *et al*<sup>[49]</sup>. Briefly, upon thawing, each

Table 1  $E_{max}$  and  $pD_2$  values of carbachol and substance P ( $n = 8$ , means  $\pm$  SE)

	Sham Control	I/R Control	CPA-I/R	ADO-I/R	DPCPX + ADO-I/R
Carbachol					
$E_{max}$	488.67 $\pm$ 47.01	157.05 $\pm$ 41.35 <sup>a</sup>	395.05 $\pm$ 32.62 <sup>c</sup>	372.21 $\pm$ 54.68 <sup>c</sup>	212.35 $\pm$ 40.09 <sup>a</sup>
$pD_2$	6.30 $\pm$ 0.19	7.02 $\pm$ 0.31	6.24 $\pm$ 0.40	6.94 $\pm$ 0.13	5.81 $\pm$ 0.18
Substance P					
$E_{max}$	255.94 $\pm$ 31.17	115.00 $\pm$ 13.36 <sup>a</sup>	148.19 $\pm$ 13.80 <sup>a</sup>	242.93 $\pm$ 46.55 <sup>c</sup>	259.61 $\pm$ 24.62 <sup>c</sup>
$pD_2$	6.51 $\pm$ 0.05	8.29 $\pm$ 1.08	7.00 $\pm$ 0.22	6.69 $\pm$ 0.28	6.51 $\pm$ 0.07

<sup>a</sup> $P < 0.05$  vs sham-operated control group, <sup>c</sup> $P < 0.05$  vs I/R control group.

sample was very finely minced with surgical blade in a petri dish containing 50 mmol/L potassium phosphate buffer (PB, pH 6.0) at a volume 20 times the tissue weight (e.g. 1 mL) followed by homogenization for 5 min in ice-cold PB by means of motor driven homogenizer. The homogenate was centrifuged at 40000 g for 15 min at 4°C. The homogenized tissue pellet was suspended in 50 mmol/L PB containing 5 g/L hexadecyltrimethylammonium bromide (HETAB) and then homogenized again. Following three freeze and thaw cycles with sonication (Bandelin Sonopuls HD2070, Bandelin Electronic GmbH&CO.KG, Berlin, Germany) between cycles, the samples were centrifuged at 40000 g for 10 min. Aliquots of supernatant (0.1 mL) were added to 2.9 mL of reaction mixture containing 0.167 mg/mL of *o*-dianisidine, and 20 mmol/L H<sub>2</sub>O<sub>2</sub> solution, which were prepared in 50 mmol/L of PB. Immediately after adding the aliquot to the mixture, the change in absorbance at 460 nm was measured for 5 min. One unit of MPO activity was defined as that degrading 1  $\mu$ mol of peroxide per min at 25°C. The activity was then normalized as unit per mg of tissue (U/mg).

### Statistical analysis

Values for the experiments dealing with contractility were normalized for per g of tissue followed by expressing them as percentage of KCl response. Each data point represents mean  $\pm$  SE. For statistical evaluation, SPSS 11.0 statistical software package programme was used (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was applied for statistical comparison of groups, followed by analysis with Tukey-Kramer test so as to determine differences between the groups. Probability value ( $P$ ) of 0.05 or less was considered statistically meaningful.

## RESULTS

### Ileal longitudinal muscle contractility

For longitudinal ileum muscle collected from sham control, CPA-treated, adenosine-treated, and DPCPX-adenosine-treated animals, mean contraction responses to 30 mmol/L KCl were measured as 0.59  $\pm$  0.01 g; 0.40  $\pm$  0.09 g; 0.50  $\pm$  0.22 g; and 0.44  $\pm$  0.21 g, respectively, which were statistically indistinguishable (Figure 1). In I/R control group however, the contractile response (0.26  $\pm$  0.08 g) was significantly reduced when compared to that in sham-operated control group ( $P = 0.012$ ).

The addition of carbachol at concentrations from 10<sup>-9</sup> mol/L to 10<sup>-2</sup> mol/L into the organ bath resulted

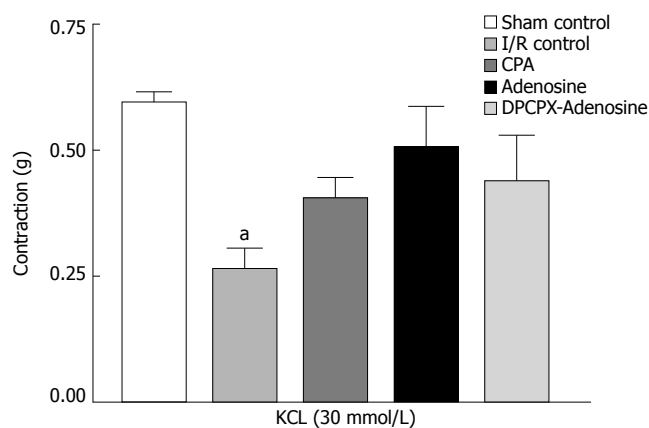


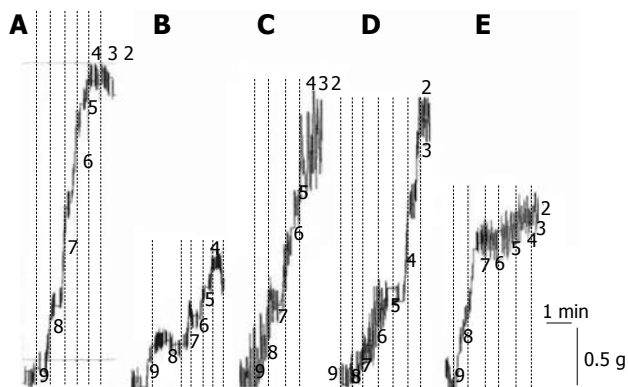
Figure 1 Mean contraction of longitudinal ileum muscle isolated from sham-operated control, I/R control, CPA-I/R, adenosine-I/R, and DPCPX-adenosine-I/R rats in response to 30 mmol/L KCl. Data are expressed as means  $\pm$  SE ( $n = 8$ ). <sup>a</sup> $P < 0.05$  vs sham-operated control group.

in a dose-dependent contractile effect on the terminal ileum segments from all groups (Figure 2), providing sigmoid curves with  $E_{max}$  and  $pD_2$  values (Figure 3).  $E_{max}$  value for carbachol was significantly lower in the I/R control group than in the sham-operated control group (157.04%  $\pm$  41.35% vs 488.66%  $\pm$  47.01%, respectively). In other words, contraction in response to carbachol was significantly reduced by induction of I/R. Statistical difference between the groups appeared to be meaningful at 10<sup>-6</sup> mol/L ( $P = 0.02$ ), reaching a maximal level at 10<sup>-3</sup> mol/L of carbachol ( $P = 0.0001$ ). The I/R-induced reduction in contractility was significantly restored by treatments with both CPA and adenosine but not by pretreatment with DPCPX. Amelioration of reduced contractions with CPA and adenosine therapies became statistically significant at millimolar doses of carbachol ( $P = 0.03$  at 10<sup>-3</sup> mol/L and 10<sup>-2</sup> mol/L).

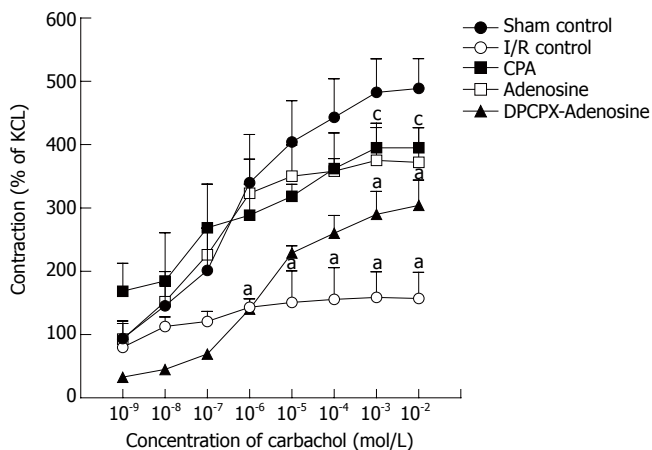
Comparison of the  $E_{max}$  values showed that average contraction of ileum samples in I/R group was just 32% of that in sham-operated control group, while those in CPA- and adenosine-treated groups were approximately 81% and 76%, respectively (Table 1). In the group pretreated with DPCPX,  $E_{max}$  was found to be approximately 60% of that in sham control group, which was statistically significant ( $P < 0.05$ ). On the other hand, no statistically significant change was detected in the corresponding  $pD_2$  values in any group (Table 1).

In response to various concentrations of substance



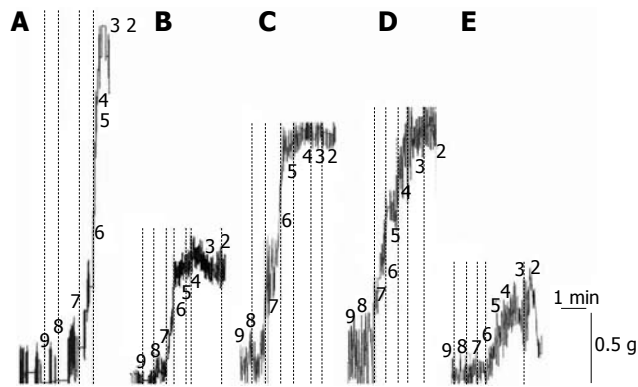


**Figure 2** Representative traces showing responses generated by various concentrations of carbachol in longitudinal ileum muscle isolated from sham-operated control (A), I/R control (B), CPA-I/R (C), adenosine-I/R (D), and DPCPX-adenosine-I/R (E) rats.

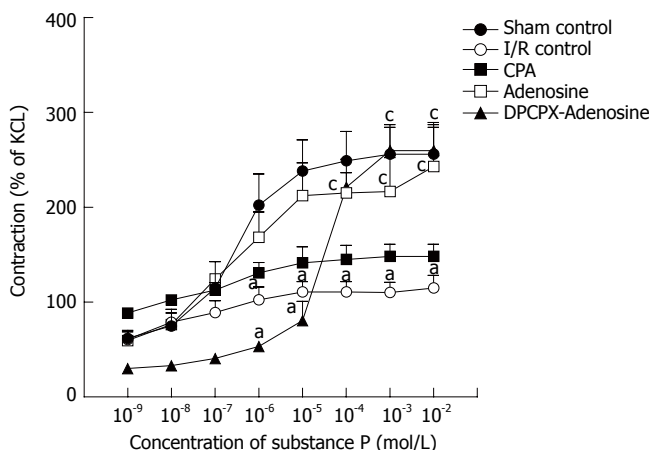


**Figure 3** Dose-response curves of carbachol in longitudinal ileum muscle isolated from sham-operated control, I/R control, CPA-I/R, adenosine-I/R, and DPCPX-adenosine-I/R rats. Data are expressed as means  $\pm$  SE ( $n = 8$ ). <sup>a</sup> $P < 0.05$  vs sham-operated control, <sup>c</sup> $P < 0.05$  vs I/R control groups.

P ranging from  $10^{-9}$  mol/L to  $10^{-2}$  mol/L, terminal ileum samples contracted in a dose-dependent fashion in all groups (Figure 4), rendering sigmoid curves with  $E_{max}$  and  $pD_2$  values (Figure 5). The contractile response induced by substance P was significantly and dose-dependently inhibited by induction of I/R. Statistical difference between sham-operated control rats and I/R control animals was significant at  $10^{-6}$  mol/L and over doses of substance P ( $P < 0.05$ ). Reduced contractility due to I/R was alleviated significantly by adenosine treatment ( $P < 0.05$ ). This effect of adenosine was completely lost once DPCPX was given prior to adenosine administration. However, the exacerbating effect of DPCPX was significantly evident in response to substance P at doses lower than  $10^{-5}$  mol/L (Figure 5). Above this concentration, as shown in the ascending part of the curve, responses in both adenosine- and DPCPX-adenosine-treated groups were statistically indistinguishable. Accordingly, there was a statistically significant difference between I/R control group and DPCPX-adenosine-treated group in response



**Figure 4** Representative traces showing responses generated by various concentrations of substance P in longitudinal ileum muscle isolated from sham-operated control (A), I/R control (B), CPA-I/R (C), adenosine-I/R (D), and DPCPX-adenosine-I/R (E) rats.

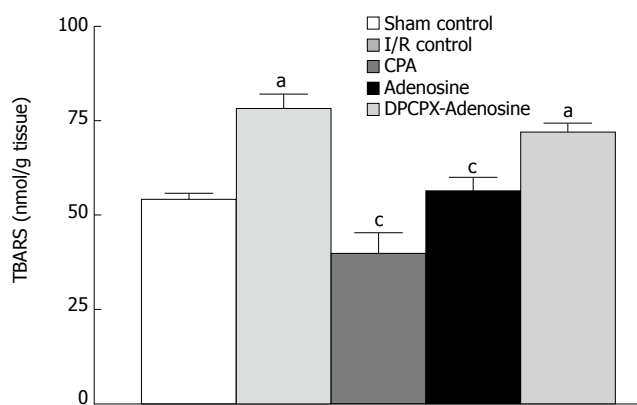


**Figure 5** Dose-response curves of substance P in longitudinal ileum muscle isolated from sham-operated control, I/R control, CPA-I/R, adenosine-I/R, and DPCPX-adenosine-I/R rats. Data are expressed as means  $\pm$  SE ( $n = 8$ ). <sup>a</sup> $P < 0.05$  vs sham-operated control, <sup>c</sup> $P < 0.05$  vs I/R control groups.

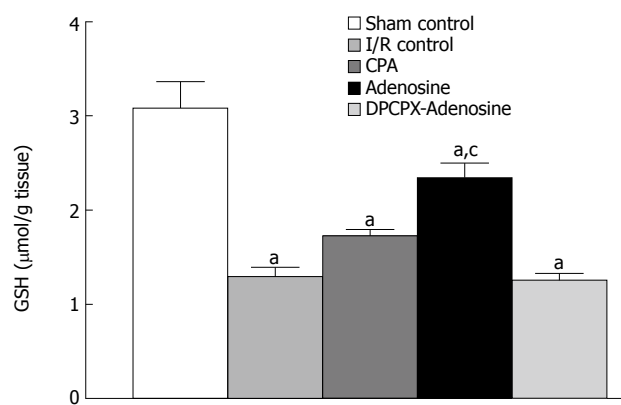
to  $10^{-4}$  mol/L ( $P = 0.022$ ),  $10^{-3}$  mol/L ( $P = 0.004$ ), and  $10^{-2}$  mol/L ( $P = 0.011$ ) of substance P. Regarding the corresponding  $pD_2$  values, no statistically significant change was detected in any group (Table 1).

### TBARS level

Average TBARS content of intestinal samples from sham-operated animals was  $54.18 \pm 3.26$  nmol/g tissue, while that from I/R control rats was  $78.27 \pm 7.60$  nmol/g tissue (Figure 6). I/R caused approximately 1.45 fold increase in TBARS content of the tissue, which was significantly different from that measured in samples from sham-operated animals ( $P = 0.002$ ). Administration of either CPA or adenosine prior to the induction of ischemia significantly reduced the elevated TBARS content to the levels observed in sham control rats. Mean values of the both groups ( $39.87 \pm 11.02$  nmol/g tissue and  $56.49 \pm 7.03$  nmol/g tissue, respectively) were significantly different from that of the I/R control group ( $P = 0.001$ ). On the other hand, in the case of DPCPX pretreatment before



**Figure 6** TBARS content of ileum samples from sham-operated control, I/R control, CPA-I/R, adenosine-I/R, and DPCPX-adenosine-I/R rats. Data are expressed as means  $\pm$  SE ( $n = 8$ ). <sup>a</sup> $P < 0.05$  vs sham-operated control, <sup>c</sup> $P < 0.05$  vs I/R control groups.



**Figure 7** GSH content of ileum samples from sham-operated control, I/R control, CPA-I/R, adenosine-I/R, and DPCPX-adenosine-I/R rats. Data are expressed as means  $\pm$  SE ( $n = 8$ ). <sup>a</sup> $P < 0.05$  vs sham-operated control, <sup>c</sup> $P < 0.05$  vs I/R control groups.

adenosine administration followed by I/R, the average TBARS content was  $72.02 \pm 4.34$  nmol/g tissue, which was not significantly different from that in the I/R control group.

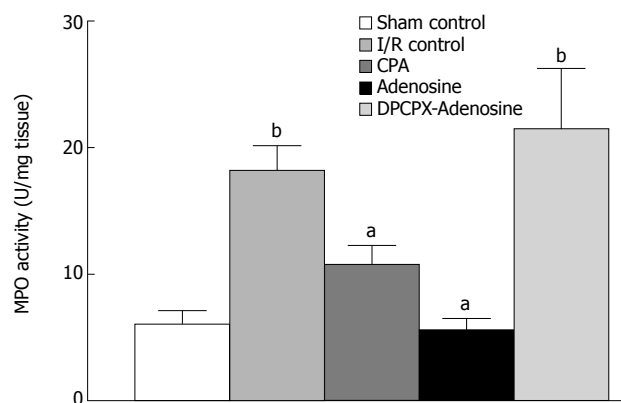
**GSH level**

As shown in Figure 7, the amount of GSH measured in tissues subjected to I/R ( $1.29 \pm 0.19$  μmol/g tissue) decreased approximately 58% compared to that measured in the tissues from the sham-operated group ( $3.08 \pm 0.27$  μmol/g tissue) ( $P < 0.001$ ). Levels of tissue GSH were statistically indistinguishable when comparing the samples from I/R control group with those from CPA-treated group. In contrast, treatment with adenosine significantly ameliorated the decreased amount of GSH. Mean GSH content was  $2.34 \pm 0.31$  μmol/g tissue, which was significantly different from that measured in I/R control animals ( $P = 0.002$ ). However, pretreatment with DPCPX prevented this effect of adenosine, reducing GSH content to the levels observed in I/R control animals.

**MPO activity**

MPO enzyme activities in the terminal ileum samples from animals subjected to sham operation, I/R, CPA treatment, adenosine treatment, and DPCPX-adenosine treatment averaged  $6.09 \pm 1.04$  U/mg tissue,  $18.19 \pm 6.57$  U/mg tissue,  $10.80 \pm 3.66$  U/mg tissue,  $5.58 \pm 2.89$  U/mg tissue, and  $21.45 \pm 9.61$  U/mg tissue, respectively (Figure 8).

I/R caused approximately a 3 fold increase in MPO activity of terminal ileum tissue compared to the basal level of the activity ( $P < 0.0001$ ), which was measured in tissues of sham control animals (Figure 8). As MPO activity of samples from animals treated with CPA or adenosine were significantly different from that in I/R group ( $P = 0.056$  and  $P = 0.0001$ , respectively), it appeared that pretreatment with DPCPX completely abolished the reducing effect of adenosine on MPO activity. Clearly, no statistical difference was observed in MPO activity between I/R control animals and DPCPX-pretreated rats, while there was a significant difference between CPA-treated animals and DPCPX-pretreated animals ( $P = 0.023$ ) as well as between adenosine-treated group and DPCPX-



**Figure 8** MPO level of ileum samples from sham-operated control, I/R control, CPA-I/R, adenosine-I/R, and DPCPX-adenosine-I/R animals. Results are the means  $\pm$  SE of 6 to 8 animals in each group. <sup>a</sup> $P < 0.05$  vs I/R control, <sup>b</sup> $P < 0.001$  vs sham-operated control.

pretreated group ( $P < 0.0001$ ).

**DISCUSSION**

The major findings of the present study can be summarized as follows: (1) I/R resulted in reduced ileal contractility in response to KCl, carbachol, and substance P as well as elevating oxidative stress and neutrophil infiltration; (2) These disturbances were significantly ameliorated by either adenosine administration or A<sub>1</sub>AR activation in the preischemic period; (3) Adenosine and A<sub>1</sub>AR-mediated protection against I/R injury seemed to be associated with decreased oxidative stress and MPO activity; (4) A<sub>1</sub>AR antagonist DPCPX diminished the injury-sparing effect of PPC with adenosine as observed in other tissues. Pharmacological blockade of A<sub>1</sub>ARs exacerbated the contractile response of small intestinal smooth muscle.

I/R results in disrupted exogenous electrical activity and contractile response of ileum<sup>[8-10]</sup>. A substantial amount of evidence indicates that the pathogenesis of I/R and I/R-induced motor alterations have been related to OFRs<sup>[1-3,6,8]</sup> and activated neutrophils<sup>[1-3,10]</sup>. Intestinal I/R sets the groundwork for an inflammatory response in

the vicinity of muscularis cells, provoking the recruitment and extravasation of leukocytes into smooth muscle syncytium<sup>[2,10]</sup>. A number of experimental studies have been conducted in order to test various pharmacological agents that might reduce reperfusion injury of the intestinal mucosa<sup>[4,5,7,9,50,51]</sup> with the intention of improving life-span after acute mesenteric ischemia.

Our results showed that intestinal I/R resulted in decreased ileal contractility in response to carbachol, substance P, and KCl; therefore influencing both receptor-mediated induction and non-receptor-mediated induction. That the  $pD_2$  values in all groups were statistically indifferent from each other however, suggest that I/R does not alter agonist-receptor interaction. Hence, the reduced  $E_{max}$  value in I/R group may be dependent partly on change in the regulation of postreceptor processes (e.g. excitation-contraction coupling)<sup>[9,46]</sup>. Furthermore, the decreased contraction response also observed in non-receptor-mediated induction strongly supports this possibility.

IPC makes reference to a phenomenon in which the harmful effects of prolonged ischemia is prevented by exposure of a tissue to brief periods of ischemia<sup>[55]</sup>. In spite of the fact that it is highly complicated in nature, IPC has been successfully applied to various animal models of intestinal I/R, resulting in attenuation of the reperfusion injury<sup>[37-40,52]</sup>. However, although IPC has been shown to be beneficial in the human heart and liver, prospectively controlled studies in both humans and animals involving IPC and PPC of the intestine are inadequate. More research focused on the application of drugs that can mimic the effects of IPC is needed to analyze the cellular and molecular events during I/R injury of the intestine so as to attenuate I/R injury<sup>[1]</sup>. Both animal and human studies have revealed that adenosine is one of the major triggers for IPC. A study done by Unal *et al*<sup>[37]</sup> has demonstrated that administration of adenosine prior to ischemia is as effective as IPC for inducing ischemic tolerance in rats. Data gathered in the present study confirms this finding and show that the treatment with adenosine significantly restored I/R-reduced contractile response. Furthermore, the treatment also provided such beneficial effects such as elevating GSH content, lowering lipid peroxidation, and reducing neutrophil infiltration. In addition, that the  $A_1AR$  antagonist DPCPX significantly blocked these protective effects of adenosine is consistent with the hypothesis that PPC with adenosine is primarily mediated via  $A_1ARs$ .

The findings of the present study revealed that non-receptor mediated (e.g. KCl-induced) and receptor mediated (e.g. carbachol- and substance P-induced) ileal contractions that were reduced significantly due to I/R, were improved remarkably and returned to sham-control levels by systemic administration of adenosine or CPA. During the process of preconditioning, adenosine is generated in the ischemic tissue. The endogenous adenosine or selective pharmacological agonists activate  $A_1ARs$ . Preischemic activation of  $A_1ARs$  has been demonstrated to prevent from I/R damage in various organs including heart<sup>[15-18,25]</sup>, kidney<sup>[26,27]</sup> and brain<sup>[28,29]</sup>. In these studies, the activation of  $A_1ARs$  has been

strongly implicated in the mediation of IPC. Adenosine therapy before induction of ischemia has been reported to attenuate ischemic injury in heart<sup>[23,24]</sup> and intestine<sup>[37]</sup>. Furthermore, pharmacological blockade of  $A_1AR$  during preconditioning eliminates the achievement of protection<sup>[15-18]</sup>. The protective effect of  $A_1AR$  activation is accomplished through the activation of PKC, leading to translocation of PKC to sarcolemmal and to mitochondrial membranes. Activated PKC then induces an increase in opening of ATP-sensitive  $K^+$  channels in heart<sup>[11,12]</sup>. Stimulation of  $A_1ARs$  also precedes the early activation of some other kinases such as tyrosine kinases, p38, MAPK<sup>[11,19,25,31,33]</sup>, ERK<sup>[32]</sup>, and Akt<sup>[20]</sup>. Additionally, in protection obtained by agonist-induced stimulation of  $A_1AR$ , elevated content or activity of many proteins have been demonstrated such as HSP 27<sup>[11]</sup> and Mn-SOD<sup>[15]</sup>. Despite the existence and involvement of relatively large number of effector molecules, it appears that they vary among tissues. In small intestine, for instance, Davis *et al*<sup>[38]</sup> report that pharmacological modulation of  $A_1ARs$  is involved in reduced expression of P-selectin, which is a downstream effector target of the adenosine-initiated, PKC dependent, anti-inflammatory signaling pathway in preconditioning. In our study, I/R of small intestine elevated the tissue TBARS content, indicating enhanced generation of OFRs; therefore, inducing lipid peroxidation. Systemic administration of adenosine or CPA appeared to be protective against I/R-induced reduction of contractility via, at least, inhibiting lipid peroxidation and neutrophil infiltration as confirmed by reduction of TBARS and MPO levels, respectively. Another significant observation reported recently is that activation of  $A_1ARs$  *in vitro* prevents cellular functions from  $H_2O_2$ -induced injury through signaling pathways related to PKC in renal proximal tubular cells<sup>[53]</sup>. The same observation has been demonstrated in other studies on heart<sup>[54,55]</sup> and kidney<sup>[53]</sup>. In these studies, activation of  $A_1ARs$  *in vivo* and *in vitro* is reported to be associated with protection against  $H_2O_2$ -induced oxidative injury by modulation of the detrimental increases in intracellular calcium concentration and by means of activation of cardiomyocyte  $K^+$  channels after  $H_2O_2$  exposure.

GSH is an endogenous antioxidant and present in all animal cells. Reacting with free radicals, it can provide protection from singlet oxygen, hydroxyl radical and superoxide anion<sup>[51]</sup>. Many published studies indicate that tissue injury, induced by various stimuli (e.g. I/R), is coupled with glutathione depletion<sup>[9]</sup>. In the present study, we showed that depleted GSH content in ischemic ileal tissue was recovered by adenosine or CPA therapies. In other words, inducing PPC with these drugs maintained GSH content during reperfusion. This effect may be related to activation of PKC since adenosine has been reported to induce the activation of antioxidant enzymes *in vitro* and since it is suggested that the stimulatory action of adenosine is likely involved in PKC-mediated phosphorylation. Such a mechanism could serve to decrease the levels of OFRs, which would otherwise be harmful to the cell. This very effect of adenosine is also evident *in vivo*, and may account for adenosine-induced reduction of lipid peroxidation in cochlea<sup>[34]</sup>. Although the

present study has not examined antioxidant enzymes or PKC, the elevated level of GSH implicates the potential involvement of a cytoprotective mechanism related to adenosine and A1 receptor activation.

Modulation of the inflammatory response following I/R injury is an important component of tissue defense, mostly because inflammation is the major component of cell death and motor alteration in intestine subjected to intestinal injury. In the initial period of I/R, generation of OFRs occurs, which is the most likely the initial factor responsible for the induction of neutrophil chemotactic activity. Afterwards an influx of leukocytes during reperfusion triggers an intricate cascade of proinflammatory events associated with cytokine/chemokine release and free radical-mediated intestinal injury<sup>[1,3]</sup>. Upon attachment to endothelium, neutrophils cause the secretion of additional OFRs, contributing to the damage. At this point, the enzyme MPO, found largely in leukocytes particularly in neutrophils, provides an opportunity to check the tissue level of the cells since it is a marker of neutrophil infiltration and accumulation into tissues<sup>[27]</sup>. In the present study, we have demonstrated that the therapy with A<sub>1</sub>AR agonist CPA prevented neutrophil infiltration into the reperfused-intestine as shown by the decrease in MPO content. This finding is in agreement with those of previous studies which reports that A<sub>1</sub>AR stimulation is associated with decreased inflammation and MPO levels<sup>[22,27,38]</sup>.

In the present study, we have demonstrated that administration of adenosine and the A<sub>1</sub>AR agonist CPA ameliorated intestinal contractile dysfunction induced by I/R. The outcome of the study suggests that preischemic administration of adenosine or CPA may protect intestine, as indicated by recovery of contractile response, possibly through decreasing oxidative stresses and reducing neutrophil infiltration. In conclusion, our findings suggest the cellular mechanism by which adenosine and pharmacological stimulation of A<sub>1</sub>ARs attenuate intestinal injury, which may indicate the possible therapeutic usage of adenosine as an adjunct for ischemia and ischemia related small bowel diseases.

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## Background

A number of experimental studies have shown that reperfusion injury in the small intestine is prevented by ischemic preconditioning (IPC). Moreover, various studies using rat small intestine have demonstrated that establishing pharmacological preconditioning (PPC) by administration of either adenosine or adenosine A1 receptor agonist mimic the protective effects of IPC. On the other hand, relatively little data is available on the role of the different adenosine receptors in mediating cytoprotection during intestinal I/R injury. There is no direct evidence which confirms the possible effects of adenosine and adenosine A1 receptor activation on I/R injury-related decreased in contractility of intestinal smooth muscle.

## Research frontiers

That adenosine exerts anti-ischaemic actions is indicated by a number of studies using adenosine receptor agonists and antagonists as well as animals overexpressing or lacking the adenosine A1 receptor. Administration of adenosine either prior to ischemia or during reperfusion has been shown to attenuate myocardial injury. Treatment with adenosine A1 receptor agonist initiates preconditioning not only in heart but also in such tissues as kidney and brain, resulting in attenuation of ischemic injury.

## Innovations and breakthroughs

IPC of the small intestine reduces postischemic leukocyte adhesion by maintaining the bioavailability of nitric oxide. Moreover, it lowers the expression of P-selectin, which is a downstream effector target of the adenosine-initiated, PKC dependent, signalling pathway in intestine. Although activation of PKC triggered by adenosine is a crucial factor for initiating the beneficial actions of IPC in most tissues, the effector of the preconditioning phenomenon appears to vary among tissues. Activated-K<sup>+</sup> channels, nitric oxide, and endogenous opioid peptides have reported to be the other downstream effectors of IPC in intestine. Furthermore, currently published studies suggest important anti-ischemic roles of the A<sub>1</sub>, A<sub>3</sub> or A<sub>2a</sub> adenosine receptors in heart.

## Applications

The therapeutic efficacy of adenosine and the adenosine A1 agonist, 2-chloro-N<sup>6</sup>-cyclopentyladenosine (CPA) should be examined for potential clinical application in the treatment of conditions related to intestinal ischemia-reperfusion injury, such as small bowel transplantation, strangulated hernias, and abdominal aortic aneurysm. In addition, it would be worthwhile to focus on the possible effector molecules (e.g. involvement of PKC, opening of mitochondrial ATP-sensitive K<sup>+</sup> channels, or activation of Akt) which underlie the mechanism (s) responsible for the beneficial effects of adenosine and CPA observed in the present study.

## Terminology

Ischemia: deficient supply of blood to a body part (e.g. any organ) that is due to obstruction of the inflow of arterial blood (for example, by narrowing of arteries as a result of spasm or disease); Ischemia-reperfusion: interruption of the blood flow to a tissue for a period of time followed by restoration of blood flow. During the ischaemic period, a sequence of events is initiated that may ultimately lead to cellular dysfunction or even cell death; Reperfusion injury: When ischemia is ended by restoration of blood flow, a second series of injurious events ensue producing additional damage. The injury produced by reperfusion is more severe than that induced by ischemia and is called reperfusion injury. The primary harmful events are the formation of cytotoxic oxidants (also commonly called oxygen free radicals) derived from molecular oxygen, oxygen free radical-mediated damage to cellular membranes via lipid peroxidation, loss of cellular calcium balance, and generation of inflammatory reaction at the site of damage; Oxidative stress: stress on the body or organism that results from the cumulative damage done by oxygen free radicals which are inadequately neutralized by antioxidants; Agonist: a chemical substance capable of combining with a receptor on a cell and initiating the same reaction or activity typically produced by the binding of an endogenous substance; Antagonist: a chemical substance that acts through receptor to reduce the physiological activity of another chemical or endogenous substance.

## Peer review

The present study is interesting, well designed, and contained novel findings. The study is set up thoroughly and the paper is well written. The conclusions are well based and are of clinical value.

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