



Intestinal Ischemic Preconditioning Modulates Oxidative Stress in Rat's Spinal Cord Ischemic Reperfusion Injury

**Salah O Bashir⁽¹⁾, Ossama A. Mostafa⁽²⁾, Mohamed S Rizk⁽³⁾, Mamduoh R Al-Ridi⁽⁴⁾
Mohamed D Morsy^{*(1)}**

⁽¹⁾Physiology Department, College of Medicine, King Khalid University, Saudi Arabia

⁽²⁾Public Health Department, Faculty of Medicine, Beni Suef University, Egypt

⁽³⁾Biochemistry Department, College of Medicine, Menoufiya University, Egypt

⁽⁴⁾Department of Physiology, Faculty of Medicine (Rabigh Branch), King Abdul Aziz University, Saudi Arabia

*** Corresponding author:**

Mohamed Darwesh Morsy

Physiology Department

College of Medicine

King Khalid University

Saudi Arabia

Tel: +966544495223

Fax: +96672418194

E-mail: morsydarwesh@yahoo.com

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Abstract

Background: Paraplegia is a major devastating and unpredictable complication of spinal cord ischemic reperfusion injury (SC-IRI). Ischemic preconditioning (IPC) is a procedure whereby a brief episode of non-lethal ischemia to an organ produces protection against subsequent detrimental ischemic-reperfusion insult to that organ. It has been shown that IPC may not only induce a local protective effect, but also induces resistant to ischemic-reperfusion damage in remote organs what is termed remote ischemic preconditioning (rIPC).

Aim: The aim of the present study was to investigate the potential protective effect of small intestinal ischemic preconditioning on subsequently induced SC-IRI in rats.

Methods: Fifty male Sprague-Dawley rats were randomly divided into five groups (n=10 each): control rats (C) which underwent no surgery; Sham rats (S) were subjected to laparotomy without induction of rIPC or clamping of the aorta; remote intestinal ischemic preconditioning group (rIPC) were subjected to 3 cycles of 8-min anterior mesenteric artery occlusion followed by 5-minute reperfusion; SC-IRI rats were subjected to laparotomy with clamping of the aorta just above the bifurcation by non-traumatic vascular clamp for 45 minutes, then the clamp was released for reperfusion for 24 hours; and SC-IRI+rIPC rats underwent 3 cycles of 8-minute anterior mesenteric artery occlusion followed by 5-minute reperfusion each. Twenty four hours later, animals underwent another laparotomy and spinal cord ischemia followed by reperfusion similar to group SC-IRI. Twenty four hours after surgery; neurological assessment was done. Serum tumor necrosis factor alpha (TNF- α), spinal cord homogenate levels of prostaglandin E₂ (PGE₂),

malondialdehyde (MDA) and advanced oxidative protein product (AOPP) and nitrite/nitrate (NOx) as well as superoxide dismutase (SOD) and catalase (CAT) activities were assessed. Small intestinal MDA homogenate levels and CAT activities were also measured.

Results: Induction of intestinal rIPC 24 hours before SC-IRI produced significant reduction of the serum TNF- α , and spinal cord homogenate levels of PGE₂, MDA, AOPP and NOx as well as a significant reduction of SOD and a significant increase in CAT activity levels compared with the SC-IRI group.

Conclusions: Remote small intestinal ischemic preconditioning ameliorated the clinical neurological dysfunction induced by ischemic reperfusion injury of spinal cord. This ameliorating effect appears to be through improvement of the oxidative stress level, lipid peroxidation and the preservation of SOD and CAT activities in the spinal cord.

Keywords: spinal cord ischemia, remote preconditioning, intestinal ischemia, oxidative stress, thoraco-abdominal aneurismal repair.

1. Introduction

Severe neurological injury still represents one of the most disastrous complications occurring after surgical repair of thoraco-abdominal aneurysms due to spinal cord ischemic reperfusion injury (SC-IRI) [1]. However, conventional strategies for preventing paraplegia due to spinal cord ischemia provide insufficient protection and cause additional side effects [2]. The pathophysiological mechanisms of neuronal damage during SC-IRI involve the production of free radicals, lipid peroxidation, intracellular calcium deposition, and apoptosis [3]. Free radicals, particularly reactive oxygen or nitrogen intermediates, are thought to be involved in inflammatory processes, exacerbating inflammation, and causing tissue damage [4]. It has been reported that ischemia induces oxidative stress, leading to induction and expression of various genes in a variety of cell types throughout the central nervous system [5]. One of those important genes is the cyclooxygenase enzyme (COX) gene. This enzyme is the rate-limiting enzyme involved in arachidonic acid metabolism, with subsequent generation of prostaglandins and thromboxanes that play important roles in sustaining the inflammatory response and inducing different neurological deficits [6]. It was also found that, elements of oxidative stress are essential for the activation of COX enzyme [7]. On the other hand, inflammatory mediators increase nitric oxide (NO) production during ischemic reperfusion injury in tissues by activation

of induced nitric oxide synthase (iNOS). Excessive NO interacts with superoxide anion to form a toxic product, peroxynitrite with subsequent oxidative injury. So, it was suggested that a vicious cycle is created between oxidative stress and inflammatory mediators [8].

Ischemic preconditioning (IPC) is a procedure whereby a brief episode of non-lethal ischemia produces protection against a subsequent detrimental ischemia-reperfusion insult (ischemic tolerance) [9]. IPC is perhaps the most powerful endogenous protective mechanism to protect against ischemic reperfusion injury in cardiac and non-cardiac tissues [10]. Recently, it was reported that, a transient ischemic episode in one organ not only augments the ischemic tolerance within the same organ but may also have a remote effect on other organs [11]. This phenomenon of cross tolerance has been termed 'remote ischemic preconditioning' (rIPC). Remote renal IPC protection is reported to exist in animals and humans [12]. Remote IPC of the small intestine elicits not only a local protective effect, but also protects remote organs like the heart during cardiac ischemic reperfusion injury [10]. Dong et al. reported that rIPC by transient hind limbs ischemia resulted in resistance of the spinal cord to ischemia and tissue damage [1]. However, little attention has been paid to spinal cord protection by remote organ IPC.

Several studies have shown that rIPC has a protective effect which follows two different time scales, classified as 'short-term' and 'long-term' effects of rIPC "second window of protection" [13]. The protective effect of short-term rIPC lasts

for 2-3 hours after ischemic insult during which adenosine, bradykinin, norepinephrine and opioids are released. These compounds prevent damage by activation of the potassium channels and increasing ATP stores [14]. The long term protective mechanism of rIPC begins 12-24 hours after ischemia and involves iNOS, COX-2 activities and heat shock proteins [13].

Small intestinal rIPC protective effects on neurological tissues have not been fully clarified and addressed. So, the aim of this study was to investigate the possible protective effect of long-term small intestine rIPC on the spinal cord against oxidative stress damage caused by ischemic reperfusion injury. This was achieved through assessment of changes spinal cord oxidative stress and inflammatory mediators' levels, associated with SC-IRI.

2. Methods

All animals received human care in compliance with the Public Health Service Policy on Human Care and Use of Laboratory Animals, published by the National Institutes of Health and were approved by the Ethical Committee of the College of Medicine, Menoufiya University, Egypt.

2.1 Experimental animals

This study followed a randomized controlled animal experiment design. At the start of the experiment, a total of 80 male Sprague-Dawley rats were obtained from the animal house of King Khalid University, weighed between 150 - 200 gm. Animals were fed on a standard chow diet, water, *ad libitum* and housed in the animal house of faculty of Medicine with a 12:12-hour light/dark cycle. Animals with any motor deficit; limping or abnormal placing/stepping reflex had been excluded. The apparent healthy animals were randomly divided into five groups (n=10 each) as follows: C control rats which underwent no surgery; Sham rats were subjected to laparotomy without clamping of the aorta or induction of IPC; intestinal IPC group were subjected to 3 cycles of 8-minute anterior mesenteric artery occlusion followed by 5-minute reperfusion without SC-IRI [14]; SC-IRI rats were subjected to laparotomy

with clamping of the aorta, just above its bifurcation by non-traumatic vascular clamp for 45 minutes, then the clamp was released for reperfusion for 24 hours. SC-IRI+IPC rats underwent 3 cycles of anterior mesenteric artery occlusion, followed by reperfusion as in sham group. Twenty four hours later, the animals of this group were subjected to another laparotomy and spinal cord ischemia was performed, followed by reperfusion as in group SC-IRI. The reason for producing the transient IPC of the small intestine 24 hours ahead of SC-IRI and reperfusion for another 24 hours in our study was to benefit from the long-term effect of IPC.

2.2 Induction of intestinal ischemic preconditioning and ischemic reperfusion injury of the spinal cord

Intestinal IPC was induced in IPC and SC-IRI+IPC groups: Feeding of the animals was stopped 12 hours prior to the start of the intestinal IPC procedure and they received only water. Each rat received 150 IU/kg heparin i.p. and was anesthetized with sodium pentobarbital (60 mg/kg i.p.). The anterior mesenteric arteries were dissected free, and a suture was placed around the arteries to facilitate occlusion. Occlusion of the anterior mesenteric arteries with a nontraumatic microvascular clamp caused ischemia. Ischemia was recognized by the absence of pulse and pale color of the intestine. Reperfusion was achieved by releasing the clamp. This procedure was done for 3 cycles (each lasting for 8 minutes of ischemia followed by 5 minutes for reperfusion). The abdomen was then closed and fluid losses were replaced by i.p. administration of 5 ml of pre-warmed (37 °C) isotonic saline after surgery [14].

Spinal cord ischemia was induced in SC-IRI and SC-IRI+IPC rats, as described by Akguna et al. [15]. Rats were initially anesthetized with i.m. ketamine (50 mg/kg), followed by a half dose as required during the procedure. The animals did not receive ventilatory support. Body temperature was monitored by a rectal probe and was maintained between 37°C - 38°C by a thermal pad and a heating lamp. The femoral artery was cannulated with a 22-gauge PE catheter, which was used to monitor distal arterial pressure (DAP)

and for intra-arterial infusion of heparin. The left carotid artery was cannulated with a 20-gauge PE catheter (Terumo, Tokyo, Japan), which was used to monitor the proximal artery pressure (PAP) and to take blood samples. Each rat received 150 IU/kg heparin injected into the femoral artery immediately after completion of arterial cannulation and before aortic occlusion. The abdominal aorta was reached through midline laparotomy. Animals were then subjected to aortic cross clamping by non-traumatic vascular clamp placed under the left renal vein and above the bifurcation of the aorta for 45 minutes. The success of occlusion was documented by an immediate and sustained decrease in DAP in the femoral artery. To maintain the PAP approximately at 40 mmHg during occlusion, blood from the carotid artery was allowed to flow into a collecting circuit filled with heparinized saline (4 U/ml of saline) positioned 54 cm above the rat. The temperature of the blood in the syringe was maintained at 37- 37.5°C. The aortic clamps were released after 45 minutes and the blood in the syringe was transfused back into the rat over a 60-second period. After completion of all procedures, the wounds were closed. Immediately after surgery, animals were rehydrated with 5 ml/kg i.p. pre-warmed isotonic saline (37°C). The abdominal incision was closed in two layers with silk suture and protamine sulfate (4 mg) was subcutaneously injected to reverse the anticoagulation effect of heparin. Animals were allowed to recover in a plastic box at 28°C for 3 hours and were then placed in their cages with free access to food and water [16]. Rats with complete hind limb paralysis for 24 hours, hematuria, or 25% reduction in body weight were killed using a lethal dose of thiopental sodium injection (75 mg/kg i.p.) for humanitarian reasons. The survival rate in IPC group was 90%, in SC-IRI group was 60% and SC-IRI+IPC was 90%. One rat of the sham group died from surgical trauma during the operation. In spite of these different survival rates we included 10 living rats in each group to be involved in the study.

2.3 Neurological assessment

After 24 hours from the second surgical procedures for group SC-IRI+IPC and after 24

hours from the surgical procedures in group SC-IRI and 48 hours after surgical procedure in group IPC, neurological assessment was done. Hind limbs neurologic function was assessed using the Tarlov Scoring System [17]. A score of 0–5 was assigned to each animal as follows: 0= no voluntary hind limb movement; 1= movement of joints perceptible; 2= active movement but unable to sit without assistance; 3= able to sit but unable to hop; 4= weak hop; 5= complete recovery of hind limb function. The placing/stepping reflex (SPR) was assessed by dragging the dorsum of the hind paw along the edge of a surface. This normally evokes a coordinating lifting and placing response which was graded as follows: 0 = normal; 1 = weak; and 2 = no stepping [18]. Sensory function was assessed by a hind limb withdrawal from a noxious stimulus (1 = withdrawal response to a stimulus applied to hind limbs and 0 = no response to a stimulus). One member of the research team, was blinded to the different groups, conducted all neurological tests.

2.4 Blood sampling and biochemical measurements

After the neurological assessment, retro-orbital blood samples were obtained through non-heparinized capillary tubes after overnight fasting. Samples were allowed to clot for 20 minutes at 37°C water bath, then, centrifuged at 14,000 rpm for 10 minutes for serum separation and used for determination of tumor necrosis factor alpha (TNF- α) level.

2.5 Determination of serum TNF- α level

TNF- α level was measured by cytotoxicity on L929 cells by enzyme-linked immunosorbent assay, using recombinant human TNF- α (BASF/Knoll, Ludwigshafen, Germany; specific activity 10⁷ U/mg) and expressed in pg/mL [19].

2.6 Preparation of small intestinal and spinal cord homogenates

After obtaining the blood samples, all rats were killed using a lethal dose of thiopental sodium injection [17]. For small intestinal homogenate preparation, 3 cm of rat jejunum were washed two times with cold saline solution and homogenized using an Omni tissue homogenizer

(Omni international, Gainesville, VA, USA) in buffer at a ratio of 1/10 (50 mM potassium phosphate buffer pH: 7.8, containing 0.5 mM/L PMSF, 10 µg/mL aprotinin). The homogenate was centrifuged at 2500x g for 10 minutes at 4°C. The supernatants were then stored at -80°C till the time of estimation of catalase (CAT) activity and malondialdehyde (MDA) level in the homogenate. For spinal cord homogenate preparation, sections of the 3, 4, 5 lumbar segments of the spinal cord were harvested, dissected out, sliced into small pieces and homogenized using an Omni tissue homogenizer. Tissues were homogenized in ice-cold lyses buffer [0.1 M potassium phosphate, pH 7.4, 1 mM EDTA, 10 µM indomethacin (Cayman Chemical, Ann Arbor, MI, USA)] using a tube pestle. Acetone was added (2x sample volume), and samples were centrifuged at 1500 x g for 10 minutes at 4°C. The supernatants were then stored at -80°C till the time of estimations [21].

2.7 Estimation of spinal cord homogenate levels of MDA and advanced oxidative protein product (AOPP) levels and Nitrite/Nitrate (NOx) as well as SOD and CAT activities

Lipid peroxidation was assessed by the measurement of secondary product MDA after precipitation of protein by addition of trichloroacetic acid then thiobarbituric acid (TBA) which reacted with MDA to form TBA reactive product, which was measured at 532 nm. MDA solution, freshly made by the hydrolysis of 1,1,3,3-tetramethoxypropane, was used as a standard [21]. Determination of AOPP was based on spectrophotometric method detection (Shimadzu Corporation, Kyoto, Japan, UV- 160A) with tissue homogenate diluted with PBS to 0.2-0.5 g/mL with characteristic absorbance at 340 nm [22]. SOD activity was assayed following the method of Kakkar et al. [23]. The sample containing 5 µg protein was mixed with sodium pyrophosphate buffer, phenazine methosulphate (PMT) and nitro blue tetrazolium (NBT). The reaction was started by the addition of NADH, incubated at 30°C and stopped by the addition of 1 mL of glacial acetic acid. The absorbance of the chromogen formed was measured at 560 nm. One unit of SOD activity is defined as the enzyme concentration required to inhibit chromogen

production by 50% in one minute per mg protein under the assay condition [23]. CAT activity was measured in homogenate by the method of Bonaventura et al. [24]. Five micrograms proteins from the homogenate were mixed with 2 mL of 7.5 mM H₂O₂ and a time scan was performed for 10 minutes at 240 nm at 25°C. One unit of CAT activity is defined as the amount of enzyme decomposing 1 µM of H₂O₂ per minute per milligram protein [24]. The method for estimation of total NOx level was based on the Griess reaction. Spinal cord homogenate nitrite/nitrate levels were measured after enzymatic conversion of nitrate (NO₃⁻) to nitrite (NO₂⁻) by nitrate reductase in the presence of NADPH. The oxidation of the coenzyme was monitored by the decrease in absorbance at 540 nm. Results were expressed as µM /L [25]. Small intestinal MDA homogenate level and CAT activity were done by the same technical manoeuvres as done for spinal cord homogenate.

2.8 Spinal cord homogenate prostaglandin E₂ (PGE₂) determination

Spinal cord homogenate levels of PGE₂ were assayed using monoclonal enzyme immunoassay (EIA) kit. The EIA kit demonstrates sensitivity from 10 to 1000 pg/ml and demonstrates little cross reactivity between structurally related PE₃ and PE₁. Absorbance (412 nm) values of standards and samples were corrected by subtraction of the background value to correct for absorbance caused by nonspecific binding [26].

2.9 Chemicals

Nitrate reductase from *Aspergillus* was supplied by Sigma (St Louis, MO, USA) that utilizes polyoxyl-35- Ricinusolle as an emulsifier; ketamine hydrochloride was supplied by Pfizer Pharmaceutical Company, Inc, USA. Thiopental sodium was supplied by Biochemie, Austria; heparin and protamine sulfate was ordered from Leo, Ballerup (Denmark); Prostaglandin E₂ EIA kit and indomethacin were purchased from Cayman Chemical, Ann Arbor, MI, USA.

2.10 Statistical analysis

Data were expressed as frequency, percentage and mean±SD. Testing significance was performed using χ^2 -test and the one-way analysis of variance (ANOVA). Post-hoc Scheffe test was applied to identify the source of statistical significance. P-values <0.05 were considered as statistically significant.

3. Results

The transient induction of intestinal IPC in rats did not produce any significant changes in small intestinal homogenate level of CAT or MDA activity compared with the control levels [Fig. 1]. Both the sham and the IPC groups did not show any significant changes in all measured parameters compared with the control group or with each others

3.1 Serum level of TNF- α and PGE₂ spinal cord homogenate level

Induction of SC-IRI in rats produced significant elevation of serum TNF- α (10.2±1.41 pg/mL) and spinal cord homogenate of PGE₂ (30.1±2.7 pg/mL) levels compared with the control groups [C (2.7±0.11 pg/mL), sham (2.8±0.63 pg/mL) and IPC (3.1±0.23 pg/mL), (P<0.001)] and [C (17.3 ±1.5 pg/mL), sham

(20.2±1.8 pg/mL) and IPC (18.1±1.9 pg/mL), (P<0.001)] respectively. On the other hand, induction of intestinal rIPC 24 hours before SC-IRI resulted in significant reduction of the serum TNF- α (6.2±0.81 pg/mL) (P<0.001) and spinal cord homogenate of PGE₂ (25.3±1.7 pg/mL) (P<0.05) levels compared with SC-IRI group or control groups (Fig. 2A and 2B), while serum TNF- α level did not show any significant changes in either the sham or IPC groups.

3.2 Spinal cord homogenate activities of SOD and CAT

Induction of SC-IRI in rats resulted in significant elevation of the SOD activity (10.1±1.0 unit/min/mg protein) and significant reduction of CAT activity [1.4±0.3 (μ mol H₂O₂ decomposed /min/mg protein)] in the spinal cord homogenate compared with the control group [(7.4±0.9 unit/min/mg protein) (p<0.05) and [4.1±0.5 (μ mol H₂O₂ decomposed /min/mg protein)] (p<0.001) respectively]. On the other hand the SC-IRI+IPC group showed a significant reduction in SOD (7.1±0.8 unit/min/mg protein) (p<0.05) and a significant increase in CAT [2.7±0.6 (μ mol H₂O₂ decomposed /min/mg protein)] (p<0.05) activities compared with the SC-IRI group (Fig. 3A and 3B).

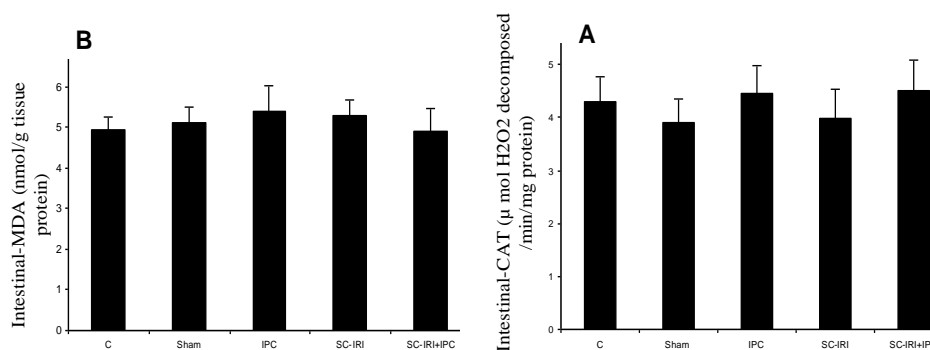


Figure 1. Effect of remote intestinal ischemic preconditioning (IPC) on catalase enzyme (CAT) activity [A] and malondialdehyde (MDA) level [B] in small intestinal homogenate. C: Control group; Sham: sham group; IPC: intestinal preconditioning without spinal ischemic cord injury group; SC-IRI: Spinal cord ischemic reperfusion injury group; SC-IRI+IPC: remote intestinal IPC in spinal cord ischemic reperfusion injury group.

Results are expressed as mean ±SD (n=10).

All differences between groups are not statistically significant.

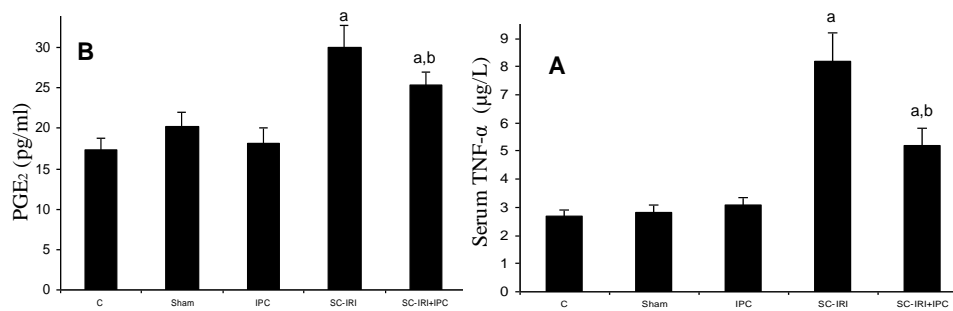


Figure 2. Effect of remote intestinal ischemic preconditioning (IPC) on serum TNF- α [A] and prostaglandin E₂ (PGE₂) [B] levels in spinal cord homogenate. Control group; Sham: sham group; IPC: intestinal preconditioning without spinal ischemic cord injury group; SC-IRI: Spinal cord ischemic reperfusion injury group; SC-IRI+IPC: remote intestinal IPC in spinal cord ischemic reperfusion injury group.

Results are expressed as mean \pm SD (n=10).

a. Significant difference, as compared with the control groups (C, Sham, IPC).

b. Significant difference, as compared with the SC-IRI group.

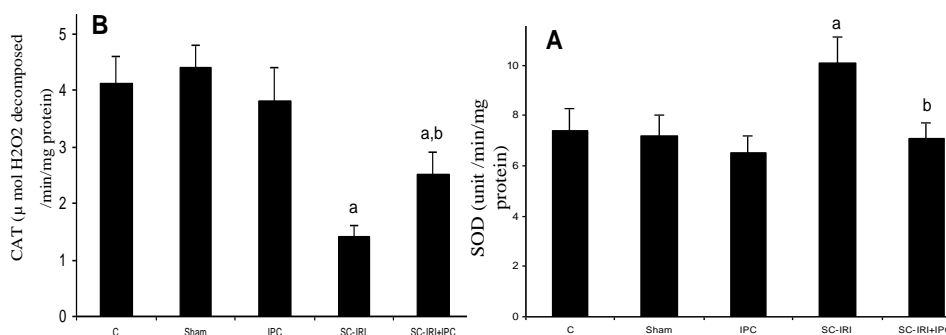


Figure 3. Effect of remote intestinal ischemic preconditioning (IPC) on superoxide dismutase (SOD) [A] and catalase (CAT) [B] activities in spinal cord homogenate. C: Control group; Sham: sham group; IPC: intestinal preconditioning without spinal ischemic cord injury group; SC-IRI: Spinal cord ischemic reperfusion injury group; SC-IRI+IPC: remote intestinal IPC in spinal cord ischemic reperfusion injury group.

Results are expressed as mean \pm SD (n=10).

a. Significant difference, as compared with the control groups (C, Sham, IPC).

b. Significant difference, as compared with the SC-IRI group.

3.3 Spinal cord homogenate levels of MDA, AOPP and NOx

Lipid oxidative product MDA [6.1 ± 0.6 (nmol/g tissue protein)], protein oxidative product AOPP (7.5 ± 0.9 ($\mu\text{mol/mg}$ protein)) and NOx levels (0.233 ± 0.029 (nmol/g wet tissue)) levels in spinal cord homogenate increased significantly in SC-IRI

group compared with the control groups [2.9 ± 0.3 (nmol/g tissue protein)], (3.8 ± 0.4 ($\mu\text{mol/mg}$ protein)) and (0.161 ± 0.018 (nmol/g wet tissue)), ($P < 0.001$)] respectively. While induction of intestinal rIPC in SC-IRI+IPC group produced significant reduction spinal cord homogenate levels of MDA (4.1 ± 0.4 (nmol/g tissue protein)),

AOPP (5.1 ± 0.7 ($\mu\text{mol}/\text{mg}$ protein)) and NOx (0.171 ± 0.021 (nmol/g wet tissue)) compared with the SC-IRI group ($p < 0.05$) (Fig. 4A, 4B and 4C).

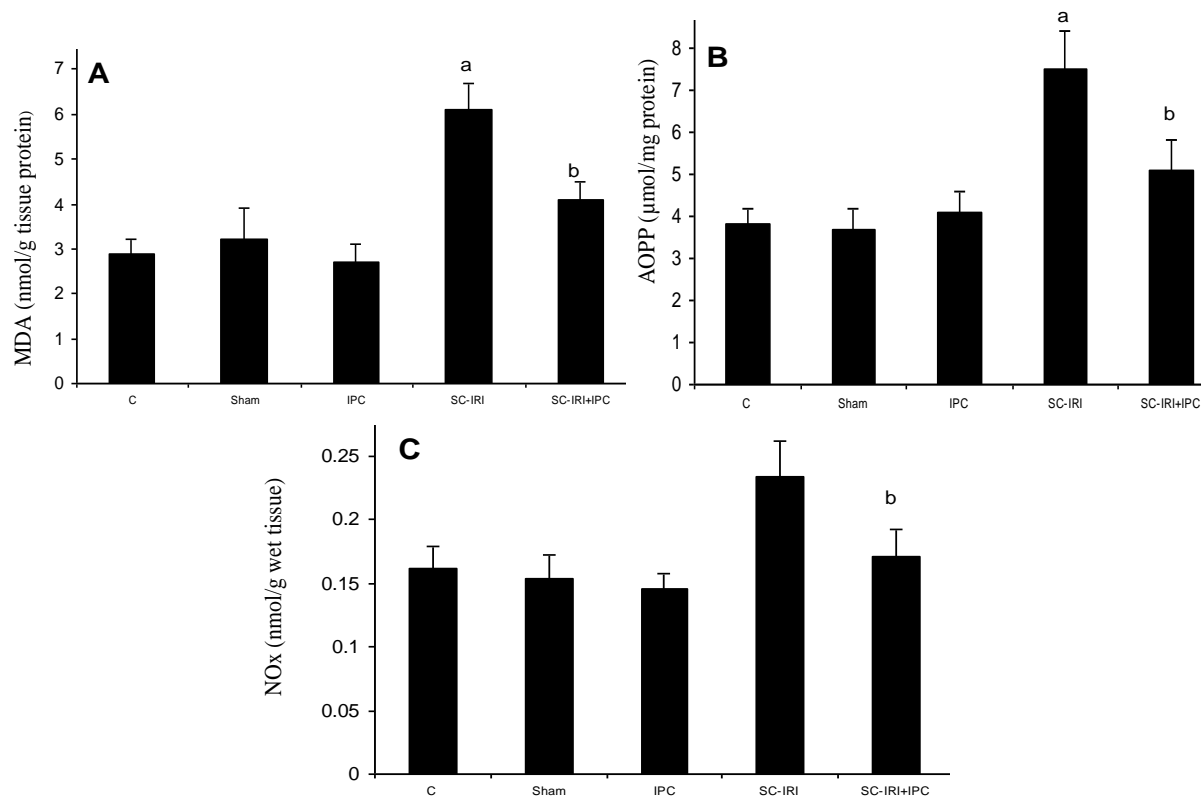


Figure (4): Effect of remote intestinal ischemic preconditioning (IPC) on malondialdehyde (MDA) [A], advanced oxidative protein product (AOPP) [B] and Nitrite/Nitrate (NOx) [C] activities in spinal cord homogenate. C: Control group; Sham: sham group; IPC: intestinal preconditioning without spinal ischemic cord injury group; SC-IRI: Spinal cord ischemic reperfusion Injury group; SC-IRI+IPC: remote intestinal IPC in spinal cord ischemic reperfusion injury group.

Results are expressed as mean \pm SD (n=10).

a. Significant difference, as compared with the control groups (C, Sham, IPC).

b. Significant difference, as compared with the SC-IRI group.

3.4 Motor, SPR and sensory assessment

Rats in the SC-IRI group showed acute flaccid paraplegia of the hind limbs up to 6 hours after reperfusion injury followed by spastic paraplegia. Their motor score (mean \pm SD) was (1.2 ± 0.7) compared with (4.4 ± 0.2) in the control group. Also sensory function was impaired in 66.6% of rats in the SC-IRI group, while SPR was lost in 33.3% and was impaired in 16.7% of SC-IRI rats. On the other hand, induction of intestinal rIPC in SC-IRI group produced recovery of motor and sensory functions and SPR (3.9 ± 1.0 , 88.9% and 77.8%, respectively) (Table 1).

4. Discussion

The major targets of this study were to demonstrate the remote effects of transient intermittent small intestinal IPC on the spinal cord ischemic-reperfusion injury and to investigate whether the intestinal tissues may suffer any significant deterioration in their oxidation markers during this procedure.

It is well documented that 40-60 minutes' ischemia produces ischemic tissue impairment which appears as early as 2 hours after reperfusion and reaches its peak 24 hours after reperfusion [9]. The evidence indicates that the spinal cord

ischemia causes remarkable dysfunctional injuries and reperfusion actually aggravates the neurological damage [27]. Therefore, in the present study we induced SC-IRI by aortic artery occlusion for 45 minutes, followed by 24 hours reperfusion. The induced SC-IRI caused significant neurological deficit both motor and sensory. The neurological manifestations were associated with strong evidence of lipid peroxidation. Evidence of oxidative stress included the significant increase in serum TNF- α level and spinal cord homogenate levels of PGE₂ and MDA in addition to AOPP and SOD activity with a significant reduction in CAT activity. The neurological deficits and their strong association with the markers of oxidative stress and inflammation have been strongly documented in

previous studies [15]. The mechanisms responsible for SC-IRI are not fully understood. However, the reduction of CAT activity is possibly responsible for a number of deleterious effects in ischemic reperfusion injury due to the accumulation of H₂O₂ [28]. Lipid peroxidation, induced by reactive oxygen species (ROS) such as superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical (OH[•]), has also been postulated to play an important role in post-ischemic acute spinal cord injury [28]. Alternatively, it has been proposed that inflammatory cells, especially neutrophils, are activated during ischemia, presumably by cytokines, and enter the spinal cord at the onset of reperfusion causing tissue damage by releasing ROS [8].

Table 1. Effect of remote intestinal IPC on SC-IRI in rats regarding scores of motor function, sensory response and placing/stepping reflex.

	Test	C	Sham	IPC	SC-IRI	SC-IRI+IPC	p-value
Motor assessment	Mean \pm SD	4.4 \pm 0.2	4.6 \pm 0.5	4.3 \pm 0.9	1.2 \pm 0.7 ^(a)	3.9 \pm 1.0 ^(b)	F= P<0.001
Sensory assessment	Normal	10 (100%)	9 (100%)	9 (100%)	2 (33.3%) ^(a)	8 (88.9%) ^(b)	χ^2 = p<0.05
	Affected	0 (0%)	0 (0%)	0 (0%)	4 (66.6%) ^(a)	1 (11.1%) ^(b)	
Placing/Stepping Reflex	Normal	10 (100%)	9 (100%)	9 (100%)	3 (50%) ^(a)	7 (77.8%) ^(b)	χ^2 = p<0.01
	Impaired	0 (0%)	0 (0%)	0 (0%)	1 (16.7%) ^(a)	1 (11.1%)	
	Lost	0 (0%)	0 (0%)	0 (0%)	2 (33.3%) ^(a)	1 (11.1%)	

C: Control group; Sham: sham group; IPC: intestinal preconditioning without spinal ischemic cord injury group; SC-IRI: Spinal cord ischemic reperfusion Injury group; SC-IRI+IPC: remote intestinal IPC in spinal cord ischemic reperfusion injury group.

Results are expressed as mean \pm SD (n=10).

P value < 0.05 is considered significant.

a. Significant difference, as compared with the control groups (C, Sham, IPC).

b. Significant difference, as compared with the SC-IRI group.

The high serum NO_x and TNF- α level in spinal cord homogenate, shown in the SC-IRI group, indicate iNOS activation, with subsequent increased inflammatory processes. The contribution of this process to the neurological damage has also been documented. Naik et al. [29] reported that, vascular neurological injuries stimulate iNOS activity with subsequent NO overproduction. This induces the formation of inflammatory cytokines, such as TNF- α and interleukin-1, with subsequent activation of COX-2 and PGE₂ overproduction. Consistent with this, Nogawa et al. [30] found that COX-2 mRNA expression in the brain peaked 12 hours after middle cerebral artery occlusion with concomitant iNOS over-expression. Another factor contributing to the ischemic reperfusion induced NO overproduction may be the reduction of the nuclear factor kappaB-dependent pro-inflammatory factors which play a major role in the activation of iNOS during the inflammatory process [31].

The small intestine is known as one of the most susceptible organs to ischemic reperfusion injury and is a primary site for overproduction of oxidant species and inflammatory cytokines after reperfusion [32]. These oxidant and inflammatory agents are most likely responsible for the remote organ injury observed after intestinal ischemic reperfusion injury [32]. However, pretreatment with brief small intestine ischemic reperfusion, not only elicits a local protective effect [9], but also remotely protects other organs like the heart and kidney from subsequent ischemic-reperfusion insults [10,15]. In this study we found that intermittent small intestinal rIPC similarly protected the spinal cord from IRI, while the small intestinal tissues did not suffer any significant deterioration of the oxidative markers (Figure 3). The neuroprotection, evident 24 hours after the SC-IRI, was demonstrated by significant improvement of motor and sensory function with preservation of the placing stepping reflex in the SC-IRI+IPC group. Serum TNF- α levels and the spinal cord homogenate levels of PGE₂, NO_x, MDA, and AOPP and SOD activity were significantly decreased, while CAT activity was significantly increased in the SC-IRI+IPC group compared to the SC-IRI. These results suggested

that, intermittent rIPC of the small intestine protects against increased oxidative stress level and inflammatory mediators via inhibition of lipid peroxidation and preservation of the endogenous antioxidants enzyme activities in SC-IRI. Our results are in agreement with Song et al, who reported that intestinal preconditioning resulted in a reduction in lipid peroxidation and preservation of antioxidant enzyme activities in rats subjected to renal ischemic reperfusion injury [14]. Dong et al, using hind limb ischemic preconditioning demonstrated a similar remote neuroprotective effect on the spinal cord [1]. Ates et al. also observed remote protective effects, employing hepatic ischemic preconditioning, in rats subjected to renal ischemic reperfusion injury [33]. A significant observation in this study is that, 48 hours after the ischemic episodes, the small intestinal homogenate levels of CAT and MDA activity were within in the normal range.

Several studies attempted to explain the mechanisms of rIPC between various organs. Although ROS are involved in the harmful effects of reperfusion on tissues after ischemia, their generation from brief ischemic reperfusion is the possible “trigger” for the initiation of preconditioning [34]. This protection could also be mediated through inhibition of the iNOS and COX-2 activity with modulation of the heat shock proteins [13]. Rüdiger et al suggested that IPC can induce energy-saving mechanisms, thereby decreasing the level of ATP degradation with subsequent accumulation of lactate throughout the ischemic period [34]. Gurcun et al. demonstrated the effectiveness of direct IPC and rIPC on the prevention of spinal cord ischemic injury and reported the protective values of reduced levels of neuron-specific enolase, malondialdehyde and nitric oxide [35]. Tang et al attributed the rIPC induced between the myocardium and the renal tissue to adenosine receptors and ATP-sensitive potassium channels [36]. Patel et al. demonstrated that intestinal rIPC resulted in reduction of the infarct size during myocardial ischemia via an opioid sensitive mechanism [37]. Beside humoral mediators, neurogenic mechanisms have been implicated as mediators of rIPC [13]. Whether the small intestinal rIPC protective effect is related to

humoral, neurogenic mechanisms or both, deserves further investigation.

5. Conclusions

Intermittent, transient, small intestinal ischemic preconditioning ameliorated the clinical neurological dysfunction induced by ischemic reperfusion injury of spinal cord. This ameliorating effect appears to be through reduction of the oxidative stress level and inflammatory mediators in the spinal cord.

As spinal cord ischemia-induced paraplegia remains a serious complication of ischemic reperfusion injury, prophylactic transient intestinal ischemic preconditioning appears to have promising potential as a preventive tool and warrants further and more exhaustive research.

Competing interests

The authors declare that they have no competing interests.

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