

Original Research Article

Pterostilbene Prevents Intestinal Ischemia Reperfusion Injury in Wistar Rats via Modulation of Antioxidant Defense and Inflammation

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Abstract

Purpose: To evaluate the protective mechanisms afforded by pterostilbene against intestinal ischemia/reperfusion (I/R) injury in Wistar rats.

Methods: Male Wistar rats were divided into 4 groups as follows: Control group; intestinal ischemia/reperfusion (I/R) group; pterostilbene only group (20 mg/kg) of body weight and pterostilbene followed by intestinal ischemia/reperfusion (I/R) treated group. The study evaluated oxidative stress markers, including reactive oxygen species (ROS) and lipid peroxide levels, protein carbonyl content, antioxidant status (superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, glutathione content) and membrane-bound ATPase activity. The levels of pro-inflammatory mediators, including nuclear factor- κ B (NF- κ B), cyclooxygenase-2 (COX-2) and inflammatory cytokines (TNF- α and IL-1 β), were also evaluated.

Results: The results showed that pterostilbene (20 mg/kg) followed by intestinal ischemia/reperfusion (I/R) significantly lowered the level of lipid peroxidation (41.33 %), protein carbonyl content (PCC, 44.18 %) and ROS (29.14 %) ($p < 0.001$) but significantly restored membrane-bound ATPase activities (Ca²⁺ATPase, 30.76 %; Na⁺/K⁺ATPase, 21.42 %; Mg²⁺ATPase, 30.06 %) ($p < 0.003$), compared with rats induced with I/R. Furthermore, pterostilbene significantly down-regulated NF- κ B and COX-2 expressions (30 %, $p < 0.05$) compared to rats with I/R injury.

Conclusion: The study reveals that pterostilbene offers significant protective activity in rats owing to its antioxidant and anti-inflammatory properties.

Keywords: Pterostilbene, Intestinal reperfusion, Ischemia, Inflammation, Antioxidant, Oxidative stress

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INTRODUCTION

Intestinal ischemic reperfusion (I/R) injury occurs during aneurysm surgery, cardiopulmonary bypass, strangulated hernias and intestinal transplantation [1]. In I/R injury, interruption of the blood supply causes ischemic injury which results in tissue damage. Restoration of blood flow to ischemic site results in reperfusion injury, which further exacerbates oxidative stress than

that of initial ischemic injury. Reports have demonstrated that alteration in the absorptive function of the intestine occurs following I/R injury [2]. Pathogenesis of intestinal ischemia reperfusion injury (IRI) are known to be mediated through reactive oxygen and nitrogen species, nitric oxide, inflammatory cytokines, complement activation and polymorph nuclear neutrophil [3]. However, an imbalance in redox homeostasis at the cellular level is the first major deleterious

effect which causes propagation of ischemia reperfusion injury IRI. Thus an effective strategy to completely protect from IRI injury could be achieved through antioxidant supplementation [4].

Pterostilbene (trans-3, 5-dimethoxy-4-hydroxystilbene) is a naturally derived compound similar in structure to resveratrol with increasing bioavailability. The methoxy group in pterostilbene is responsible for oral absorption and lipophilic properties. Therapeutic performance might be due to increased bioavailability of pterostilbene (80 %) which is much higher when compared to that of resveratrol (20 %) [5]. Pterostilbene is an excellent antioxidant [6] with plethora of pharmacological effects including anti-proliferative, anti-cancer and anti-inflammatory activities *in vitro* and *in vivo* [7]. The major protective role of pterostilbene is mediated through enhanced antioxidant defense mechanisms against oxidative stress conditions [8]. The present study was aimed at determining the protective effect of pterostilbene on intestinal ischemia reperfusion injury-induced oxidative stress and inflammation in Wistar rats.

EXPERIMENTAL

Animals

Male Wistar rats weighing 150–250 g were used for the present study. The rats were housed under optimum conditions of 21 ± 1 °C and relative humidity of 50–70 % with alternating dark and light cycle. The animals were fed with standard rat pellets and water (*ad libitum*). After the period of acclimatization, they were randomly divided into 4 groups with six animals per group. The rats were maintained in accordance with the Chinese legislation guidelines and the international guidelines on the protection, care and handling of laboratory animals and all the protocols were approved by the Ethical Committee of Third Affiliated Hospital of Harbin Medical University, China (approval Ref. no: CLG/TAHMMU/2014/00752).

Experimental groups

Group I (Control): The control rats were operated with surgical steps; however, the animals were not subjected to intestinal ischemia reperfusion (I/R). Group II (Intestinal I/R): The rats were subjected to intestinal ischemia for 30 min followed by reperfusion for 60 min. Group III (Pterostilbene): Pterostilbene (20 mg/kg body weight) was administered for 5 consecutive days through gavage. Group IV (Pterostilbene +

Intestinal I/R): Pterostilbene (20 mg/kg body weight) was administered for 5 consecutive days through gavage. Later, the rats were subjected to intestinal ischemia for 30 min, followed by reperfusion for 60 min.

After the treatment schedule, the rats were allowed to fast for 12 h. Following which the animals were anaesthetized and dissected intra-peritoneally (1500 mg/kg). During the whole process of dissection, the body temperature of the animals was maintained at 37.5 °C, with the help of heating lamp. The intestinal I/R were performed according to methods of Guneli *et al* [9]. Briefly, the abdominal region of the rats was sterilized with povidine iodine solution and then shaved. The intestinal region was subjected to midline laparotomy and intestinal I/R injury were created in superior mesenteric artery (SMA) which was occluded with a traumatic micro vascular clamp for 30 min. The abdominal region was closed and on completion of ischemia, the clamp was removed and reperfusion was induced for 60 min. After the complete I/R injury the jejunal segment was removed and the animals were sacrificed by exsanguination. The blood collected was centrifuged and serum was stored at -70 °C. The tissues were rinsed with ice cold saline and blood was completely removed. The tissues were homogenized using Tris-HCl buffer (50 mM, pH 7.4) at 4 °C and centrifuged at 3000 rpm for 30 min. The supernatant was removed and aliquoted in small volumes and stored at -70 °C for biochemical analysis.

Estimation of oxidative stress

Lipid peroxidation: The lipid peroxide levels were determined as described previously [10]. The lipid peroxidation is observed by reaction between MDA (Malondialdehyde), thiobarbituric acid & lipid peroxides resulting pink color formed is measured spectrophotometrically at 532 nm. Results were expressed as nanomol TBA (Thiobarbituric acid) reactants formed/g wet tissue.

Protein carbonyl content (PCC)

The reaction between carbonyl group and 2, 4-dinitrophenylhydrazine results in the formation of protein carbonyl content. These were measured spectrophotometrically at 370 nm. The results were expressed as nanomoles of carbonyl/ mg of protein [11].

Reactive oxygen species generation

The tissue homogenate was incubated with 2', 7'-dichlorofluorescein diacetate DCF-DA at 37 °C

for 15 min. After 15 min, it was centrifuged at 10,000 rpm, 30 min. The supernatant was discarded and the pellet was re-suspended in PBS solution (phosphate buffered saline) and incubated for 60 min at 37 °C. The levels of ROS were measured spectrophotometrically at an excitation wavelength (485 nm) and emission wavelength (528 nm). The results were calculated as percentage of ROS generation when compared with control group [12].

Determination of antioxidant enzyme activities

GSH level

The tissue homogenate was treated with 5 % meta-phosphoric acid followed by which the supernatant was collected for determining total glutathione (Glutathione assay kit; Trevigen Inc., Gaithersburg, MD). The concentration of GSH was determined from the standard GSH concentration curve. The results of GSH levels were expressed as nmol/mg of protein.

Total (Cu–Zn and Mn) SOD activity

Ethanol phase of the tissue homogenate was extracted using ethanol-chloroform mixture (5:3, v/v) and used for determining SOD activity. The assay was carried out according to the principle of nitro blue tetrazolium (NBT) reduction assay [13]. 1 U of SOD activity = amount required for 50 % inhibition of NBT reduction. The SOD activity is expressed as U/ mg of protein.

Catalase (CAT) activity

The activity was determined according to the method described by Aebi [14]. The reaction mixture contained sample in 30 mM H₂O₂ in a 50 mM phosphate buffer pH 7.0. The activity was estimated by decreased in absorbance of H₂O₂ at 240 nm.

GPx activity

The GPx was performed as described Paglia et al [15]. Oxidized glutathione (GSSG) is reduced by glutathione reductase and NADPH. The oxidation of NADPH to NADP⁺ is measured by decrease in absorbance at 340nm. GPx activity is expressed as U /mg of protein.

Glutathione-S-transferase (GST) activity

The GST activity was determined as described Habig et al [16]. The assay involves reaction between 1-chloro-2, 4-dinitro benzene (CDNB) and reduced glutathione which ultimately results

in formation of dinitro phenyl thioether. The formed product is measured spectrophotometrically at 340 nm and the enzyme activity was calculated. 1 U = Amount of enzyme producing 1 mmol of CDNB-GSH conjugate/min.

ATPase activities (Na⁺/K⁺, Ca²⁺, and Mg²⁺ ATPases)

The ATPases activity was determined by measuring the levels of inorganic phosphorous (Pi) liberated. The phosphate reacts with ammonium molybdate to form phosphomolybdate. The reaction between ANSA and phosphomolybdate results in formation of blue color complex, measured at 620 nm. Tissue Na⁺/K⁺ ATPase were determined according to the method of Bonting [17]. Tissue Mg²⁺ ATPase was determined according to the method of Ohnishi [18] and Ca²⁺ ATPase was determined according to the method of Hjerten and Pan [19]. The results were expressed as nmoles Pi-released/min/mg of protein.

Evaluation of serum TNF- α and IL-1β levels

The serum interleukins TNF-α and IL-1β levels were determined using an ELISA kit (R&D system Inc, MN, USA). The levels of interleukins were expressed as pg/ml.

Western blot analysis

For NF-κB expression study, nuclear extracts were isolated. The tissue samples were washed with ice-cold PBS thrice. The samples were homogenized in lysis buffer containing 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.75 mM spermidine, 0.15 mM spermine, 20 mM PNPP, 20 mM b-glycerophosphate, 1 mM Na₃VO₄, 1 mM PMSF and protease inhibitor cocktail. The homogenate was incubated in ice for 20 min. Followed by which NP- 40 (10 %) was added and incubated for 10 min. After incubation time, centrifugation was carried out at 15,000 rpm for 20 min. The supernatant was discarded and the pellet were resuspended in nuclear extraction buffer containing 20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.75 mM spermidine, 0.15 mM spermine, 20 mM PNPP, 20 mM b-glycerophosphate, 1 mM Na₃VO₄, 1 mM PMSF and 1:100 protease inhibitor. The samples were subjected to 3 cycle of freezing and thawing. The extract was centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant containing nuclear extracts were aliquoted and stored at -80 °C until further analysis. For COX-2 expression, tissues were homogenized in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150

mM NaCl, 5 mM EDTA, 50 mM NaF, 1 % Triton X-100, 1 mM sodium ortho vanadate, 1 mM phenyl methanesulfonyl fluoride, 1 mg/mL aprotinin, 4 µg/ml pepstatin A, and 5 µg/mL leupeptin). The homogenate was then centrifuged for 20 min at 13,000 rpm at 4 °C. The supernatant was analyzed for protein concentration according to the method described by Lowry [20].

Protein from different samples at a concentration of 50 µg was separated using SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5 % nonfat milk in Tris-buffered saline (TBS) and subsequently incubated with NF-κB, COX-2 (1:500) or β-actin, GAPDH primary antibody, followed by horseradish peroxidase-conjugated goat, anti-rabbit or -mouse IgG secondary antibody and bands were visualized with an enhanced chemiluminescence (ECL) system according to the manufacturer's instructions. Densitometric analyses of the Western blot bands were performed using optical density scanning and ImageJ software.

Statistical analysis

Data were analyzed with SPSS 11.0 (SPSS Inc. Software, Chicago, Illinois, USA) statistical software using one-way analysis of variance (ANOVA) and with Tukey-Kramer test so as to determine comparison between groups, and differences between groups, respectively. All values were expressed as mean ± SD and $p < 0.05$ was considered statistically significant.

RESULTS

Pterostilbene ameliorates oxidative stress

The study shows a significant rise ($p < 0.001$) in the lipid peroxide, PCC and ROS generation ($p < 0.001$) in rats with I/R. However, rats treated with Pterostilbene (20 mg/kg body weight) followed by intestinal I/R (group 4) showed a significant decline the levels of lipid peroxidation (41.33 %; $p < 0.001$), PCC (44.18 %; $p < 0.001$) and ROS (29.14 %; $p < 0.001$) generation when the results were compared with rats induced with I/R (group 2). Rats treated with pterostilbene alone showed non-significant difference in lipid peroxide, PCC and ROS when compared to control rats (Fig. 1).

Pterostilbene enhances antioxidant status during Intestinal I/R injury

Table 1 shows a significant decrease in antioxidant enzyme activities of GSH, SOD, CAT, GPx and GST in rats induced with I/R injury when compared to sham operated rats. Treatment with pterostilbene followed by I/R injury significantly enhanced the antioxidant status when compared to rats subjected to I/R injury alone. There was a non-significant change in the levels of antioxidant status in palone treated rats when compared to sham operated rats.

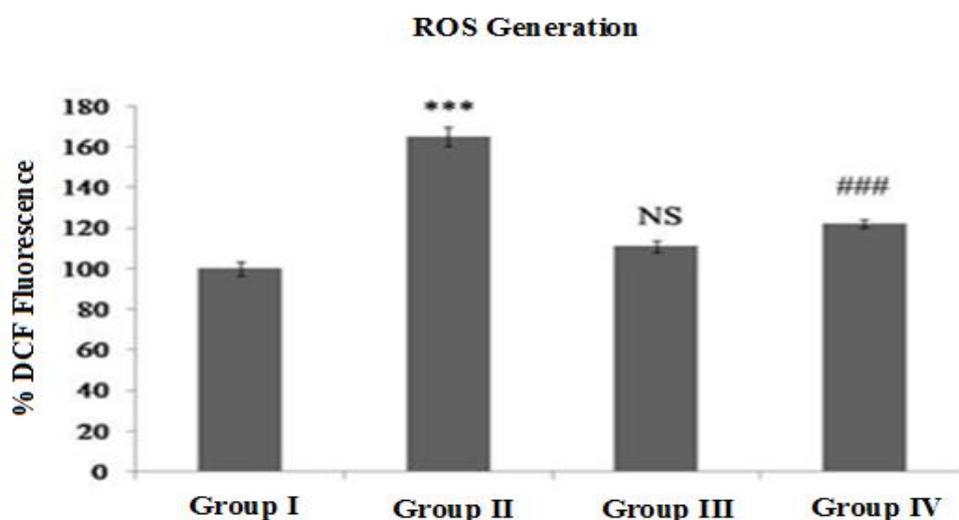


Fig 1A: Pterostilbene inhibits ROS generation. Results shown indicate ROS generated (%). ***Indicates Significant rise ($p < 0.001$, compared with control group; ###significant reduction ($p < 0.001$, compared with intestinal I/R injury rats; NS = no significant difference

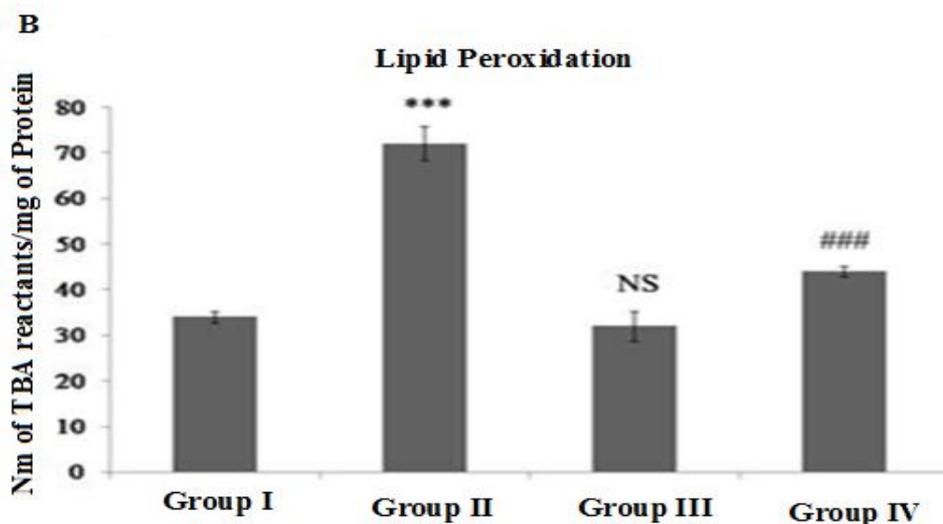


Fig 1B: Pterostilbene inhibits lipid peroxidation. The results are expressed as nano moles of TBA reactants formed/mg of protein; ($p < 0.05$, compared with control group; ### significant reduction ($p < 0.001$, compared with intestinal I/R injury rats; NS = no significant difference)

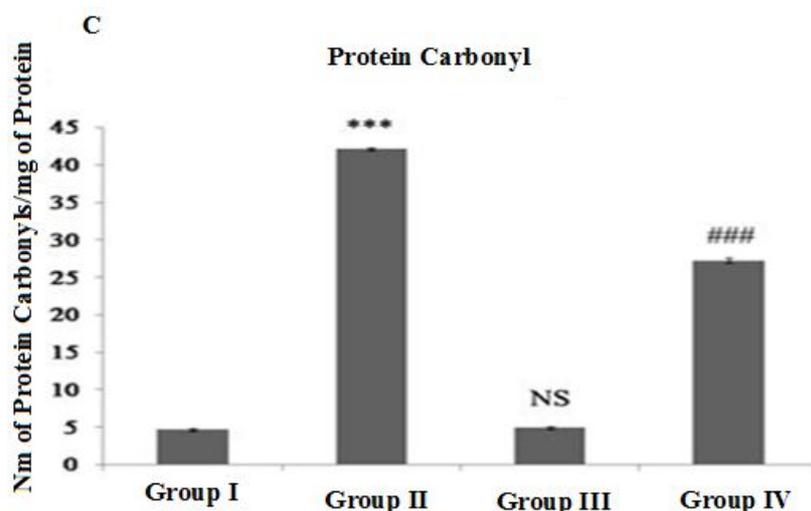


Fig 1C: Pterostilbene reduces protein carbonylation. The results are expressed as nanomoles of protein carbonyl formed/mg; ### significant reduction ($p < 0.001$) compared with intestinal I/R injury rats; NS = not significant. Results are given as the mean \pm SEM ($n = 6$)

Table 1: Effect of Pterostilbene on enzymic and non-enzymic antioxidant status in intestinal Ischemic reperfusion injury rats

Parameter	Group I	Group II [£]	Group III ^{££}	Group IV ^{£££}
GSH	65 \pm 2.19	21 \pm 1.78***	61 \pm 1.90 ^{NS}	55 \pm 1.10 ^{###}
SOD	167 \pm 3.21	34 \pm 1.0***	172 \pm 2.98 ^{NS}	145 \pm 2.75 ^{###}
CAT	4.8 \pm 0.89	1.76 \pm 0.12***	4.6 \pm 0.71 ^{NS}	3.48 \pm 0.9 ^{###}
GPX	1.7 \pm 0.001	0.2 \pm 0.001***	1.5 \pm 0.001 ^{NS}	1.18 \pm 0.001 ^{###}
GST	51 \pm 2.45	13 \pm 1.12***	50 \pm 1.89 ^{NS}	42 \pm 1.01 ^{###}

Group I (sham); Group II (Intestinal I/R injury rats); Group III (Pterostilbene); Group IV (Pterostilbene + Intestinal I/R injury rats): Results are given as the mean \pm SEM for 6 rats in each group. For GSH levels the results are expressed as nanomoles of GSH/g of tissue. For antioxidant enzyme activities the results are expressed as U/mg of protein; [£] significant differences when compared with sham group (Group 1) (** $p < 0.001$); ^{££} no significant differences when compared with sham group (Group 1) NS $p < 0.004$; ^{£££} significant differences when compared with Intestinal I/R group (Group II) (### $p < 0.001$)

Pterostilbene restores membrane bound-ATPase activities

Rats with intestinal I/R injury showed a significant decline ($p < 0.03$) in the activities of Na^+/K^+ , Ca^{2+} , and Mg^{2+} ATPases when compared to sham operated rats. However, the activities were normal with non-significant change in rats treated with pterostilbene treated rats alone. Pterostilbene treatment followed by I/R injury significantly reversed enzyme activities with increase (Na^+/K^+ ATPase-21 %, Ca^{2+} ATPase-30.76 %, and Mg^{2+} ATPases-30.06 % $p < 0.03$) when compared to rats with I/R injury alone (Fig 2).

Pterostilbene down regulates NF- κ B, COX-2 expression

The effect of pterostilbene on NF- κ B, COX-2 expression during intestinal I/R injury was determined by western blot analysis (Fig. 3). The results show a significant increase ($p < 0.001$) in the expression levels of NF- κ B, COX-2 during rats induced with I/R injury. Treatment with Pterostilbene significantly down regulated the NF- κ B (58.33 %; $p < 0.05$), COX-2 (70 %; $p < 0.04$) expressions when compared to rats with I/R injury.

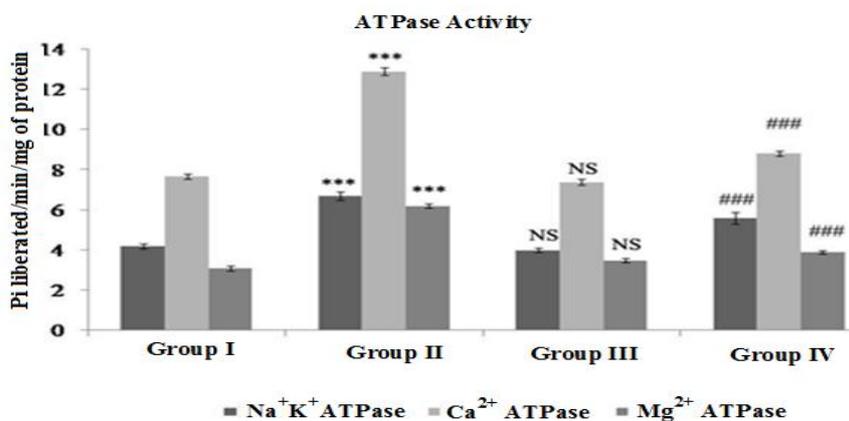


Figure 2: Pterostilbene maintains ATPase enzyme activities in ischemic injury in Wistar rats. Results are expressed as phosphate ion (Pi) liberated/min/mg of protein; *** indicates significant change ($p < 0.03$), Vs sham group. ### Significantly maintains all ATPase ($p < 0.03$), when compared to intestinal I/R injury group. NS = not significant when compared to control group; Results are given as mean \pm SEM ($n = 6$)

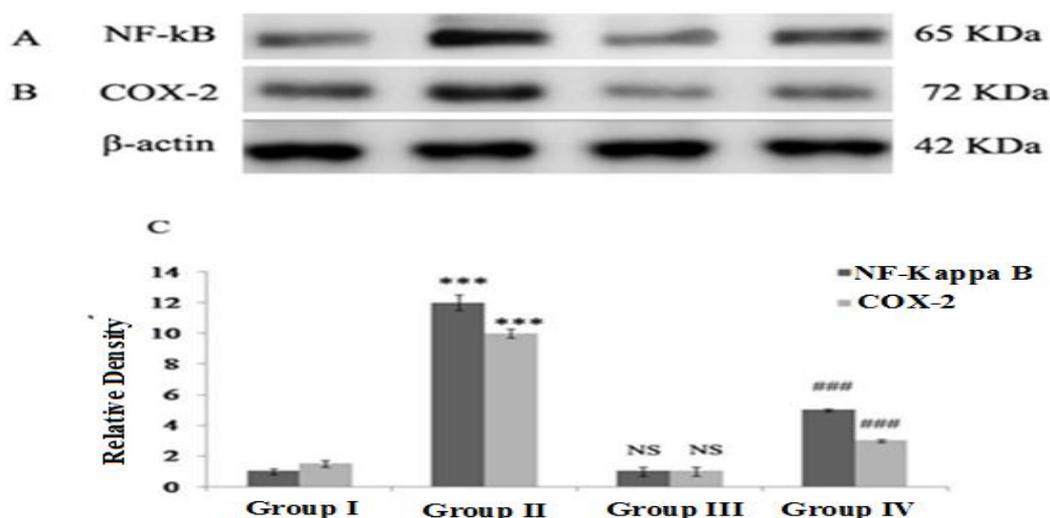


Figure 3: Pterostilbene exhibits anti-inflammation through down regulation of NF- κ B and COX 2 expressions in Intestinal Ischemic injury in wistar rats A. Western blot analysis of NF- κ B expression; B. Western blot analysis of COX- 2 expression C. Densitometric analysis of NF- kb and COX- 2 expressions. Statistical analyses were carried out by Students t-test. The results are expressed as mean \pm SEM. *** $p < 0.001$ compared with control rats; ###significant reduction was observed ($p < 0.05$), compared to rats with intestinal Ischemic injury. NS = not significant

Pterostilbene downregulated Interleukin levels (TNF- α and IL-1 β)

The results show that the levels of TNF- α and IL-1 β were significantly higher ($p < 0.001$) in rats induced with I/R. Treatment with pterostilbene followed by I/R injury (group 4) significantly down regulated the levels of TNF- α (31.81 %, $p < 0.05$) IL-1 β (52.94 %, $p < 0.05$) when compared to I/R (group 2) rats. There was a non-significant level of interleukin release in rats treated with pterostilbene alone when compared to sham operated rats (Fig 4).

DISCUSSION

Oxidative stress and its associated inflammation play a major role in intestinal I/R injury. Oxidative stress during I/R injury ensues as a result of imbalance between ROS generation and its scavenging mechanisms [21]. Thus, for an efficient protection against I/R injury, there must be an effective strategy for combating the initially generated reactive species. The present study shows for the first time, the protective role of pterostilbene in intestinal I/R injury in wistar rats. The data suggest that anti-oxidative and anti-inflammatory properties of pterostilbene might be the related to its protective role.

The present study shows that intestinal ischemia resulted in a significant increase in ROS

generation and various oxidative stress mediators such as lipid peroxides and protein carbonyls. However, treatment with pterostilbene offered protection against intestinal I/R injury-induced oxidative stress. The present study results are in line with Guven *et al* [22], clearly showing the imbalance in redox homeostasis in I/R injury. The antioxidant effects of pterostilbene have been largely studied which is in line with the present research findings. Significant increase in SOD activity during pterostilbene treatment has been shown in earlier studies on anti-proliferation against breast cancer cells [23].

In addition to ROS and toxic mediators during exacerbation of oxidative stress, there is decrease in the inbound defense mechanisms. This ultimately results in inactivation various proteins and enzymes present in cellular membrane as a result of lipid peroxide effects, membrane bound ATPases included [24]. ATPase are involved in the normal ionic regulation which is highly responsible for normal tissue functioning. It is important to maintain proper ionic homeostasis for normal functioning of tissues. Estimation of enzyme activities of ATPases in the present study clearly shows that intestinal ischemia significantly inhibits these enzyme activities which were completely restored by treatment with antioxidant, pterostilbene.

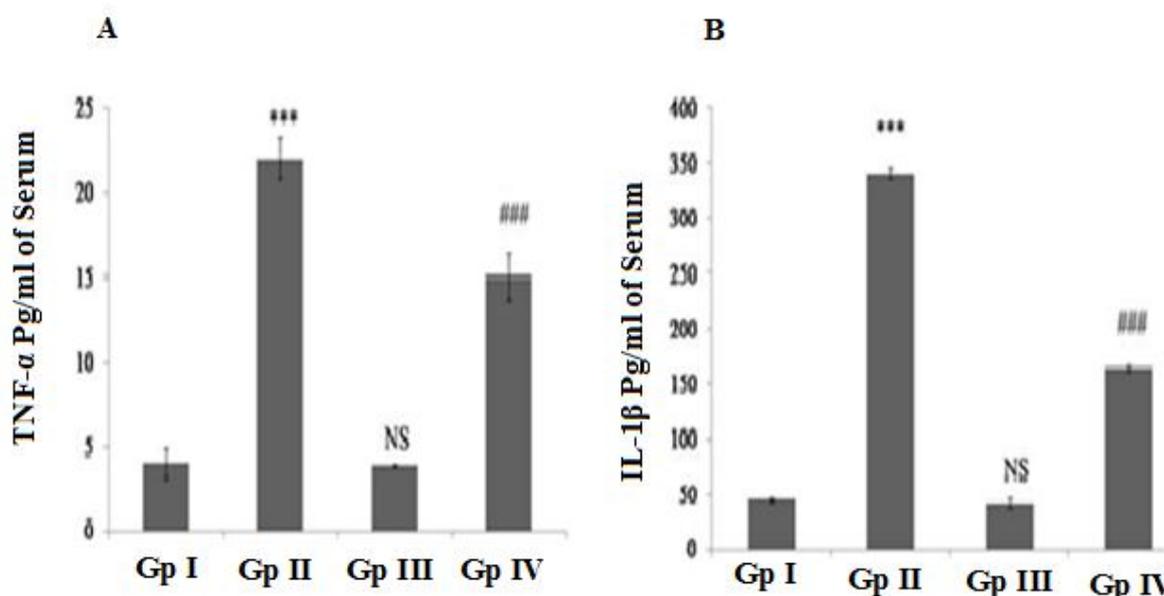


Fig 4: Pterostilbene exhibits anti-inflammation through downregulation of inflammatory cytokines (TNF- α and IL-1 β). A = effect of pterostilbene on TNF- α levels; *** $p < 0.03$ significantly different compared with control rats. #### $p < 0.05$, significant changes compared with Intestinal Ischemic injury were observed. B = effect of pterostilbene on IL-1 β levels; #### $p < 0.05$ compared with rats with intestinal ischemic injury; results are given as mean \pm SEM ($n = 6$)

Thus the present study shows that the antioxidant effect of pterostilbene plays as a central mediator in the prevention of oxidative stress and protection against membrane bound ATPase activity. The present study also shows that I/R injury resulted in significant up regulation of inflammatory proteins NF- κ B, COX-2 and pro-inflammatory cytokines TNF-alpha and IL-1 β when compared to sham operated rats.

CONCLUSION

The present investigation highlights the protective role of pterostilbene in IIR injury in Wistar rats. The protection shown by pterostilbene is associated with the inhibition of oxidative stress and enhanced antioxidant status. Further, pterostilbene maintains ionic homeostasis by regulating ATPase enzyme activities. Pterostilbene, in addition, ameliorates IIR injury-induced pro-inflammatory genes and inflammatory cytokines. Thus, this study provides some insight into pterostilbene-mediated cytoprotection against IIR injury but there is a need for further elucidation of the exact mechanisms of action.

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