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Research article

Possible protective role of verapamil on ischemia/reperfusion induced changes in the jejunal mucosa of adult male albino rat: Histological and biochemical study

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Key words: intestine, Verapamil, I/R, oxidative stress.

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Abstract

Background: The small intestine is extremely sensitive to ischemia-reperfusion (I/R) injury and a range of microcirculatory disturbances which contribute to tissue damage. Previous studies have shown that verapamil plays an important physiological role in the microvascular environment. This study aimed to evaluate the protective effects of verapamil in ischemia/reperfusion (I/R) – induced mucosal injury in the jejunum. Fifty adult albino rats were divided into five groups: **group I** (Control untreated); **group II** (Sham-operated); **group III** (sham-operated +verapamil); **group IV** (Ischemia/Reperfusion) and **Group V** (I/R + verapamil). At the end of the reperfusion period the jejunum was extracted and prepared for histological and immunohistochemical examination. Distribution of myeloperoxidase (MPO) stained cells and the levels of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH,) were also determined in all dissected tissues. Morphometric study was also done and statistical results were analyzed. Intestinal IR caused severe intestinal mucosa injury especialy epithelial cell damage. Group (I/R) revealed villus structural alterations, cellular infiltration and hemorrhage. MPO and goblet cells showed a significant increase in the number with elevation of oxidative stress marker. Co-administration of verapamil ameliorated these histological changes and significantly decreased mucosal damages compared to group I/R.
Conclusion: Verapamil can protect against intestinal I/R probably through inhibition of oxidative stress and neutrophil infiltration. So it can be used safely in mesenteric occlusive diseases, since it induces improvement of circulation and relief of concomitant structural changes.

Introduction

The small intestine is the largest component of the digestive tract and the major site of digestion and absorption. In addition to receiving chyme from the stomach, the duodenum, receives bile from the gall bladder and digestive enzymes from the pancreas. In humans, the duodenum is tube connecting the stomach to the jejunum. The mucosa of the small intestine is highly modified and is formed of multiple highly specialized cells. The predominant cell type of the epithelium is the enterocyte or absorptive cell [1].
The condition by which the deprivation of blood flow leads to insufficient oxygen and nutrient supply to the tissue is called ischemia. Reperfusion injury refers to the tissue damage caused when blood flow is restored after an ischemic period for a minimum 10 min. The intestinal ischemia/reperfusion (IR) injury induces injury to the intestinal mucosa, serious impairment of the local microvasculature, increased vascular and mucosal permeability, and multiple organ failure [2].

The small intestine is extremely sensitive to I/R injury and a range of microcirculatory disturbances which contribute to its tissue damage [3]. Surgical pathologies often meet I/R injury and consider it as a quite dangerous condition. I/R cause severe clinical pathologies by destruction close and far tissues. So, intestinal I/R is considered as a clinicopathologic entity closely associated with a variety of clinically severe conditions, including major surgery with decreased intestinal blood flow, acute blood loss, severe burns, sepsis, intussusceptions, transplantation, mesenteric arterial occlusion and hemodynamic shock [4,5]. Also, it may be attributable to potential complications during various medical and surgical procedures.
Intestinal I/R injury is an important factor associated with a high morbidity and mortality in both surgical and trauma patients [6]. It induces multiple organ dysfunction syndromes (MODS), which is associated with damage of oxygen radicals, energy metabolism and overload of intracellular calcium, leukocyte adhesion and endothelial cell injury [7]. In MODS, intestinal I/R is trigger that

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cause various remote organs as lung, liver, and kidney affected; moreover, humoral mediators are generally thought to play the central role in the reperfusion phase of this entity [8].

During ischemic and the reperfusion phases, certain cascade occurs. This cascade consequently leads to increased bowel permeability, gut barrier dysfunction, and transmigration of bacteria and bacterial products into the systemic pool, where upon a systemic life-threatening occurs [9]. The loss of fluid and protein from the vascular bed due to increased vascular permeability will also result in multiple organ failure [10-11]. The respiratory and renal systems have been considered a common dangerous consequence of I/R.

Verapamil is a voltage-dependent calcium channels blocker. It is used in treating high blood pressure and angina pectoris because it induces relaxation of the vascular smooth muscle and vasodilatation [12]. Voltage-gated calcium channels (VGCC) regulate rapid and transient changes in the intracellular calcium within excitable cells. Calcium entering the cell through these channels serves as a second messenger, initiating intracellular events such as contraction, secretion, protein phosphorylation, gene expression, neurotransmitter release and action potential firing patterns [13-14]. By preventing the calcium influx, verapamil can help in the decrease of progression of IR injury. Some study has been reported that it can protect against renal I/R in rats [15].

It was documented that verapamil has independent cytoprotective effects on 60-min warm ischemia/ reperfusion injury of rat kidneys. The mechanism of cytoprotection is not restricted to the suppression of lipid peroxidation but nearly complete protection of reperfusion injury can be obtained by such an intervention [16-17]. Tucci *et al.* [15] concluded that administration of verapamil before the ischemic episode provides only partial- and short-lasting functional protection of mitochondrial function and this protection is superimposed on by renal perfusion solution before ischemia. Also, other studies, emphasized that verapamil improve both histopathological and biochemical parameter of liver I/R [18].

I/R injury is a phenomenon that attracts the interest of researchers, which through experimental models seek to understand its pathophysiology and possible therapeutic measures. The current study aimed to investigate the possible protective effect of ischemic preconditioning by verapamil on intestinal IR injury induced in rats and to record its effect on oxidative stress and infiltration by inflammatory cells.

Experimental

Materials and methods

Chemicals

Drugs: Verapamil (El Nasr Pharmaceutical Chemicals Company, Cairo, Egypt): dissolved in distilled water immediately before use.

Kits for measurement of tissue malondialdehyde (MDA) (Bio-diagnostic Company Giza, Egypt).

Animals used

Total of 50 adult healthy male Wister rats weighing 200–250 g were used. They were purchased from the Animal House, Faculty of Medicine, Zagazig University, Egypt. The animals were housed for at least two weeks before experiments period to be acclimatized to the laboratory conditions under a 12 h light/dark cycle. Food and water were provided ad libitum. The animal experiments were carried out in accordance with the ethical guidelines of animal welfare and the ethical recommendations of the Faculty of Medicine, Zagazig University, Egypt.

Experimental design

Rats were classified into five groups (10 rats each):

Group I **(Control untreated group)**: Rats of this group were received 1 mL distilled water orally for three consecutive days and were maintained alive until the end of the experiment.

Group II **(Sham operated)**: Laparotomy was performed in the animals without clamping the Superior Mesenteric Artery (SMA). The animal's abdomens were covered with plastic wrap to prevent dehydration then their intestines were manipulated. They received 1 ml distilled water (the vehicle for verapamil and kept under anesthesia for 3 hours (the duration of the intestinal I/R method). These rats also were maintained alive until the end of the experiment.

Group III **(Sham- Verapamil)**: All the steps were performed as in group II, except that verapamil 10 mg/kg/day were administered orally to rats.

Group IV **(Ischemia/reperfusion; IR)**: Rats of this group were subjected to intestinal ischemia for 60 min followed by reperfusion for 2 h [19].

Group V **(IR-Verapamil treated):** Rats of this group received verapamil in a dose of 10 mg/kg/day orally for three consecutive days before prolonged ischemia/ reperfusion as group III, with the last dose administered 1 h prior to ischemia [20].

Technique of Intestinal I/R

All rats were fasted for 18 h before the experiments, but they were allowed free access to water until 20–30 min before the start of the experiment. All experiments were started between 10 and 11 a.m. The rats were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg).

Then, they were placed in the supine position and secured in the dissection tray. The abdominal region was shaved

and cleaned with povidone-iodine 10% antiseptic solution. Throughout the procedure, rats were placed on a heating pad to maintain normal body temperature. A 2–3 cm abdominal mid-line incision was applied to rats. In I/R group, the superior mesenteric artery was exposed with care and occluded with an atraumatic microvascular clamp, for 60 min. At the end of this period, the clamp was removed, mesenteric artery pulsation was observed and the intestine reperfused for 120 min. During I/R period, the area of operation was covered with a worm moist dressing to prevent hypothermia.

At the appropriate time, all rats were sacrificed and immediately, jejunum segments were extracted from the animals. The extracted specimens' were cleaned with cold isotonic saline and immediately frozen for biochemical measurements. Several other samples (taken from the same region) were excised and processed for histological studies [21].

Histological study

For light microscopy, intestinal specimens were fixed in a 10% formalin solution for 24 h, processed and embedded in paraffin wax by routine protocol. Five μm thick sections were stained with hematoxylin and eosin and Mallory trichrom stains (H&E) [22].

Immunohistochemical study

Immunohistochemistry (IHC) was performed to detect the location and the distribution of caspase-3 positive cells using a streptavidin-biotin method by (Santa Cruz Biotechnology kit, USA). Sections were deparaffinized in xylene and rehydrated in decreasing concentrations of alcohol followed by washing with water. Background staining was blocked with powdered skim milk (3% in phosphate buffered saline). Endogenous peroxidase was blocked by hydrogen peroxide 3% then it was blocked with normal goat serum at 37°C for 15 min. Slides were incubated with polyclonal antibodies of caspase-3 (Diluted to 1 : 100, Santa Cruz Biotechnology) at 37°C for 60 min. After washing with PBS, the slides were incubated with a biotinylated horse peroxidase-conjugated secondary antibody and 0.1% DAB substrate, using the standard streptavidin-biotin-based method. Incubation with PBS instead of the primary antibody served as a negative control. The brown granule was marked as a positive expression for caspase3 (400-fold magnification) [23].

Biochemical analysis

For the biochemical analysis, tissues were washed two times with cold saline solution, placed into glass bottles, and stored in a deep freezer at −80°C until processing. The frozen intestinal tissues were homogenized (10%w\v) in ice-cold 0.1M Tri-HCL buffer (pH 7.4). The homogenates were centrifuged at 3000 rpm for 15 min at 4ºC and the supernatant was used for biochemical analysis. MDA, GSH and SOD, and were measured in intestine tissue samples [24].

Detection of content of MDA in the intestine

Analysis of tissue malondialdehyde (MDA) level as an indicator of lipid peroxidation was performed by the spectrophotometry method. MDA content was determined by spectrophotometric measurement of the color produced during TBA reaction with MDA at 535 nm. Homogenate (0.1 mL) was taken to detect MDA content. Briefly, 0.1 mL 8.1% SDS, 0.8 mL acetic acid buffer, 0.8 mL 0.8% TBA and 0.2 mL distilled water were added into the sample tubes and one standard tube (containing 0.1 mL tetraethoxypropane). MDA level is expressed as nmol\g tissue protein [25].

Detection of activity of superoxide dismutase (SOD)

Measurement of SOD activity depends on the inhibition of nitro blue tetrazolium (NitroBT) (NBT) reduction by xanthin-xanthin oxidase after addition of superoxisd dismutase. One unit of SOD was defined as the amount of enzyme causing 50% inhibition in the nitroblue tetrazolium reduction rate [26-27]. Results were expressed as U per milligram of protein.

Detection reduced glutathione (GSH)

GSH plays a major role in protecting cells against damage from ROS. The activity of GPX was determined essentially as described by Rotruck *et al.* [28]. One unit was the amount of enzyme that converted 1 μmol of GSH to the oxidized form of glutathione (GSSH) in the presence of H_2O_2/min). Results were expressed as U/mg tissue protein [29-30].

Measurement of myeloperoxidase (MPO) activity

Myeloperoxidase (MPO) is a specific enzyme released by activated PMN [31]. MPO activity in the jejunal mucosa, an index of polymorphonuclear leukocyte accumulation, was determined using a modification of the method described by Grisham *et al.* [32]. A total of 2 ml of mucosal homogenate was centrifuged at 20,000 x g for 20 min at 4°C. The resulting pellets were re-homogenized in an equivalent volume of 0.5% hexadecyltrimethyl ammonium bromide (HETAB).

Morphometric analysis

Image analysis system was used to count the mean number of MPO positive nuclei mean area percentage of collagen fiber and mean area of caspase3 reaction. They were observed under 400 x magnifications. Five non overlapping fields in slides of each animal of each group were examined and the mean number of MPO positive

nuclei, caspase immunoreactive cells/ crypt and mean area of collagen fiber were measured.

Statistical analysis

Data were expressed as mean ± SD and analysis of variance was performed using SPSS 11.0 software. One-way analysis of variance (ANOVA) was used for multiple comparison. Differences were considered significant when P was < 0.05.

Results and discussion

Results

Histological and immunohistochemical study

Group I, II, III (Control Groups)

Examination of sections obtained from rat jejunum of the control untreated, Sham-operated and sham-verapamil treated groups stained with hematoxylin & eosin showed the same histological picture which was identical to the known histological structure of the jejunum. The wall was formed of mucosa, submucosa, musculosa and serosa. The jejunal mucosa was formed of epithelium, lamina propria and muscular is mucosa (Figure 1A). The villi appeared as finger-like projections with a core of loose connective tissue and invaginations in the lamina propria forming simple tubular glands (crypts of Libierkühn). The epithelial cells covering the villi were tall columnar cells with basal oval nuclei and acidophilic cytoplasm (enterocytes). Goblet cells appeared as clear empty spaces in between the columnar cells (Figure 1B & 1C). Caspase positive immuno stained cells was minimal in this group (Figure 1D). Normal distribution of goblet cells with minimal amount of collagen fibers appeared in all control groups (I, II, & III) (Figure 4A & 4D).

Group IV (Ischemia/ Reperfusion (I/R) Group)

Light microscopic examination of sections obtained after I/R, revealed certain histological changes. These changes were represented mainly as broadening, fusion and distortion of some villi. Obliterated crypts were also seen (Figure 2A). Epithelial desquamation and sloughing appeared in some area leaving denuded areas. Loss of brush border in some areas and inflammatory cellular infiltration in lamina propria were seen (Figure 2B). Wide subepithelial edema spaces under the tips of the villi and haemorrhage in lamina propria were also observed (Figures 2C & 2D). Enterocytes showed degeneration with vacuolaion of the cytoplasm and rounded nuclei. Caspase3 immunoreaction was increased in this group (Figure 2E). Increased distribution of goblet cells with large amount of collagen fibers also appeared in this operated groups (IV) (Figure 4B & 4E).

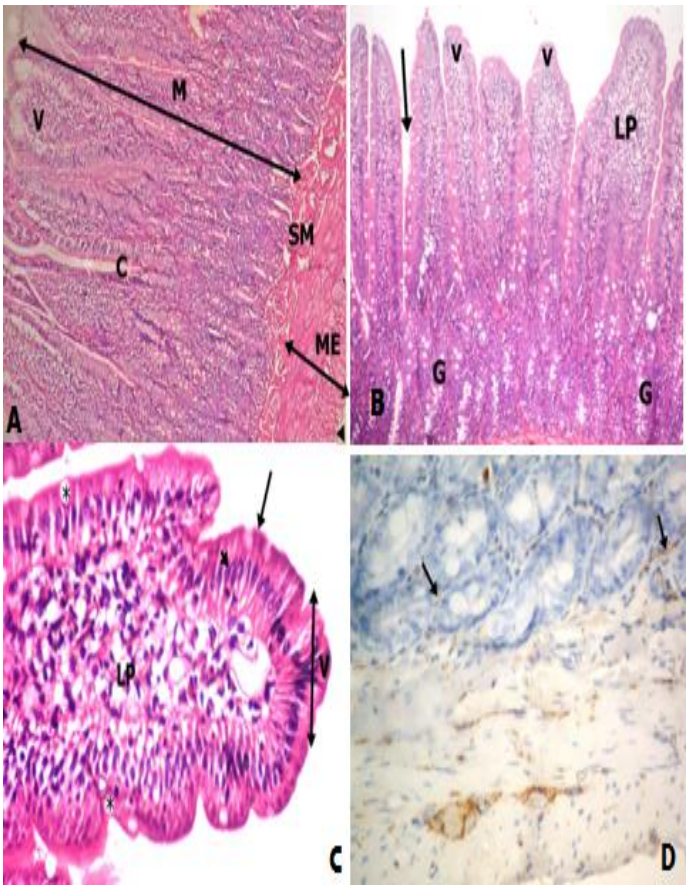


Figure 1. (A) A photomicrograph of jejunum of control group showing mucosa (M), submucosa (SM), musculosa (ME) and serosa (arrowhead) (Hx. & E. Mic. Mag. × 200). (B) Showing jejunal mucosa with finger-like villi (V) with CT core (LP). Notice simple tubular crypts of Liberkühn (arrow) opening at the base of the villi and intestinal gland (G). (C) Higher magnification of one villus is covered with simple columnar cells (arrow) with oval nuclei (arrow head) and acidophilic cytoplasm. The villus shows a core of lamina propria (LP). Notice the presence of goblet cells (*) in between the columnar cells (D) Few MPO immune stained cells (arrow) (Hx. & E. Mic. Mag. × 400).

Group V (Verapamil-treated)

Administration of verapamil at the onset of reperfusion period ameliorated some of structural changes associated with I/R. Examination of sections from this group showed that the villi and the crypts were almost similar to those of the control group. The villi was covered by tall columnar cells of enterocytes with oval basal vesicular nuclei and regular continuous brush border with goblet cells (Figures 3A & 3B). Few vacuolation in enterocytes and cellular infiltration with dilated capillary could be observed (Figure 3C). Caspase3 immunoreaction was decreased and appeared moderately (Figure 3D). Return of nearly normal distribution of goblet cells with moderate amount of collagen fibers also found in treated group (V) (Figure 4C & 4F).

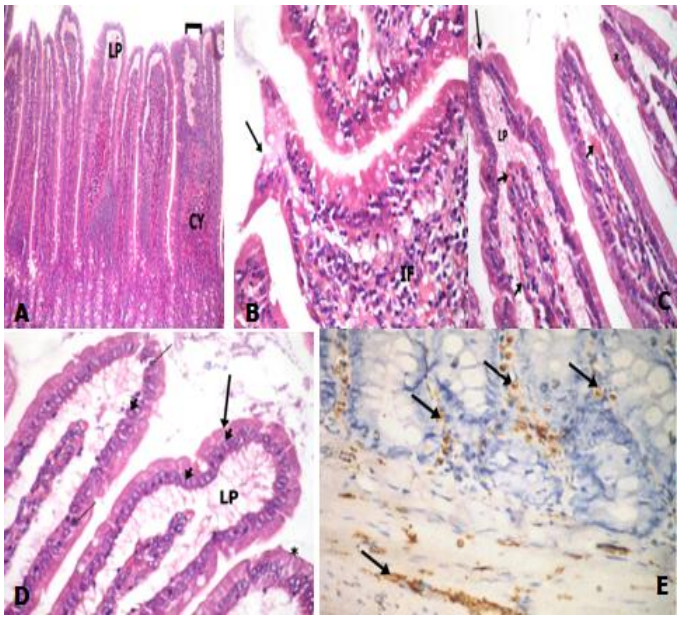


Figure 2. (A) A photomicrograph of rat jejunum of group (IV) showing broad fused villi ([ ) and obliterated crypts (CY). (Hx. & E. Mic. Mag. × 200). (B) showing sloughing of the surface epithelium of the tip of the villi leaving denuded areas (arrow). Some surface area of villi has loss of brush border (*). Notice cellular infiltration (IF) in lamina propria. (C, D) Showing intestinal villi with degenerated epithelial cell (arrow) with wide subepithelial edema space (LP) in lamina propria. Notice haemorrhage (short arrow) in the lamina propria and rounded nuclei of enterocyte (arrowhead). (E) Showing increased MPO stained cells (arrow) (Hx. & E. Mic. Mag. × 400).

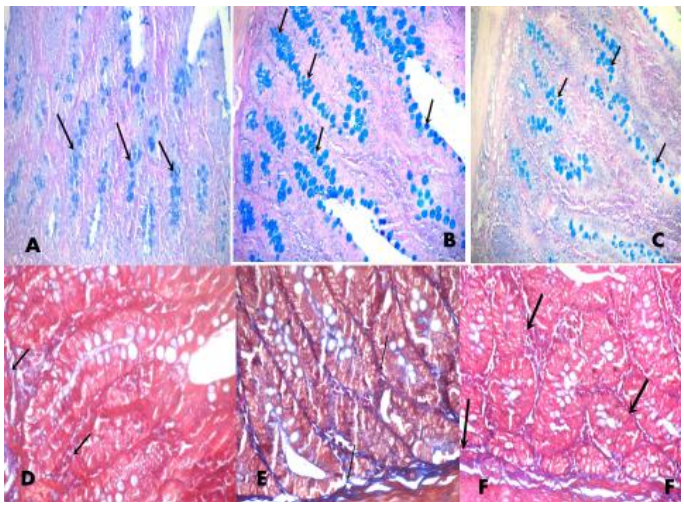


Figure 4. (A) A photomicrograph of rat jejunum of group (V) showing apparently normal number of goblet cells (arrow). (B) Showing increased number of goblet cells. (C) Showing decreased number of goblet cells (PAS stain Mic. Mag. × 400). (D) Little amount of CT in control jejunum (arrow). (E) Showing increased amount of CT in operated group (arrow). (F) Showing moderate amount of CT in treated group (Mallory's trichrom stain Mic. Mag. × 400).

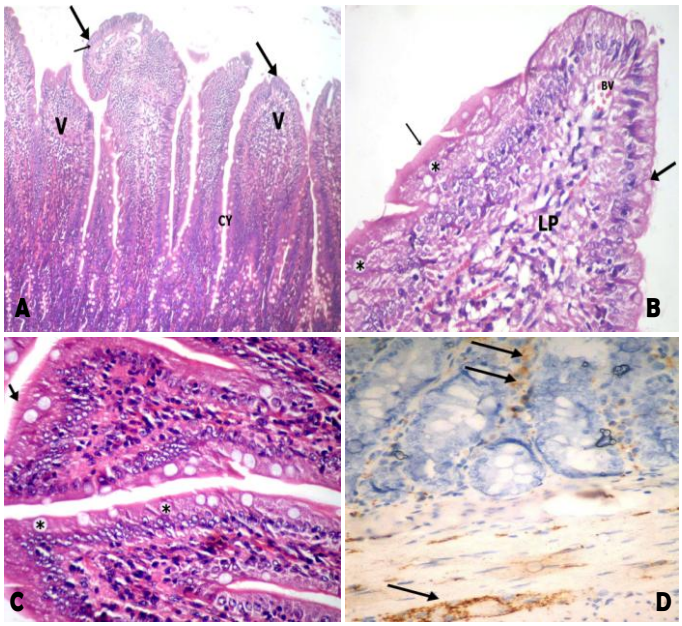


Figure 3. (A) A photomicrograph of rat jejunum of group (V) showing apparently normal villi (V) covered by simple columnar cell (arrow) and normal crypts (CY). (Hx. & E. Mic. Mag. × 200). (B, C) Showing normal C.T core of the villus (LP) with blood vessel (BV). It is covered by tall columnar cells of enterocytes with oval basal vesicular nuclei and regular continuous brush border (thin arrow) with goblet cells (*). Vacuolation of few enterocytes (thick arrow) is also seen. (D) Showing few MPO immunostained cells. (Hx.&E. Mic. Mag. × 400).

Biochemical results

Changes of MDA in jejunum

MDA level in the jejunal mucosa of I/R group was found to have a significant increase ($P<0.05$) when compared with control group. As for the verapamil-administered group, a significant decrease in the MDA level was observed in (Table 1 & Chart 1).

Changes of activity of SOD & GSH in jejunum

The activity of intestinal SOD and GSH decreased significantly with I/R injury groups. Compared with the control groups, the group IV exhibited a significant decrease in the level of both enzymes ($P<0.05$); however, a significant increase was determined in the level of GSH and SOD activity in the group V (Table 1 & Chart 2).

Changes in MPO stained cell in jejunum

MPO activity in the jejuna tissue in the group IV was found to have a significant increase ($P<0.05$) when compared with control groups. However, a significant decrease in the MPO activity was observed in group V in comparison with group IV ($P<0.05$) (Table 2 & Chart 3).

Table 1. The mean MDA, SOD and GSH levels of different groups.

Group	MDA (nmol/g protein)	SOD (U/mg protein)	GSH (U\mg protein
I (control-untreated)	1.97 ± 0.12	5.03±0.44	1.15 ± 0.20
II (Sham operated)	1.95 ± 0.09	4.51 ± 0.55	0.95 ± 0.09
III (Sham+virapmil)	1.82 ±0.07	4.88 ± 0.62	1.08 ± 0.11
IV (IR)	2.34 ± 0.10**	2.78 ± 0.27**	0.51± 0.12**
V (Virapmil treated)	1.70 ± 0.21*	4.91 ± 0.40*	1.11 ± 0.10*

*significantly different, ** highly significant difference

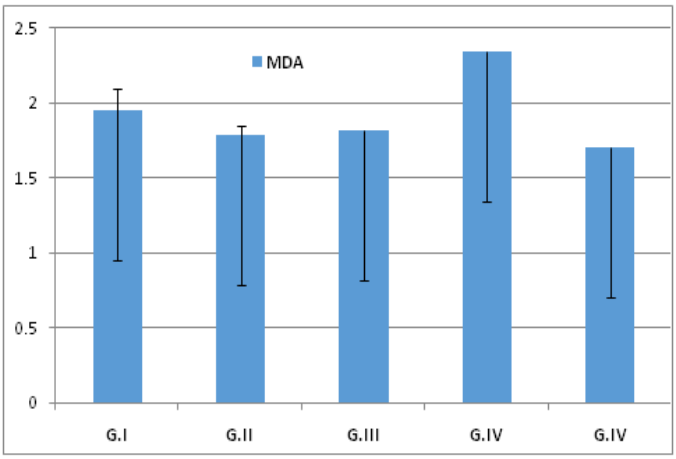


Chart 1. Level of MDA (nmol/mg protein) in all groups.

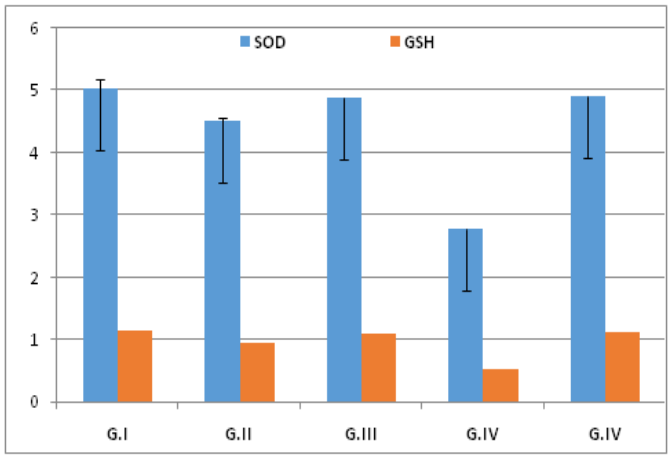


Chart 2. Level of SOD & GSH (U/mg protein) in all groups.

Table 2. The mean ± SD of goblet cells, MPO positive cells in the jejunum among the different groups.

Groups	MPO positive cells	Goblet cells
I (control-untreated)	5.82 ± 1.35	35.2 ± 1.9
II (Sham operated)	6.96 ±1.57	34.5 ±1.5
III (Sham+virapmil)	6.82 ± 1.93	35.6 ± 1.2
IV (IR)	12.25 ± 3.08**	74.3 ±1.7**
V (Virapmil treated)	6.52 ± 1.70*	40.5 ±1.4*

*significantly different, ** highly significant difference

Table 3. The mean ± SD of area % of fibrosis and caspase 3 immunoreaction in the jejunum among the different groups.

Groups	Area % of collagen fiber	Area % of caspase reaction
I (control-untreated)	6.8 ±2.7	28.2 ±4.18
II (Sham operated)	7.4 ± 2.5	26.8 ±3.3
III (Sham+virapmil)	7.8 ± 2.0	25.3 ± 4.5
IV (IR)	36.8 ± 7.9**	55.2 ± 5.1**
V (Virapmil treated)	13.6 ± 3.7*	32.1 ±2.1*

*significantly different, ** highly significant difference

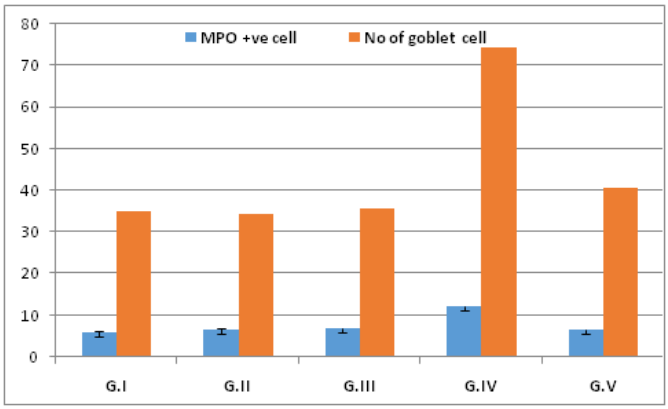


Chart 3. MPO positive and No of goblet cells in all groups.

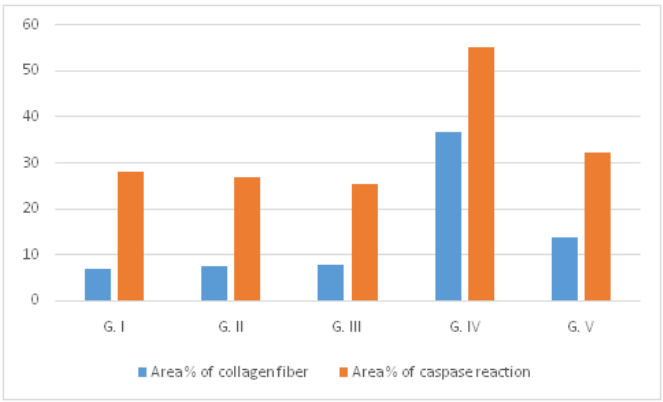


Chart 4: Area % of collagen and caspase immunoreactions in all groups.

Immunohistochemical results

Caspase-3 protein expressions were detected immunohistochemically by counting positive cells in jejunum after 60 min ischemia and 120 min reperfusion for each in four regions/image/ protein using high power magnifications. Positive cells of caspase-3 was found to be highly expressed in I/R groups when compared with untreated, sham and verapamil-treated groups (I, II, III) ($P<0.01$), and decreased significantly ($P<0.01$) after verapamil treatment when compared to I/R group (Table 3 & Chart 4).

Discussion

Jejunum is involved in digestion of food which also entails absorption of nutrients. These roles are greatly enhanced by the epithelial lining of the digestive tract, whose main functions are to provide a selectively permeable barrier between the contents of the tract and the body tissue, to facilitate the food transport and digestion, to promote the absorption of the products of this digestion and to produce hormones that affect the activity of the digestive system [33].

Syndromes of mesenteric ischemia (MI) remain clinically challenging, despite decades of surgical experience. It is a critical circulatory condition, is caused by an arterial or venous thrombosis or embolism [34-35]. The causes and major complications of mesenteric ischemia are well recognized by all surgeons. Nevertheless, diagnosis and effective treatment is often delayed in these patients [36]. The overall mortality rate of MI has remained at 60% to 80% over the last 25 years and the incidence of this disease is increasing [37, 38].

MI comprises a group of pathologic processes that have a common end point intestinal necrosis [38]. The intestinal epithelium is probably one of the most sensitive tissues to I/R injury in the body [39]; intestinal ischemia rapidly progresses to severe metabolic derangements, infiltration of inflammatory cells, loss of villi and epithelial cells, and mucosal destruction, culminating in irreversible bowel necrosis [37].

In the present study, jejunal mucosa of I/R group showed structural damage to the small intestinal mucosa. This histological damage is in the form of boarding and fusion of villi, loss of villus epithelium leaving denuded area. Degeneration of epithelial cells with nuclear alterations into rounded ones. Subepithelial space in LP with invasion by inflammatory cells were recorded in affected mucosa of this group. These results were coincided with the results of [40-41]. Prolonged jejunal ischemia (45 min) followed by reperfusion results in damage intestinal barrier integrity, which is accompanied by significant translocation of endotoxins. These phenomena result in an inflammatory response that is characterized by complement activation, endothelial activation, neutrophil sequestration and the release of pro-inflammatory mediators into circulation [42].

Particularly, villi and intestinal mucosa are one of the most sensitive tissues to I/R [43-44]. Severe damage in the architecture of the small intestine was observed and structural investigations confirmed the damage. Epithelial cells of mucosal villi were completely denuded accompanied with an infarction of the submucosa and muscularis layers, as well as an increase in Chiu's scores in the intestinal mucosa [45]. Chiu has shown that ischemia of the small intestine causes necrosis which begins at the tips of the villi and gradually progresses towards the serosal surface.

The intestinal mucosa in the small intestine, it is the first area to be severely affected in cases of ischemia. This ischemia could produce edema at submucosa, followed by loosening of the superficial mucosa and it could produce ulcers and/or bleeding in the intestinal villous [46-47]. When the intestinal barrier is lost, its content could go to the blood flow, with synthesis of inflammatory local factors, aggravating the lesions and mobilization of defense cells, beside to the risk of bacterial translocation. The maintenance of ischemia increases the inflammatory edema, causing derangement of the tissue microenvironment and facilitating the lesions dissemination to a more extensive area of intestinal mucosa. The involvement of the muscular layer will disturb the peristaltic movements with consequent stagnation of the intestinal content and increase of the local pressure [48-49].

Also, Brasileiro JL *et al.* [50] & Jia *et al.* [51] reported that Intestinal ischemia is a fatal clinical condition and intestinal ischemia-reperfusion (II/R) causes a decrease in vital nutrients, especially O2. As a result, damage of epithelium with vascular and inflammatory mediators increase, which causes adhesion, migration and activation of leukocytes [52]. According to Sola *et al.* [53] 90 minutes of ischemia by clamping the mesenteric artery and 30 minutes of reperfusion leads to histologic lesion grade 5 in the intestine according to the modified classification by Chiu *et al.* 14. On the other hand, Santos *et al.* [54] demonstrated that the degree of histological injury obtained can vary between 3 and 4 only.

The main function of goblet cells is to produce mucins for the maintenance of the mucous blanket. Goblet cells reside throughout the length of the intestine and are responsible for the production and maintenance of the protective mucous blanket by synthesizing and secreting high-molecular-weight glycoprotein [55]. In this study, it was observed that increase the number of goblet cells in operated group with significant difference from control groups. The involvement of goblet cell mucins in the pathophysiology of intestinal neoplasia and inflammatory bowel disease has been reported Menke *et al.* [56]. Recent study demonstrated the involvement of goblet cells in the process of restitution in the small intestine

subjected to superficial ischemia–reperfusion injury. They observed accumulation of goblet cells among enterocytes in ischemic injury and concluded that this increase is not due to its proliferation. The villous contraction due to the contraction of subepithelial myofibroblasts effectively minimizes the denuded surface area on the basis of findings with both an in vivo and vitro [57]. It was clearly demonstrated that goblet cells show resistance to injury. Matovelo *et al.* [58] & Ikada *et al.* [59] reported features of goblet cells are less sensitive to noxious effects than are absorptive cells perhaps because of more limited exposure. There are differences in the integrin family of cell surface receptors between goblet cells and absorptive cells [60].

It is well known that ischemia could cause tissue necrosis but if a reperfusion is done the blood flow could be reestablished, as well as recovery of tissue oxygenation in the area, bringing indispensable nutrients to tissue repair. However and paradoxally the liberation of free radicals could increase the local lesions and provoke lesions far away from the ischemic area. Therefore, the histopathological tissue alterations consequent to blood flow deprivation would be detected microscopically, by light microscopy or by imunohistochemical techniques, showing tissue alterations in the area submitted to ischemia and reperfusion [61-62]. At those areas the integrity of cell membrane could suffer with hypoxia and the cell itself could miss the ideal amount of energy production as well as accumulate, direct or indirectly, metabolites and inflammatory mediators causing cellular dysfunction [63-64].

The I/R injury of the small intestine is the serious and common condition as it causes severe local and common tissue damage [65]. Intestinal structural disorders and obstruction are the most common problems in the repair process after I/R. In the present study, immunostaining of caspase 3 revealed increase reaction in I/R group as compared with control and Verapamil-treated one. It has recently been found that blood flow repair or reperfusion after ischemia exposes the risk of late cellular necrosis in ischemic tissues, thereby the recovery of organ functions may be restricted. Reperfusion injury occurs after the intestinal ischemic period and increases tissue destruction caused by hypoxia [66].

In recent years, a number of studies have indicated that the intestinal mucosa is the first affected site under shock or other low blood flow conditions [67-68]. Apoptosis, a form of cell death distinct from necrosis, appears to play a key role in the intestinal injury response to the ischemia and anoxia associated with these conditions. The resultant cell death may then lead to loss of the gut barrier function and the translocation of bacteria [69]. To date, however, the exact cellular mechanisms involved in the pathogenesis of intestinal ischemia and anoxia injury remain unclear. Ischemia and anoxia result in an increased Ca^{2+}-influx through plasma membrane calcium channels resulting in accumulation of large quantities of intracellular Ca^{2+}[Ca^{2+}]i [70-71]. Such an increase in [Ca^{2+}]i has been proposed to be the major mediator leading to apotosis in various cell types.

Myeloperoxidase (MPO) activity is commonly used to measure the extent of inflammation in intestinal tissues subjected to I/R injury. In the present study, intestinal I/R caused an elevation in tissue MPO activity, indicating the presence of enhanced leukocyte recruitment in the inflamed tissue.This results concomitant with Amara *et al.* [72]. I/R-induced intestinal injury was confirmed biochemically by an increase in myelo-peroxidase (MPO) activity as a measure of neutrophil infiltration, and histopathologically by the presence of a diffuse transmural inflammatory infiltrate (in the form of mononuclear cells and PMN) and complete sloughing of the epithelial surface and focal pseudo-membrane formation [73]. Occlusion and re-perfusion of the splanchnic arteries can precipitate circulatory shock mainly through an increase in vascular permeability, causing the activation and adhesion of polymorpho-nuclear neutrophils (PMN), release of pro-inflammatory substances and formation of free radicals [74]. Activated neutrophils contributed to tissue damage through release of free radicals, proteolytic enzymes, stimulation of cytokine release from local cells, thus promoting further neutrophil recruitment and, plugging of capillaries causing no-flow phenomenon [75-76].

The present work also showed that the changes in MPO are attended by the concomitant MDA increase and reduction in the activities of the antioxidant enzyme glutathione. Glutathione is an endogenous antioxidant found naturally in all animal cells. It has the capability of reacting with free radicals and the provisions of glutathione precursors are protective for different types of free radical mediated cellular injury [77]. Intestinal ischemia causes a decrease in vital nutrients and reactive oxygen species (ROS) and reactive nitrogen species (RNS) increase, causing biomolecules such as membrane lipids, nucleic acids, enzymes and receptors in the tissues to be damaged. ROS adhere to membrane-associated polyunsaturated fatty acids after this peroxidation starts. Increased vascular leakages (protein and liquid leakages) may cause multi-organ dysfunction syndrome due to the increased response to the local and systemic response [78-79]. During resuscitation, oxygenated blood, which goes to ischemic tissues, may paradoxially increase the degree of damage due to the increase in free oxygen radicals [80].

In the current study, biochemical results revealed increased in MDA and decrease In SOD and GSH. MDA level is considered a reliable indicator of an ischemia-reperfusion event and it increased with the severity of the injury [81]. Other studies reported that Ischemia and consecutive reperfusion causes oxidative stress, which is characterized by an imbalance between reactive oxygen

species (ROS) and the anti-oxidative defense system. Reperfusion of ischemic tissue has been shown to worsen acute ischemic injury via the release of ROS [82]. I/R injury in the small intestine causes local production of the ROS which are known to play an important role in gut epithelial damage [83]. Lipid peroxidation increase in lung, liver and small intestine in animals with intestinal I/R, was evidenced by significantly increased MDA levels in all these organs [84].

In the current study, the significant low tissue MDA levels and increased SOD and GSH in I,II,III and V groups compared with IV showed that verapamil prevents lipid peroxidation in intestinal I/R. These results also clearly showed the preventive effect could be obtained by this drug, a fact suggested by statistically different MDA levels between groups I,II,III,V and IV. The antioxidant effect of verapamil is supported by the findings recorded by Kuo S *et al.* [85]. They explained its action against oxidative damage via inhibition of lipid peroxidation and by restoration of cytosolic catalase and glutathione peroxidase activities. Previous investigators showed that verapamil attenuates cardiac injury in rats by reducing oxidative stress and apoptosis [86].

Results of this study revealed attenuation also of the hisopathological alteration in intestinal mucosa. Similarly, another study demonstrated that verapamil protects against I/R injury of intestine because of its anti-inflammatory and anti-apoptotic actions [17, 87]. The present work revealed that administration of verapamil at the beginning of reperfusion protected the mucosa from the damaging effect of ischemic/reperfusion injury. Mucosa of jejunum in group V after administration of verapamil became more or less like control except the appearance of slight vacuolation of few enterocytes and small subepithelial spaces. Ischemia/Reperfusion showed focal degeneration in crypt bases that was not seen in the tissue of animals received verapamil.

However, a significant decrease in MPO level was seen in the I/R plus virapamil group. Some researchers reported the anti-inflammatory properties of virapamil against I/R induced tissue injury. Also verapamil administration significantly decreased the polymorphonuclear neutrophils infiltration when compared with the I/R group. This finding was in agreement with the significant alleviation of the intestinal morphological changes by verapamil. Recently, it has been reported that cellular calcium may have an important role in ischemic injury, which consists of damage during ischemia and impairment at the time of reperfusion.

Intestinal ischemia is clinically complex to diagnose and treat and an early diagnosis is necessary to avoid mortality. At the same time, there is no treatment algorithm. Because of these reasons many researchers were intended to evaluate the injury and treatment options. The main goal of treatment is to stop the ischemia and inflammatory process at an early time and therefore decrease the degree of injury. Therapeutic strategies aimed at ameliorating IR injury have focused on artificially increasing in the quantity or activity of scavengers and antioxidants in the ischemic tissues. They help to increase the disposal of ROS and the degree of reperfusion injury in the same time will decrease purposely.

Therefore, a calcium channel blocker may exert a protective effect and accelerate the recovery of reperfusion. A number of different calcium channel blockers using different treatment regimens have been tested on some organs, including myocardium [88], liver [20] and kidney [15]. But few investigators have tested calcium channel blockers on intestinal ischemia and reperfusion (I/R), and there is no information about the effects of calcium antagonist on the small intestine after the onset of ischemia.

Conclusion

Pharmacological preconditioning by verapamil showed more significant protection against intestinal (jejunum) IR injury. The degree of protection of either ischemic or pharmacological preconditioning is correlated with intense inflammatory cellular infiltration and lipid perioxidation. Further studies are needed to investigate the effect of different types of calcium channel blockers and angiotensin converting enzyme inhibitors on intestinal IR. Further studies are also needed to examine these effects in different animal species, with different doses and durations and study of possible side effects of these drugs, especially in presence of intestinal insult.

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