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Simvastatin attenuates intestinal ischaemia/ /reperfusion-induced injury in rat

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Ischaemia/reperfusion (I/R) injury is commonly seen in the field of intestine surgical interventions, shock, trauma, and many other clinical conditions. Simvastatin is known to have antioxidant and anti-inflammatory properties. This study investigated the effect of simvastatin administration in a warm intestinal I/R model on TNF-α, antioxidant enzymes and intestinal tissue morphology.

Thirty-six male wistar rats underwent laparotomy under general anaesthesia. Simvastatin was administered from four days before ischaemia induction. The rats were divided in to three groups ($n = 12$): the sham goup, the I/R group, and the I/R + simvastatin group. Intestinal ischaemia was induced by superior mesenteric artery ligation with microvascular clamps for 60 minutes, and after ischaemia, blood perfusion was released into the tissue and a reperfusion phase was started, which lasted for 3 hours. After 3 hours, the animals were sacrificed and serum and tissue obtained for biochemical and histological study.

In the simvastatin treated group, intestinal tissue injury, TNF-α level, and tissue malondealdehyde levels were significantly lower than in the I/R group ($p < 0.05$). Glutathion peroxidase and superoxide dismutase levels were significantly higher in the simvastatin treated group than in the I/R group ($p < 0.05$).

Simvastatin pretreatment reduced intestinal I/R injury and was associated with down--regulation of serum TNF-α and tissue malondealdehyde level, and simvastatin administration maintained cellular antioxidant enzyme contents compared to the I/R group after 3 hours reperfusion time. (Folia Morphol 2009; 68, 3: 156–162)

Key words: simvastatin-Intestine, ischaemia/reperfusion, injury

INTRODUCTION

Ischaemia/reperfusion (I/R) injury in the intestine occurs when the intestinal tissue is deprived of oxygen and other nutrients necessary to maintain cellular function. Mesenteric ischaemia is a clinical entity with a mortality rate between 60% and 100% that usually requires surgical resection of the necrotic intestinal segment [4, 9].

It is a well-known phenomenon that reperfusion is as dangerous for tissues as ischaemia, particularly

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in the heart and the intestines. Intestinal ischaemia is a result of systemic factors (hypovolaemia, hypotension, hypoxia, or sepsis) or local factors (cardiopulmonary by-pass, organ transplantations, abdominal aortic aneurysm repair, embolectomy for acute mesenteric occlusion, or repair of traumatic vascular lacerations). It can lead to life-threatening complications associated with remote organ injury [14, 51]. Restored circulation results in the formation of free oxygen radicals and other acute phase reactants. Cellular death occurs via the lipid peroxidation of the cell wall [14]. The consequences of mesenteric ischaemia are devastating to patients and usually result in diarrhoea, malabsorption, short bowel syndrome, and even death [1]. In addition, I/R injury has been a key problem with respect to successful organ preservation in small intestine transplantation [33].

The gastrointestinal tract is one of the most sensitive organs to ischaemia and reperfusion [30]. Thus, there is great interest concerning methods to verify protective mechanisms in extensive small intestine lesions that can shorten the patient's life. For effective transplantation treatment, we must identify and protect small intestine morphological changes after I/R injury. In this regard, many authors have studied small intestine morphological aspects to determine what might correlate to the pathogenesis of the resulting injury and protective mechanisms [11, 19]. Although there have been advancements in the treatment of ischaemic injury, an ideal treatment has not been defined, and new options should be considered.

The mechanisms of intestinal mucosa injury after intestinal I/R are complex. Reactive oxygen species (ROS)-induced lipid peroxidation is known to be one of the major factors causing intestinal I/R injury, and the administration of free radical scavengers appears to prevent intestinal mucosa from intestinal I/R injury [27, 42]. During reperfusion, reintroduction of molecular oxygen into the ischaemic tissue results in the production of ROS such as superoxide anion, hydrogen peroxide, peroxinitrite, and hydroxyl radicals [11], particularly, hydroxyl radicals typically cause biological damage by stimulating the free chain reaction known as lipid peroxidation [5, 44, 46].

Inflammatory pathways play an important role in pathogenesis of intestinal I/R, through production of inflammatory cytokines, involvement of the complement system, and neutrophil infiltration [4, 9, 14, 30, 33, 42] at the site of damage [5, 18, 24, 28, 29, 41].

3-hydroxy-3-methylglutaryl coenzyme A (HMG--CoA) reductase inhibitors, or statins, are a class of drugs particularly beneficial in combating this risk factor. Therapeutic doses of statins potently reduce serum cholesterol levels in humans [40]. They have been shown to have properties independent of their cholesterol-lowering ability, referred to as pleiotropic effects. It has been shown that pretreatment with statins decreases tumour necrosis factor alpha (TNF-α) production [21, 38, 49] and enhances superoxide dismutase (SOD) levels [6, 54]. It is effective in preventing reperfusion injury after I/R in experimental models of I/R in the liver [12], heart [43] and kidneys [52].

The current study was performed to clarify whether simvastatin can prevent intestinal mucosa I/R injury and tissue anti-oxidant enzyme content, and to investigate its effects on TNF-α release during intestinal I/R in an *in vivo* rat model.

MATERIAL AND METHODS

Surgical procedure

The study was carried out with 36 male Wistar rats (purchased from the central animal house of Tabriz medical school, Tabriz, Iran) weighing between 220 and 260 g. The rats were kept at room temperature and provided with free access to standard chow and tap water. This research was done in accordance with university guidelines for the care of laboratory animals.

Under ketamine (50 mg/kg) and xylazine (10 mg/kg) anaesthesia, a median laparotomy was performed and the blood supply to the intestine was interrupted for 60 minutes by occlusion of the superior mesenteric artery using a microvascular clamp. Intestinal ischaemia was confirmed by observing the loss of pulsation of the mesenteric artery and its branches, as well as paleness of the jejunum and ileum. Afterwards the intestines were returned to the abdomen, which was then closed with two small clamps. At the end of 60 minutes of ischaemia, the clamp was gently removed to allow reperfusion of the blood flow for 3 hours, which was confirmed by observing the pulsation of the artery and its branches on the intestine [36].

Experimental design

The rats were divided into three groups of twelve animals as follows:

— sham operation (sham) group (n = 12): animals were subjected to laparotomy without vascular occlusion;

— ischaemia/reperfusion (I/R) group (n = 12): animals were subjected to 60 minutes of intestinal ischaemia and sacrificed 3 hours after reperfusion;
— ischemia/reperfusion + simvastatin (I/R + simvastatin) group (n = 12): as in the I/R group, but also treated with simvastatin (10 mg/kg/day) administered orally since 4 days before I/R induction.

At the time of sacrifice, under anaesthesia, venous blood samples were obtained for serum TNF-α level, then a laparotomy was performed and the intestine was removed. Intestinal tissue samples were obtained and frozen at –70°C for measurement of glutathione peroxidase (GPx), SOD, and malondialdehyde (MDA). Intestine samples were also fixed in buffer formalin for histological analysis.

GPx assay

For tissue biochemical analysis, the intestinal tissue was homogenized in 1.15 KCL solution. GPx measurements were performed as described by Paglia et al. [37]. GPx was catalyzed by the oxidation of reduced glutathione in the presence of cumene hydroxyperoxide. GPx activity was designated as unit for mg/protein of intestinal tissue.

SOD assay

SOD activity was determined as described by Sun et al. [50]. This method depends on the inhibition of nitroblue tetrazolium (NBT) reduction by xanthine--xanthine oxidase used as a superoxide generator. SOD activity was expressed as the amount of enzyme that causes 50% inhibition of the rate of NBT reduction. SOD activity was designated as unit for mg/protein of intestinal tissue.

Serum MDA measurement

Serum MDA levels were studied using thiobarbituric acid (TBA), as described previously [23, 55].

Histological assessment

Formalin-fixed and paraffin-embedded tissue sections were cut at 5 μm and stained with haematoxylin and eosin for histological examination. Light microscopy was used to assess the degree of intestinal tissue damage, which was performed by a pathologist who was blinded to the treatment given. Tissue samples were scored by using the following grading scale:
— grade 0 — normal mucosa;
— grade 1 — development of subepithelial space at the apex of the villous and capillary congestion;
— grade 2 — extension of subepithelial space with moderate lifting of the epithelial layer from the lamina propria;
— grade 3 — massive epithelial lifting down the side of the villi and a few tips may be denuded;
— grade 4 — denuded villi with lamina propria and dilated capillaries exposed;
— grade 5 — digestion and disintegration of lamina propria, haemorrhage, and ulceration [17].

Elisa assay for TNF-α

Plasma levels of TNF-α were measured by an enzyme-linked immunoabsorbent assay (ELISA) using a rat TNF-α immunoassay kit (Blender systems, Austria).

Statistical analysis

Data were analyzed using SPSS software 13. All data are reported as mean ± SD. Statistical comparisons of the groups were performed by one-way ANOVA analysis of variance followed by Tukey post-test. $P < 0.05$ was considered significant.

RESULTS

GPx and SOD

GPx and SOD levels in the intestinal I/R group were lower in comparison to the sham group and the intestinal I/R +simvastatin group, while the levels of these enzymes were significantly lower in the I/R + simvastatin group compared to the sham group ($p < 0.05$, Table 1).

MDA

Intestinal I/R increased intestinal tissue MDA levels, and it was higher in the intestinal I/R group compared to the other groups, and the MDA levels in the intestinal I/R + simvastatin group was significantly higher than in the sham group ($p < 0.05$, Table 1).

TNF-α

At 3 hours post intestinal I/R, the serum TNF-α level was significantly increased compared to the sham group. Serum TNF-α level was significantly lower in the intestinal I/R + simvastatin group compared to the intestinal I/R group ($p < 0.05$, Table 1).

Histological assessment

One hour intestinal ischaemia followed by 3 hours reperfusion resulted in the development of subepithelial space at the apex of the villous and capillary congestion, extension of the subepithelial space with moderate lifting of the epithelial layer

Table 1. The effect of simvastatin on intestinal antioxidant enzyme content, MDA level and serum TNF-α in the rat intestine after ischaemia and reperfusion

	Sham	Intestinal I/R	Intestinal I/R + simvastatin
GPx [U/mg protein]	3.60 ± 0.45	1.93 ± 0.17	2.96 ± 0.24
SOD [U/mg protein]	2.56 ± 0.62	0.94 ± 0.37	1.78 ± 0.27
MDA [nmol/mL]	1.64 ± 0.34	3.90 ± 0.54	2.72 ± 0.28
TNF-α [pg/mL]	23 ± 5.05	95 ± 15.70	72.14 ± 16.02

The rat intestine was pretreated with simvastatin prior to ischaemia; intestine was subjected to 60 minutes ischaemia followed by 3 hours of reperfusion. The values are shown as a mean ±SD for rats in each group and a difference of ($p < 0.05$) was considered significant; GPx — glutathione peroxidase, SOD — superoxide dismutase, MDA — malondialdehyde, TNF-α — tumour necrosis factor alpha

Table 2. The effect of simvastatin on intestinal tissue injury index after ischaemia/reperfusion

	Sham	Intestinal I/R	Intestinal I/R + simvastatin
Intestinal tissue injury index	0.66 ± 0.51	4.16 ± 0.75	3.14 ± 0.69

The rat intestine was pretreated with simvastatin prior to ischaemia; intestine was subjected to 60 minutes ischaemia followed by 3 hours of reperfusion. The values are shown as a mean ±SD for rats in each group and a difference of ($p < 0.05$) was considered significant.

from the lamina propria, massive epithelial lifting down the side of the villi, and denuded villi with lamina propria and dilated capillaries exposed, digestion and disintegration of lamina propria, and haemorrhage and ulceration in the intestinal tissue. The grade of tissue injury in the intestinal I/R + simvastatin group was significantly lower than in the intestinal I/R group ($p < 0.05$, Table 2).

DISCUSSION

In the present study, we demonstrated that simvastatin suppressed pro-inflammatory cytokine TNF-α levels in plasma of rats undergoing intestinal I/R. The histological study revealed severe intestinal mucosal damage, suppressed GPx and SOD levels, and increased MDA levels in the I/R animals; however, these findings were significantly ameliorated in the simvastatin treated animals. The small intestine is extremely sensitive to ischaemic insult, and in some clinical circumstances gives rise to mesenteric hypoperfusion followed by I/R injury. Despite the existence of evidence detailing the pathogenesis of the intestinal I/R injury, the exact mechanism of this complex process is still unknown [44]. Intestinal I/R injury occurs in a biphasic manner characterized by different time frames and mechanisms: (1) an early phase that immediately follows the transient ischaemia and lasts 2 to 3 hours; and (2) a late phase which begins 12 to 24 hours from the transient ischaemia and lasts for about 3 to 4 days [29, 51]. In our study, a period of 60 minutes of ischaemia following 3 hours of reperfusion was especially chosen to assess the changes in the early phase of reperfusion injury, which is in a clinically relevant time frame. The alterations in intestinal motility caused by I/R are directly related to the length of both ischaemia and reperfusion time [39]. The functional alterations caused by I/R have been previously identified [31]. These changes consist of reversible alterations in smooth muscle contractility and intestinal transit, as well as characteristic changes in electrical activity during ischaemia [2, 25]. The structural damage caused by ischaemia is aggravated by the restitution of blood flow. The physiopathology of intestinal mucosal damage by I/R is not completely understood. Nevertheless, it is believed that cytotoxic substances such as free radicals, nitric oxide, serotonin, and complement, as well as neutrophil infiltration and nuclear transcription factors, play important roles [7]. Macrophages have been also implicated in the initial damage caused by intestinal I/R [10]. I/R has also been shown to induce apoptosis [35]. Bacterial translocation and mucosal barrier dysfunction have been implicated in the damage caused by I/R in the gut [4, 8, 20]. I/R injury causes maldistribution of blood flow, damage to endothelium, coagulation abnormalities, and aggregation of platelets and neutrophils. The activation of neutrophils leads to the release of ROS including superoxide anion (O_2^-) and H_2O_2. A growing amount of circumstantial evidence implicates oxygen-derived free radicals (especially O_2^- and H_2O_2) and high-

-energy oxidants (e.g., peroxynitrite, $ONOO^-$) as mediators of I/R injury [57]. It has been shown that the toxicity ascribed to the O_2^- is initially caused by the superoxide's direct or indirect interaction with biological targets such as lipids, catecholamines, and DNA. Moreover, simultaneous generation of nitric oxide (NO) and O_2^- favours the production of a toxic reaction product, $ONOO^-$, and this product may account for some of the deleterious effects associated with NO production [26]. Therapeutic strategies aimed at ameliorating I/R damage include antioxidant enzymes such as SOD and GPx, free radical scavengers such as mannitol and α-tocopherol, and agents which prevent the generation of radicals such as allopurinol and deferoxamine. Because of the significant role played by oxygen-derived free radicals in the pathogenesis of I/R, studies on the application of free oxygen radical scavengers to limit the damage to tissue and organs have been attempted [32, 51]. What is more, in this study, we aimed to investigate whether simvastatin can be effective in preventing the reperfusion injury of intestinal damage after 60 minutes of warm superior mesenteric ischaemia in rats. We examined organ GPx and SOD levels as markers of antioxidative function. GPx and SOD reduce oxidative stress resulting from gut I/R and prevent tissue injury [3, 16]. However, in the present study, simvastatin treatment improved small intestinal GPx and SOD levels within our experimental time frame. Modulation of the inflammatory response following I/R injury is an important component of tissue defence, mostly because inflammation is the major component of cell death and motor alteration in intestine subjected to intestinal injury.

Lipid peroxidation results from the reaction of reactive oxygen metabolites, especially the hydroxyl and hydroperoxyl radicals with the membrane bound polyunsaturated fatty acids with a loss of a carbon radical and its rearrangement for formation of a conjugated diene. This conjugated form reacts immediately with oxygen to form peroxide radicals. Peroxide radicals initiate a chain reaction by removing a hydrogen atom from the other fatty acids. The product of this reaction is tissue MDA and hydroperoxide [34]; in this study simvastatin administration decreased intestinal tissue MDA in the simvastatin-treated group compared to the I/R group.

TNF-α is an inflammatory cytokine that may be an important mediator in the development of reperfusion-induced tissue injury and lethality [47]. Grewal et al. [15] demonstrated that the treatment of rats with anti-TNF antibodies could prevent neutrophil influx and tissue injury. It is well known that the serum levels of TNF-α are elevated after intestinal reperfusion and that these pro-inflammatory cytokine levels reflect mortality [22, 45, 48, 56]. The biological properties of TNF-α are primarily released from macrophages during the early phase of an inflammatory response, and they stimulate endothelial cells and macrophages to release IL-6 and IL-8 [13, 53]. In the present study, a significant reduction in the serum TNF-α level was observed 3 hours after reperfusion in the simvastatin-treated animals.

CONCLUSIONS

The results showed that simvastatin administration attenuates intestinal I/R injury through inhibition of TNF-α and MDA production and maintaining intestinal tissue GPx and SOD levels. As simvastatin is a safe drug, it may be used as a protective therapeutic in attenuating intestinal I/R injury, although more studies should be conducted to understand the mechanisms involved in this process.

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