OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2017.

Research Article
Protective Effect of Mesna on Intestinal Ischemia-reperfusion Injury by Nitric Oxide and Arginase in an Experimental Rat Model

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Abstract

Background and Objective: An ischemia-reperfusion (I/R) injury of small intestine is a serious and common condition that is a result of the blockage of the Superior Mesenteric Artery (SMA) because of significant clinical problems. This study aimed to evaluate the effects of the antioxidant mesna on the intestinal nitric oxide (NO) levels and arginase activity in the I/R injury at biochemical and immunohistochemical levels and to determine the tissue malondialdehyde (MDA) level as a marker of oxidative damage. **Methodology:** In the study, 10-12 week-old male Wistar albino rats weighing 200-250 g were used. The animals were grouped as group A-Sham-control group, group B-Intestinal I/R group and group C-mesna (n = 7). The I/R were performed by binding and opening the SMA after 120 min. In the mesna group, mesna (150 mg kg^{-1}, ip) was given immediately after opening the SMA. Following surgical procedures, the inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) were shown in the tissue. The levels of NO and MDA and the activity of arginase were measured. The levels of MDA and the NO, arginase activity, immunoreactivity of iNOS and eNOS were compared to the control group. Statistical analysis was done using SPSS 19 and descriptive statistics were expressed as arithmetic Mean±Standard deviation. **Results:** It was observed that the values were significantly increased ($p<0.05$) in the I/R group and decreased in the mesna group ($p<0.05$). Mesna has demonstrated its significant therapeutic effects on biochemical parameters in the I/R process by decreasing the NO level, equilibrating the iNOS and eNOS expressions and decreasing the arginase activity. **Conclusion:** It has been observed that mesna was helpful against the intestinal I/R injury because of its antioxidant properties considering the biochemical parameters. Mesna has demonstrated its significant therapeutic effects on biochemical parameters in the I/R process by decreasing the NO level, equilibrating the iNOS and eNOS expressions and decreasing the arginase activity.

Key words: Arginase, ischemia-reperfusion, intestine, mesna, nitric oxide, synthase

Received: Accepted: Published:

Citation: Sanal Filiz, Ulucam Enis, Inan Mustafa, Cerkezkayabekir Aysegul and Bakar Elvan, 2017. Protective effect of mesna on intestinal ischemia-reperfusion injury by nitric oxide and arginase in an experimental rat model. Int. J. Pharmacol., CC: CC-CC.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The I/R injury of the small intestine is the serious and common condition and also the result of obstruction of the Superior Mesenteric Artery (SMA) due to many reason. The I/R injury causes severe local and common tissue damage[1]. Small intestinal ischemia is formed by external factors such as volvulus, invagination, drowned inguinal hernia, tumor, fibrotic band and external factors such as mesenteric circulation disorder, arterial thrombosis, embolism, Henoch-Schönlein purpura, disseminated intravascular coagulation, systemic hypertension, vasoconstriction, blood viscosity disorders, arteriosclerosis and hypotension[1-3].

Intestinal structural disorders and intestinal obstruction are the most common problems in the healing process after I/R. Because of the high mortality and morbidity risks associated with these problems, researchers investigate to regulate blood flow to the ischemic tissue to prevent tissue necrosis and recovery of organ function[3,4]. However, 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[5].

Investigations showed that Reactive Oxygen Species (ROS) in periods of reperfusion play a significant role on the basis of I/R pathologies[1,6]. The most common form of ROS are the superoxide and hydroxyl radical, singlet oxygen and hydrogen peroxide (H_2O_2) in I/R process[4]. These reactive compounds damage structural molecules such as DNA, proteins and lipids. Lipid peroxidation and protein oxidation are not able indicators of oxidant injury[7]. Nitric oxide (NO) is a highly reactive substance and synthesis from L-arginine by nitric oxide synthases (NOS). Three isoforms of NOS are known: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS)[8]. While eNOS and nNOS are constitutively expressed, iNOS is expressed in reply to growth factors and cytokines[9]. The nNOS is the predominant NOS in the normal intestine and this suppresses the expression of iNOS[4]. Possible sources of NO in the intestine include intrinsic intestinal tissue (neural plexus, smooth muscle, mast cells and epithelium), leukocytes and monocytes[10]. Nitric oxide (NO), free radical, has cytotoxic and cytoprotective properties. It was explained that NO has beneficial effects in the intestine. The NO may contributed to preservation normal vascular permeability and helps scavenging oxygen free radicals[4]. The NO can cause to production of highly active radicals such as peroxynitrite anion and nitrogen radical in the organism. These radicals can damage tissues and can cause transmission disorders by inactivating various ion pumps. It is also known that NO directly inhibits the mitochondrial respiratory chain[11].

Arginase is an important enzyme for nitrogen metabolism. It catalyzes the hydrolysis of arginine to urea and ornitine and consists of two isoforms: Arginase I (Liver type) is expressed in the liver; arginase II (Non-hepatic type) is primarily expressed in the kidney and small intestine and in low levels in other tissues[12]. Arginase induction is primarily responsible for down regulating NO production through the depletion of arginine[13]. For this reason, arginase may regulate NO levels and contribute to the oxidative stresses formed during I/R. Arginase and NOS competitively consume L-arginine. The arginase/NOS competition for arginine catabolism can lead to beneficial or adverse consequences[14]. Arginase may moderate oxidative stress during I/R injury by consumption of L-arginin. Thus, increased arginase activity can lead to limit NOS activity because of decreased level of L-arginine as NOS substrate, but also it causes a decreased inhibitor effect on xanthine oxidase activity and then it results in more superoxide radical production and tissue damage[15]. It was reported that L-arginine deficiency may result in induction of NOS from the synthesis of NO to the production of superoxide. These data suggest that the arginase pathway may be an important regulator of NOS-mediated NO formation and arginase is known to play an important regulatory role for this process[14,16].

Mesna (2-mercaptoethane sulfonate) is a small, synthetic, highly water-soluble molecule. Mesna is a thiol molecule and it has been administered during oxazaphosphorine chemotherapy treatment to avoid the risk of hemorrhaging. Besides its uroprotective action, there is evidence that mesna holds potent antioxidative properties as well[17,18]. These properties of mesna's have been demonstrated that in acetominophenol induced oxidative damage in kidney tissue[19]. Ypsilantis *et al.*[20] have reported that mesna treatment of I/R-induced oxidative damage in a time dependent manner leading to a reduction. Experimental studies have demonstrated the efficient use of mesna for the prevention or reduction of I/R-induced injuries in the intestine, kidneys, stomach and liver[17, 21-26] .

Mesna is an effective oxygen radical scavenger because of it sulfhydryl groups[17,27]. It was hypothesized that the administration of mesna can decrease oxidative stress after a period of ischemia. To test this hypothesis, this study is aimed at evaluating the effects of the antioxidant mesna on the intestinal NO and tissue malondialdehyde (MDA) levels as a marker of oxidative damage and arginase activity and eNOS and iNOS immunoreactivity in I/R injuries.

MATERIALS AND METHODS

Mesna was purchased from Sigma Chemical Co. The Quantichrom Arginase Assay Kit (DRAG-200) and BIOXYTECH Nitric Oxide Assay Kit were obtained from Bio-Rad Co. and BIOTECH Co. respectively. All of the other chemicals and reagents used were analytical grade.

The experimental protocol was certified by the animal ethics committee of University. In the study, 10-12 week-old male Wistar albino rats weighing 200-250 g were used. Food, water, light and temperature conditions were provided standard. Food was with held 12 h before the experiment and water was given *ad libitum*. All surgical processes were applied under (ketamine 50 mg kg^{-1} and xylazine 10 mg kg^{-1} intramuscular) anesthesia.

The animals were grouped as group A-Sham-control, group B-Intestinal I/R and group C-mesna (n = 7). The I/R injuries were performed by binding and opening the Superior Mesenteric Artery (SMA) after 120 min. In group C, mesna (150 mg kg^{-1}, ip) was given immediately after opening the SMA[22]. The intestinal I/R procedure was applied using the surgical technique from Guneli *et al.*[23]. Following the surgical procedures, intestinal tissue samples were obtained for biochemical parameters and immunohistochemical inspection.

Biochemical analyses

Sample preparation: The samples were stored in a deepfreeze (-80°C) until the time of analysis. Tissue homogenates for the estimation of tissue MDA, NO levels and arginase activity were prepared at +4°C.

MDA concentration assessment: The concentration of MDA was measured by a colorimetric method. Samples were homogenized with 150 mmol L^{-1} ice-cold KCl for the measurement of MDA. The MDA concentrations in the small intestine tissue were assayed in the form of thiobarbituric acid-reacting substances. About 0.2 mL of 8.1% sodium dodecyl sulphate, 1.5 mL of acetate buffer (3 M, pH: 3.5) and 1.5 mL of 0.82% thiobarbituric acid were added to the supernatant (0.25 mL). This mixture was heated to 95°C for 45 min. After cooling with tap water, 0.5 mL of 35% sodium dodecyl sulphate was added and the mixture was heated to 95°C for 10 min. It was then shaken and centrifuged at 2500 rpm for 10 min at 25°C. The absorbance of the final solution was read at 532 nm. Malondialdehyde was quantified using standard graphic and expressed as µmol L^{-1} MDA.

Nitric oxide (NO) assessment: The concentrations (mM) of NO were measured in the small intestine tissue samples using the BIOXYTECH Nitric Oxide Assay Kit, according to the producer's instructions.

Arginase activity: The arginase activity of the tissue samples was measured using the Quantichrom Arginase Assay Kit. The kit provides a cromogen that forms a colored complex with the urea produced in the arginase reaction. One unit of arginase activity was defined as the enzyme amount that converts 1 µmol L-arginine to ornithine and urea per minute at pH: 9.5 and 37°C.

Immunohistochemistry: Sections were incubated at 56°C overnight, deparaffinized in xylene and dehydrated in a graded series of ethanol for eNOS and iNOS expression in the small intestine. They were treated with citrate buffer (10 mM, pH: 6,0) for 40 min for antigen retrieval in the pre-incubating chamber. After cooling down for 20 min at room temperature, sections were immersed in 3% H_2O_2 for 5 min to inhibit the endogenous peroxidase activity. They were then washed with Phosphate Buffered Saline (PBS). Ultra V Block was used for the non-specific binding of antibodies for 10 min and then sections were incubated with anti-eNOS antibody (1:50) and anti-iNOS antibody (1:100) for 60 min at +4°C. The sections were rinsed with PBS and then treated with biotinylated anti-goat antibody. Negative controls included staining tissue sections with omission of the primary antibody. The antigen-antibody complex was defined using a streptavidin-biotin-peroxidase kit. After washing with PBS, they were incubated with 3,3-diaminobenzidine (DAB) to visualize immunolabeling and counterstained with Mayer's hematoxylin. At least 10 randomly selected vision areas from each animal in each group were scored to describe the eNOS and iNOS expression. The intensity of the immunolabeling was recorded as (1) Mild, (2) Moderate, (3) Strong and (4) Very strong and graded from 0 to 4.

Statistical analysis: The statistical analysis was done using SPSS 19 and descriptive statistics were expressed as arithmetic Mean ± Standard deviation. The Kruskal Wallis test and Mann Whitney U were selected for multiple and paired comparisons, respectively. Statistical significance was defined as $p<0.05$[28].

Table 1: MDA, NO levels and arginase activity in saline-or mesna-treated animals in I/R

Groups	MDA (μmol L^{-1})	NO (μM)	Arginase (U L^{-1})
Sham-Control	0.80±0.10	28.15±8.90	17.46±1.66
IR	1.96±0.56[a]	40.25±5.69[d]	37.34±3.13[a]
Mesna	1.47±0.17[b,c]	26.56±8.34[e]	24.90±2.22[c,e]

a: SC vs. IR ($p<0.01$), b: IR vs. mesna ($p<0.05$), c: SC vs. mesna ($p<0.01$), d: SC vs. IR ($p<0.05$), e: IR vs. mesna ($p<0.01$), Mean was given with standard deviation (Mean±SD) (n = 7), MDA: Malondialdehyde, NO: Nitric oxide

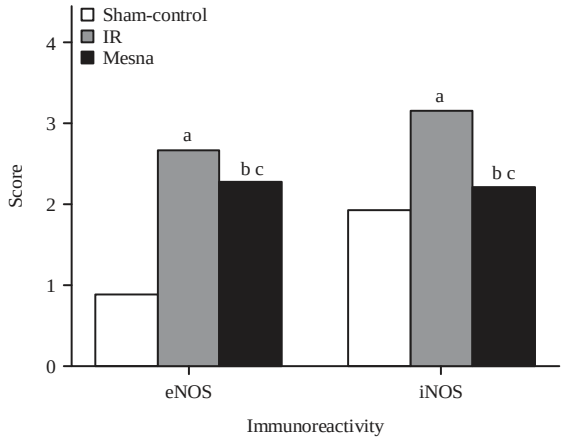

Fig. 1: Expression scores of iNOS and eNOS immunoreactivity in small intestine

Mean was given with standard deviation bar (n = 7), a: Sham-Control vs. IR ($p<0.01$), b: IR vs mesna ($p<0.05$), c: Sham-Control vs. mesna ($p<0.01$)

RESULTS

The levels of NO and MDA and the activity of arginase were measured and the results are shown in Table 1. The MDA and NO levels in the I/R group were significantly higher than the sham-control group rats, respectively $p<0.01$ and $p<0.05$. These levels were higher than the sham-control, in mesna group (150 mg kg^{-1}, ip) but the elevation in MDA and NO levels was significantly decreased ($p<0.01$) in the mesna group compared to the I/R group. In both the I/R and mesna groups, arginase activity was found to be significantly increased ($p<0.01$) when compared to the sham-control group, while mesna treatment reversed these elevations in arginase ($p<0.01$).

The eNOS and iNOS expression were determined by immunohistochemical staining. The Immunohistochemical activity was scored as the differences in intensity between the groups in the distribution patterns of eNOS and iNOS immunolabeling of the intestinal tissue. The immunoreactivity of the I/R group's iNOS and eNOS were compared with those of the control group; it was observed that the values were significantly increased ($p<0.05$) in the I/R group and decreased in the mesna group ($p<0.05$) (Fig. 1). Microphotographs of eNOS and iNOS immunoreactivity in the intestine are shown in Fig. 2 and 3.

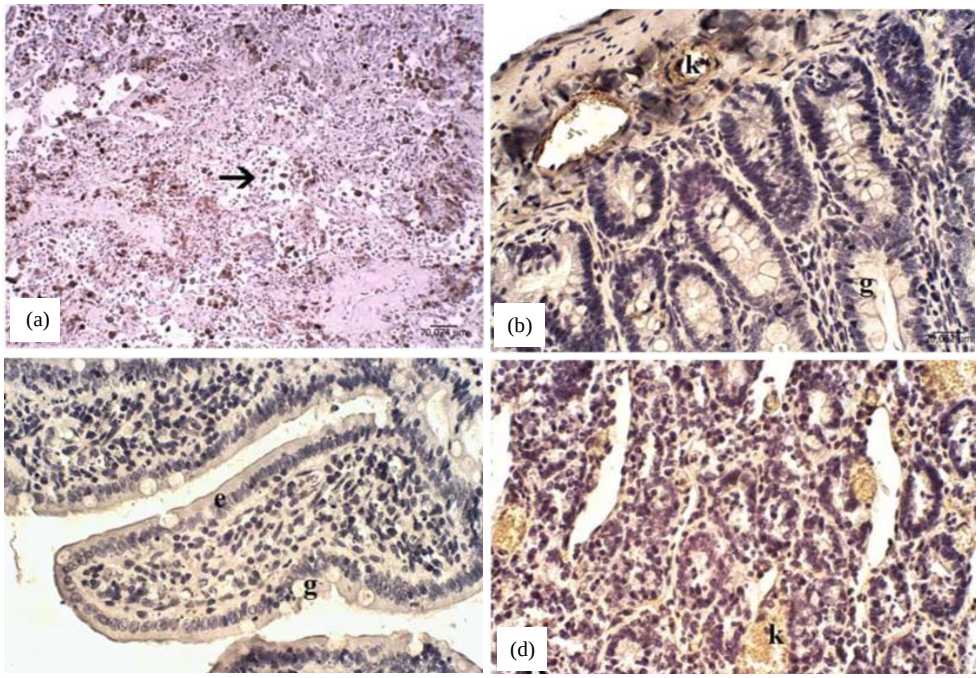

Fig. 2(a-d): Photomicrographs of small intestine for eNOS immunoreactivity. The dark brown dots represent e-NOS-positive, (a) Positive control, esophagus arrow: eNOS immunoreactivity, (b) Control small intestine, g: goblet cell k: capiler, (c) I/R group e: epithelium, g: goblet cell and (d) MESNA group; k: capiler, bar = 20,062 μm

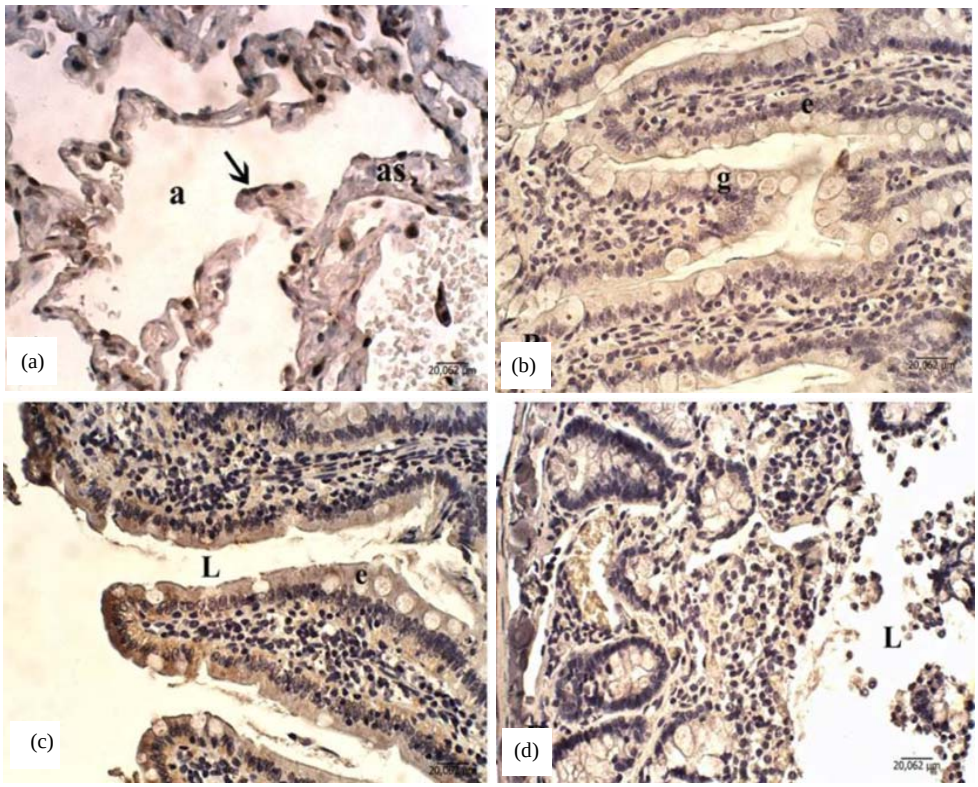

Fig. 3(a-d): Photomicrographs of small intestine for iNOS immunoreactivity. The dark brown dots represent e-NOS-positive, (a) Positive control, lung, arrow: iNOS Immunoreactivity, (b) Control small intestine, e: epithelium, g: goblet cell, (c) I/R group, e: epithelium, L: lumen and (d) MESNA group, L: lumen, bar = 20,062 μm

DISCUSSION

Oxidative stress plays an important role in the intestinal I/R process. In the intestine subjected to I/R, the activated neutrophils induce the production and release of ROS and cytotoxic proteins. The inflammatory cascades start and prolong the radicalic I/R injury[23].

Mesna can be regarded as antioxidant molecules that protect against the toxic effects of free radicals. Some researchers reported that the antioxidant properties of mesna ward against I/R-induced tissue injuries[18,22,27]. When mesna is used for prophylactic purposes, it is reported that hepatocyte regeneration capacity can be sustained by reducing the acute inflammatory response and diminished hepatic tissue injury in the non-ablated liver parenchyma[25]. It has been reported that caspase-3 activity in the spinal cord following ischemia/reperfusion injury decreased indicating a protective effect by a single dose of 150 mg kg^{-1} mesna in rabbits[24]. Besides, it was reported that mesna might scavenge ROS in reperfusion process and reduce reperfusion injury by mesna administrating before 5 min from reperfusion in acute renal ischemia[17]. Ypsilantis *et al.*[29] found that mucoprotective effect of mesna is largely related to its anti-apoptotic activity. Many experimental studies related mesna demonstrated that the efficiency of antioxidant in intestinal I/R injury by scavenging toxic oxygen metabolites[20,27,30].

It was obtained similar protective results in induced I/R injury in small intestine in other related studies which the treatment of other antioxidants. Tunc *et al.*[7] reported a-lipoic acid and N-acetylcysteine and erdosteine to be effective against intestinal I/R injuries in the small intestine tissue of rats. In their study, antioxidants played an important role in reducing intestinal I/R injuries. Remifentanil, an analgesic agent, was reported to protect intestinal epithelial cells from apoptosis when used in intestinal ischemia reperfusion injury[31]. Administration of naringin after I/R in small intestine showed that naringin reduced NO levels in intestine, balanced iNOS and eNOS expression levels and increased arginase activity[32]. It was reported *Parquetina nigrescens*, a flavanoid, might stimulate the defense mechanism against intestinal I/R resulting ROS due to its antioxidant properties and significantly reduced $p<0.05$ MDA levels[33]. Similarly, curcumin,

an antioxidant, reduced MDA level and increased total antioxidant capacity in the small intestine and also remote organs during I/R injury[34]. Prx6, a peroxiredoxin-like protein was reported to be a novel therapeutic agent for intestinal I/R damage due to its antioxidant properties. It was reported Prx6 reduced cellular apoptosis levels and minimize the damage in I/R injury of intestine[35].

This study demonstrated that mesna can effectively protect against severe ischemic damage in the small intestine of rats. In the present study, I/R caused significant increases $p<0.05$ in intestinal MDA, the end-product of lipid peroxidation. Mesna treatment inhibited the increase in MDA. The reason may be to capture the reactive hydroxyl and peroxyl radicals by mesna. These results demonstrated that mesna partially reduced the amounts of MDA and but did not reverse it back to control levels. Thus, mesna has a protective effect against I/R-induced oxidative organ damage by preserving the cellular integrity. Similarly, it has been suggested that mesna may protect tissue from damage caused by cisplatin-treated in ovaries by reducing MDA levels[36].

This study showed that I/R injury of the intestine elevated NO production in the small intestine. Treatment with mesna significantly decreased $p<0.05$ the concentration of intestinal NO levels when compared to control group. The NO levels were correlated with MDA levels. This result is similar to those in other studies that report high levels of NO as a damaging factor responsible for the pathogenesis of the intestine in inflammatory I/R response[37,38].

The NO is an important metabolism molecule in terms of its signaling role in different processes but it also can be protective or damaging in intestinal I/R injuries. The eNOS-derived NO has been reported to have a protective role during the starting phase of I/R in the small intestine. Studies suggested that eNOS can switch to having a "Dysfunctional" role during oxidative stress. For instance, superoxide anions were shown to react with NO released by eNOS, thereby turning into cytotoxic oxidant peroxynitrite[9,37]. A functional change of eNOS may play a role in intestinal I/R. It was reported that mesna has protective effect against IFS induced urothelial damage. Investigators suggest that this protection may be due to decreased production of IL 1β and proinflammatory cytokines in bladder by reducing the expression of iNOS and NO and peroxynitrite production[39]. In the present study, an increase in intestinal eNOS expression was found with 120 min ischemia followed by 120 min reperfusion. These findings showed that intestinal I/R injuries may be related to an increase in eNOS-associated NO production, producing peroxynitrite. However, this study showed that the treatment with mesna led to a decrease in the increased levels of eNOS expression induced by I/R. Therefore, the role of mesna might be to reduce peroxynitrite and cause a reduction in intestinal I/R injuries. These results demonstrated that the pronounced inhibitory effect of mesna on increased levels of eNOS-mediated NO may be the mechanism that accounts for its protective role against intestinal I/R injuries. An organ can be protected by low levels of NO production in the early stages of an injury, whereas increased and prolonged iNOS-mediated NO production during the later stages could result in organ injury or potentiate an injury. The increased expression of iNOS was shown to contribute to I/R injuries in the heart, brain, pancreas, kidney and intestine. Many studies showed that the inhibition of iNOS increases the functional improvement in reperfused kidneys in agreement with reports on other tissues[5,9,37].

iNOS knockout mice displayed significant resistance $p<0.05$ to the impairment of the mucosal barrier and bacterial translocation after intestinal I/R injuries, further supporting the role of iNOS in reperfusion injuries in the intestine[4].

The constitutive forms of NO synthase (nNOS and eNOS) are critical to the normal physiology and inhibition of these enzymes inducing tissue damage, because a considerable amount of NO and peroxynitrite is produced during tissue injury, iNOS inhibition is thought to be beneficial[40]. Cuzzocrea *et al.*[41] provided contrary evidence that the production of peroxynitrite was not related to the induction of iNOS but was associated with the inhibition of eNOS. There is plenty of published arguments that show iNOS enzymatic activity is increased in human Inflammatory Bowel Disease (IBD) and colitis and that this correlates with iNOS expression; iNOS activity is regulated *in vitro* and *in vivo* by enzyme abundance[42]. Ozguven *et al.*[39] demonstrated that ketamine and mesna combination have protective effect against ifosfamide (IFS) induced hemorrhagic cystitis in an experimental model in rats. They found that there was not significant change between iNOS and eNOS expressions IFS administrated groups compared with control in Mesna and ketamine combination[39].

Our results indicated that arginase activity was increased in the I/R group versus the sham-control group. These results are similar to the findings of Horowitz *et al.*[43], who demonstrated that increased arginase activity in IBD submucosal tissues. Recently, the arginase enzyme gained attention in studies focusing on the pathophysiology of intestinal diseases. Gobert *et al.*[44] demonstrated that the arginase enzyme exerts a protective role in the *Citrobacter rodentium* model of colitis. Earlier studies have demonstrated

that an important role of the protected bioavailability of NO is to protect from ischemia-reperfusion injuries. Upregulation of arginase that competes with NOS for their common substrate, L-arginine, may be an important mechanism by which NO bioavailability is reduced during I/R[45].

Limitations in the study include the selection of timing administration and duration of the MESNA, which probably does not correlate with an actual clinical situation because 120 min is too short a time fame to make a diagnosis of mesenteric ischemia. In future studies, different start times of mesna administration relative to the onset of ischemia should be studied. The other issue is about the number of experimental groups of rats. The therapeutic effects of mesna is well known and it is still using as a medicine, so it did not include another control group administered mesna as a vehicle. It was assumed that it is also important for the animal rights according to Helsinki declaration.

It has been observed that mesna was helpful against intestinal I/R injuries because of its antioxidant properties considering the biochemical parameters. This study findings showed that mesna, as another antioxidants and thiol-containing molecule, is a capable agent for protecting intestinal tissue against oxidative damage and preventing intestinal disorders caused by I/R. This study confirmed that I/R stimulates acute intestine injury formation of free radicals and the depletion of antioxidant defense. Our results showed that mesna constitutes protective effects on I/R-induced intestinal injuries, possibly by several mechanisms.

CONCLUSION AND FUTURE RECOMMENDATION

Present study showed that Mesna have a significant protective effect on intestinal I/R injury at 150 mg kg^{-1} single dose and intra peritoneal application procedure. This is the first time it was declared that evaluation of NO level and immunoreactivity of iNOS and eNOS together will make a prominent contribution to related studies. This study underlines the potential therapeutic role of mesna as an antioxidant agent for protection from intestinal I/R injury. Therapeutic effect of mesna should evaluate by detailed further studies with different dosages and alternate time protocols for better understanding the use of clinical applications.

SIGNIFICANCE STATEMENTS

This study discovers treatment of mesna that can protect small intestine from I/R injury by the axis of NO level, arginase activity and iNOS and eNOS immunoreactivity. Present study will help the researcher to provide further data for the argument on the protective and destructive roles of NO, which is already controversial issue. Thus, a new theory on a mechanism of protection by mesna which is carried out by decreasing eNOS level and then reducing NO production may be arrived at.

ACKNOWLEDGMENT

This was a part of study of research project number TUBAP-2010-147 supported Trakya University Foundation of Scientific Research.

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