The effect of colchicine and low-dose methotrexate on intestinal ischemia/reperfusion injury in an experimental model

Özlem Boybeyi a,⁎, Yasemin Dere Gunal a, Pinar Atasoy b, Ucler Kisa c, Mustafa Kemal Aslan a

a Kırkkale University, Medical Faculty, Department of Pediatric Surgery, Kırkkale, Turkey
b Kırkkale University, Medical Faculty, Department of Pathology Kırkkale, Turkey
c Kırkkale University, Medical Faculty, Department of Biochemistry, Kırkkale, Turkey

A R T I C L E   I N F O
Article history:
Received 9 November 2013
Received in revised form 30 December 2013
Accepted 24 January 2014

Key words:
Intestinal ischemia
Ischemia/reperfusion injury
Colchicine
Methotrexate
Experimental

A B S T R A C T
Aim: Intestinal ischemia/reperfusion (I/R) injury is a serious clinical condition. Colchicine and low-dose methotrexate have anti-inflammatory features. An experimental model was conducted to investigate the effect of colchicine and methotrexate on intestinal I/R injury.

Methods: Twenty-four rats were included. Only laparotomy was done in control group (CG, n = 6). In experimental groups, superior mesenteric artery was occluded. After 1 h ischemia, reperfusion (1 h) was started by de-occlusion. 30 min before reperfusion, saline in sham group (SG, n:6), colchicine (1 mg/kg) in colchicine group (CNG, n:6), and methotrexate (0.1 mg/kg) in methotrexate group (MTXG, n:6) were infused intraperitoneally. Small intestines were harvested for evaluation of intestinal mucosal injury (Chiu score) and oxidative stress markers (nitric oxide: NO, malondialdehyde: MDA, superoxide dismutase: SOD).

Results: Biochemically, MDA levels were significantly low in CG compared to SG, CNG, and MTXG (p < 0.05). NO levels were significantly low and SOD levels were significantly high in CG compared to MTXG (p < 0.05). Histopathologically, Chiu score was significantly low in CG compared to SG, CNG, and MTXG (p < 0.05), and significantly high in MTXG compared to SG and CNG (p < 0.05).

Conclusion: The present experimental model caused I/R injury in rat intestines. Contrary to literature, it was found that methotrexate worsens and colchicine does not attenuate intestinal I/R injury.

© 2014 Elsevier Inc. All rights reserved.

Ischemic injury of intestines is a medical and surgical emergency with high mortality rate [1,2]. This serious clinical condition can be a consequence of a mesenteric emboli, midgut volvulus, invagination, necrotizing enterocolitis or small bowel transplantation [1,2].

It causes both structural and functional damage associating with severe intestinal cellular injury. During this process, inflammatory factors and cytotoxic substances are released in intestinal tissue [2–4]. The return of oxygenated blood to the ischemic tissue worsens the injury by release of free oxygen radicals and aggravated inflammatory reactions [2,3]. Therefore, resuscitation of this fatal inflammatory reaction is as important as restoration of the blood flow.

Colchicine (CN) is an anti-inflammatory agent which regulates cytokine production, decreases the tumor necrosis factor-α (TNF-α), and inhibits leukocyte functions [5]. It has been used for several diseases such as gout, familial Mediterranean fever, and Behçet's disease [5]. Recently, it was reported that it has beneficial effects in necrotizing enterocolitis [5]. However, its effect on intestinal ischemia reperfusion (I/R) injury has not been studied previously.

Methotrexate (MTX), a folic acid antagonist, inhibits DNA and RNA synthesis by inhibiting dihydrofolate reductase enzyme [6]. It has been used in treatment of several neoplastic diseases in high doses.

However, in low doses, it has anti-inflammatory effect which allows it to be used in the treatment of some inflammatory diseases such as rheumatoid arthritis, psoriasis, and Crohn's disease [6]. But, its effects on intestinal I/R injury have not been reported so far.

Therefore, we conducted an experimental intestinal I/R model to investigate the effect of CN and MTX on intestinal I/R injury.

1. Materials and methods

The study was approved by the Local Ethical Committee and the experiments were performed under the recommendations of the laboratory animal care committee.

Twenty-four Wistar albino adult rats, weighing 250 ± 50 g were included to the study. The rats were kept in standard cages in 22 °C room temperature and 12 h day/night cycle with tap water and standard food ad libitum.

The rats were randomly divided into 4 groups including six animals in each. In CG, after anesthetization, 2 cm of small intestine 15 cm proximal to ileocecal valve was sampled without any intervention except laparotomy (CG, n: 6). Intestinal ischemia and reperfusion (I/R) were performed in other groups by occluding and

⁎ Corresponding author. Tel.: +90 5057634173.
E-mail address: ozlemboy80@yahoo.com (Ö. Boybeyi).
methotrexate (MTX) in MTX group (MTXG, n: 6) were injected intraperitoneally.

1.1. Experimental protocol

The rats were anesthetized with intraperitoneal ketamine hydrochloride (50 mg/kg, Ketalar, Eczacibasi, Istanbul, Turkey). The rats were kept warm and allowed spontaneous breathing during surgery. The abdominal wall skin was shaved and cleaned with 10% povidone iodine. Then, a midline laparotomy was performed and SMA was exposed (Fig. 1). In experimental groups, SMA was occluded with 0/0 catgut suture. The ischemia was accepted to be achieved when the small intestines became purple in color and the pulse of intestinal arteries stopped. The drugs were not like crystalloid liquid and so were given intraperitoneally to achieve a systemic response without causing venous emboli. Since we planned to give the drugs intraperitoneally before reperfusion, we closed the abdominal wall after occlusion of SMA.

After 1 h of ischemia, the catgut nodes were opened carefully to de-occlude SMA and reperfusion period began. The reperfusion period lasted for 1 h in all experimental group animals. Thirty minutes before reperfusion period, intraperitoneal injections of 1 ml serum physiologic in SG, 1 mg/kg colchicine (Colchicum-Dispert, Dr.F.Frik, Istanbul, Turkey) in CNG, and 0.1 mg/kg MTX (Methorexate, Kocak Farma, Istanbul, Turkey) in MTXG were infused into the peritoneal cavity.

At the end of each procedure, the rats were sacriﬁced by exsanguination. Small intestinal samples (2 cm of small intestine from 15 cm proximal to ileoceacal valve) were harvested in all groups for histopathological and biochemical examinations.

1.2. Biochemical examination

All samples were kept at −80 °C. Tissue was homogenized (Labor Technique, Mülheim, Germany) with 0.9% NaCl solution 1 ml in ice, and then it was centrifuged at 1500 g for 10 min at 4 °C. Supernatants were used for malonyl dialdehyde (MDA), total nitrite/nitrate (NO), superoxide dismutase (SOD) and protein determinations. Protein level was measured using the method of Lowry et al. [8].

1.3. Determination of NO

NO levels were measured by a spectrophotometric method as described by Miranda et al. [9]. Nitrate was reduced to nitrite with vanadium (III). The nitrite level was measured by using Griess reagents which reflect the total amount of nitrate and nitrite in the sample. Standards were accepted as serial dilutions of Na nitrate (Merck, Germany). The results were expressed in μM/mg protein.

1.4. Determination of MDA

MDA levels indicate lipid peroxidation and were measured by the method described by Armstrong and Al-Awadi [10]. The calibration curve was prepared with 1, 1, 3, 3-tetraethoxypropane (Sigma, St Louis, MO) standards of 1- to 25-nmol/L dilutions. The results were expressed in nM/mg protein.

1.5. Determination of SOD

SOD levels were determined by using quantitative enzyme linked immunosorbent assay kit which is commercially available (Cayman Chemical Company). The results were expressed in U/mg protein.

1.6. Histopathological examination

Intestinal samples were ﬁxed with 10% formalin and embedded in parafﬁn. Tissues were sectioned in 4–5 μm pieces. Then they were stained with routine hematoxylin and eosin stain. The specimens were examined under a light microscope (Leica, Germany) by the same pathologist who was blind to the study. Histopathologic ﬁndings were graded according to Chiu scoring system (intestinal mucosal injury score) [11] (Table 1).

1.7. Statistical analyses

Results were analyzed with Statistical Package for the Social Science version 15.0 (SPSS 15.0). All data were expressed as median with inter-quartile ranges. The difference between two groups was evaluated with Kruskal Wallis test. The p values lower than 0.05 were considered as signiﬁcant.

2. Results

The results of biochemical and histopathological examinations are given in Table 2. The median levels of NO were signiﬁcantly low in control group animals (CG) compared to MTX treated ones (MTXG) (p < 0.05). The median levels of MDA were signiﬁcantly low in CG compared to SG, CNG, and MTXG (p < 0.05). The median levels of SOD, indicator of the anti-oxidant activity, were signiﬁcantly high in CG compared to MTXG (p < 0.05). These results suggest that the present intestinal I/R model caused oxidative damage in intestinal tissue, but not more than the one caused by MTX (Fig. 2).

The harvested intestines were fragile, edematous and discolored in experimental groups macroscopically. The samples were examined histopathologically for intestinal injury and graded with Chiu score (Table 2). The Chiu scores were consistent with the macroscopic examination. The scores were signiﬁcantly low in CG compared to SG, CNG, and MTXG (p < 0.05). The Chiu scores of MTXG were signiﬁcantly higher than those of SG and CNG (p < 0.05). The microscopic images of intestines from each group are given in Fig. 3.

Fig. 1. The experimental model. Left: Small intestines and Right: Superior mesenteric artery (SMA) being exposed.
Intestinal ischemia remains both diagnostic and therapeutic challenge for clinicians because of difficulty in early diagnosis and in management of the ongoing inflammatory process [1,3]. Absence of established guidelines for management of intestinal ischemia is another challenge. As a consequence of these, intestinal ischemia reperfusion (I/R) injury, its pathophysiological explanations, and management strategies have been widely investigated so far. The common issue in these studies is the importance of restoration the blood flow of intestines as soon as possible and attenuation of the damage and ongoing inflammatory process occurred due to I/R injury. The investigated strategies were anti-oxidants, anti-inflammatory agents, and many other agents such as infliximab, lazarooids, dexmedetomidine, heparin sodium, arginine, allopurinol and L-NAME [2–4,12–14]. Despite the advance on this issue, there still is no consensus on medical management of this life-threatening clinical condition.

In the present study, intestinal ischemia was achieved by occlusion of superior mesenteric artery (SMA) with a catgut suture differently from the previous studies. We could easily establish intestinal ischemia and reperfusion by this way. The closure of the abdominal wall between ischemic and reperfusion periods was easily secured since there was no space occupying tool – vascular clamp – in the abdomen. So the injections were performed efficiently. The biochemical and histopathological examinations also revealed that I/R model caused intestinal injury in experimental groups. The present I/R model caused mucosal edema, capillary congestion, epithelial shedding from villi and increased cellularity in lamina propria in intestinal wall of samples. These findings were graded with Chiu score and consistent with literature data [2,11].

Colchicine (CN) is a commonly used anti-inflammatory agent [5]. Its anti-inflammatory effect is not fully understood. But it is widely accepted that it regulates cytokine production, such as down-regulation of tumor necrosis factor-α (TNF-α), and inhibits chemo- tactic actions of neutrophils [5]. Yurtutan et al. examined its effect in necrotizing enterocolitis (NEC) [5]. They treated NEC-induced pups with CN and showed favorable effects of CN in NEC [5]. Although CN is known to have gastrointestinal side effects and narrow therapeutic index, Yurtutan et al. did not observe any side effects with CN [5]. Therefore, we used CN in management of experimental I/R injury of intestines. We aimed to evaluate the acute effect of single dose CN in the present study, since intestinal ischemia is an emergent condition. Unfortunately, we could not demonstrate a favorable effect of CN in intestinal I/R injury in the present study. CN did not attenuate the oxidative damage and did not alter the oxidative stress markers in our study. Furthermore, it did not alter the histopathological damage that occurred after intestinal I/R injury. Although long-term treatment with multiple doses of CN was shown to have favorable effects [5], a single dose of CN was shown not to have such an effect in the present study revealing that a single dose of CN might be insufficient to attenuate the I/R injury. In addition, direct effect of CN on un-injured intestines has not been studied so far, and so does in the present study. Therefore, further studies with different doses of CN are needed to examine its effect on normal and injured intestines by I/R injury seperately.
Methotrexate (MTX) is used in low doses for treatment of some inflammatory diseases [6]. Although MTX is well-known to have gastrointestinal side effects [15], a weekly dose of 0.1 to 0.3 mg/kg was shown to have anti-inflammatory effect in previous studies [6]. Therefore, we used MTX with a dose of 0.1 mg/kg in I/R injury-induced rats to evaluate its effect in I/R injury. However, we observed that the macroscopic appearance of the intestine samples in MTX group was disappointing. The biochemical and histopathological results confirmed this observation revealing that MTX worsens I/R injury even in low doses. However, whether this worsening effect of MTX also occurs in normal intestines should be examined in future studies.

In conclusion, I/R injury in rat intestines was easily established by occlusion of superior mesenteric artery with a catgut suture in the present study. A single dose of colchicine did not cause any favorable effect in acute phase of the intestinal I/R injury. Methotrexate worsened the intestinal I/R injury in acute period even in low doses. Although these results are contrary to literature, further studies with different doses, at different time periods of injury, and examining the additive, synergistic or antagonistic effects of these drugs are needed to confirm our results.

Acknowledgment

This study was presented in 31th Congress of Turkish Pediatric Surgeons, in 2013, Eskisehir, Turkey.

Authors thank to Tutku Soyer, M. D. Associate Professor from Hacettepe University, Medical Faculty, Department of Pediatric Surgery, Ankara for the statistical analysis and evaluations of the results.

References