Direct intraperitoneal resuscitation with lidocaine, methylene blue and pentoxiphylline combination does not decreases inflammation after intestinal ischemia-reperfusion injury in rats[1]

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DOI: http://dx.doi.org/10.1590/S0102-865020160050000007

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ABSTRACT

PURPOSE: To evaluate the effects of an intraperitoneal solution of methylene blue (MB), lidocaine and pentoxyphylline (PTX) on intestinal ischemic and reperfusion injury

METHODS: Superior mesenteric artery was isolated and clamped in 36 adult male Sprague Dawley rats. After 60 minutes, clamp was removed and a group received intraperitoneally UNITO solution (PTX 25mg/kg + lidocaine 5mg/kg + MB 2mg/kg), while the other group was treated with warm 0.9% NaCl solution. Rats were euthanized 45 min after drug administration. Lung and bowel were collected for histological evaluation (using Park's score) and determination of myeloperoxidase (MPO) and malondialdehyde (MDA) levels.

RESULTS: Control samples showed lymphoplasmocytic infiltrate and crypt necrosis of villi. MPO and MDA measurements shown no differences between treated and control groups.

CONCLUSION: The combination of lidocaine, methylene blue and pentoxyphylline administered intraperitoneally at the studied dose, did not decreased histological lesion scores and biochemical markers levels in intestinal ischemia/reperfusion injury.

Key words: Ischemia. Reperfusion. Lung. Histology. Rats.

Introduction

Intestinal I/R injury is a common pathologic event associated with many intestinal pathologies, whenever the intestine undergoes a reduction with subsequent restoration (usually during surgery) of blood flow[1-5]. Spontaneous I/R can occur in humans and if the diagnosis is made within 24h after the onset of symptoms and aggressive treatment initiated, acute mesenteric ischemia has about a 50% survival rate, whereas this rate drops to 30% or less when diagnosis is delayed[6]. In experimental models, it has been shown that the extent of mucosal damage is a direct function of time elapsed from the onset of mesenteric artery occlusion with first histological changes after 30 min and more prominent destruction of the villi after 60 min[7]. After revascularization, mucosal regeneration via cell migration occurs rapidly, even after 90 min of ischemia[8].

Several authors investigated the I/R phenomenon and tried therapeutic approaches but in the majority of these it was not possible to identify a single compound completely capable to prevent and solve I/R damages, but the combination of some drugs seemed to suggest a synergic effect that enhance protection, acting on different phases of this process[5].

Pentoxifylline (PTX), a methylxanthine derivative known for many years for its rheological properties, has proven to be a potent inhibitor of tumor necrosis factor (TNF) production. It has been suggested that PTX can enhance the chemotactic response of neutrophils, but may inhibit phagocytosis and the superoxide production by neutrophils and monocytes. It has also been shown that this drug has beneficial effects in patients with lung I/R injury. The potential local effects are important for acute lung injury manifestation by inflammatory mediators that are blood- and lymph-borne, and oxygen-free radical activations[10].

Methylene blue (MB) is an inexpensive drug used to treat diseases such as methaemoglobinemia and Alzheimer's and has been proposed for use in vasoplegic shock during sepsis, cardiopulmonary bypass surgery and liver transplantation. However, MB is known to enhance key biochemical pathways in mithocondria, and cycling between oxidized and reduced MB forms might block oxidant production[11].

Lidocaine is widely used as a local anesthetic, but has been recently administered systemically in horses to treat post operative ileus. It is known that local anesthetic agents modulate the inflammatory response via mechanisms unrelated to sodium channel blockade. Lidocaine has been shown to reduce cytokines release and inhibit neutrophil function. Additionally, in studies evaluating its effect on I/R injury in other organs, lidocaine has been shown to reduce lipid peroxidation attributable to oxidant release and inhibit neutrophil adhesion and migration[12]. It has been also demonstrated to inhibit neutrophils migration[13].

Taking into consideration the previous studies, it was hypothesized that a mixture of pentoxifylline, lidocaine and methylene blue could be useful in prevention and treatment of intestinal I/R injury. For this purpose we used an animal model of intestinal I/R in which the superior mesenteric artery occlusion-reperfusion simulates the I/R injury that may occur in clinical situations, and investigated the effects of the combination of drugs on bowel and lungs.

Methods

The study was approved by the Bioethical committee of the University of Turin and by the Italian Ministry of Health.

Thirty-six Sprague-Dawley adult male rats, body weights of 300 grams (270-310 grams), were kept in standard conditions and fed standard rat chow for 5 days before the experiment. Twelve hours before surgery they were fasted but water allowed ad libitum. Anesthesia was induced by a single intramuscular injection of xylazine (0.025 ml/kg) and ketamine (25 mg/rat), and the abdomen shaved and aseptically prepared with 10% povidone iodine. Upon a 2.5-cm midline laparotomy, the superior mesenteric artery was isolated at its origin and occluded with an atraumatic microvascular clamp for 60 min. To ensure a complete ischemia, collateral arteries were blocked using an encircling ligature with Prolene USP 4-0 suture. At the end of ischemia period the clamp and ligatures were removed and reperfusion was confirmed by restoration of the pulsations and the normal gut color. The animals were randomly assigned with a random calculator (www.random.org) to two groups: treated (T) and control (C). In group T, 18 rats were treated with 1 ml of the solution (UNITO solution) composed by PTX (25mg/kg), lidocaine (5 mg/kg) and MB (6 mg/kg) diluted in warm 0.9% NaCl solution administered intraperitoneally. In group C, 18 rats were administered 1 ml of warm 0.9% NaCl solution intraperitoneally. Experiments were made in 6 different sessions involving 3 rats per each group. After 45 min of reperfusion, rats were euthanized by cervical vertebrae dislocation.

Samples collection

Lungs and bowel samples were collected and stored in formalin or at -80°C for biochemical analysis.*Histology*

Samples were fixed in buffered formalin, embedded in paraffin, and 4-µm tissue sections were stained with hematoxylin-eosin (HE). The slides were independently evaluated by two histopathologists blinded to the group assignation. To evaluate tissue damages, Park's score was used[14] (Chart 1).

CHART 1 - Description of the histological scoring system after Park and Chiu[8].

0	Normal mucosa
1	Subepithelial space at villus tips
2	Extension of subepithelial space with moderate lifting
3	Massive lifting down sides of villi, some denuded tips
4	Denuded villi, dilated capillaries
5	Disintegration of lamina propria
6	Crypt layer injury
7	Transmucosal infarction
8	Transmural infarction

Biochemical analysis

The biochemical analysis consisted in myeloperoxidase (MPO) and malondialdehyde (MDA) activity evaluation according, respectively, to the methods proposed by Muià *et al*.[15] and Hei et *al*.[16], but introducing some minor modifications.

MPO

MPO activity was measured both in lung and intestine. Aliquots of 500 mg of frozen tissues were homogenated in potassium phosphate buffer (PPB) (10 mM and pH 7) and 0.5% (w/v) hexadecyltrimethyl-ammonium bromide (CTAB, Sigma Aldrich, Italy), and centrifuged at 20.000 x *g* for 30 min at 4°C. Fifty µL of surnatant were incubated with 950 µL of tetramethylbenzidine (TMB plus, Kem-EN-Ten Diagostic, Denmark) and rate of change in absorbance was measured at 650 nm.

MDA

MDA content was measured in intestine samples. Tissues homogenate (50 µL) were incubated with 50 µL 8.1% sodium dodecyl sulfate (SDS, Sigma Aldrich, Italy), 400 µL acetic acid buffer (Sigma Aldrich, Italy), 400 µL 0.8% thiobarbituric acid (TBA, Sigma Aldrich, Italy) and 100 µL distilled water.

Aliquots of of 1,1,3,3-tetraethoxypropane (Sigma Aldrich, Italy) were used as positive control.

Then all the tubes were incubated at 95°C for 1 h. After cooled at -20 for 5 min, 1 mL n-butyl alcohol (Sigma Aldrich, Italy) was added into the sample, which was centrifuged for 10 min at 800 x *g*. The supernatant of the samples was taken to detect absorbance at 533 nm with spectrophotometer. To determine the concentration we used a molar extinction coefficient for MDA of 1.56×10^5 $cm^{-1}M^{-1}$. Results were expressed as nmol MDA/100mg protein[17].

Statistical analysis

All data are presented as mean ± standard error (SEM). Normality of data was assessed with the Kolmogornov-Smirnoff test. Differences for histological score comparison were analyzed with a Mann Whitney test. Unpaired T-test was performed to analyze biochemical results. Statistical analyses were performed by GraphPad InStat (vers. 3.05) statistical software (GraphPad Inc.). The Grubbs test was used to reveal potential outliers, which were excluded. A value of $p<0.05$ was considered to be statistically significant.

Results

During the experiment, two rats of treated group died during recovery from anesthesia.

Histology

Histological score for the control group was 3.4 ± 0.6817 while in the treated group was 2.47 ± 0.6537. The difference was not statistically significant (p=0.3242).

Malondialdehyde level in the intestine was not significantly different (p=0.2884) between control (88.3 ± 27.234 nmol/100mg) and treated I/R group (80.28 ± 14.802 nmol/100 mg).

The MPO activity in the intestine did not differ significantly (p=0.6834) between treated (1.156±0.35 mU/g) and control group (1.349±0.39 mU/g).

The MPO activity in the lung was not significantly different (p=0.5686) between control (3.06±0.497 mU/g) and treated I/R group (3.235±0.583 mU/g).

Discussion

The results of the present study highlighted that the UNITO solution (mixture of PXT, MB and lidocaine) is not

effective against I/R injuries when administered intraperitoneally. Analysis of the data showed that MPO and MDA increased in all the tested samples without significant differences between groups.

In the authors' best knowledge, this was the first time that this combination was used. The authors' hypothesis was that these substances could have a synergic effect on I/R bowel and lung injuries. This hypothesis was supported by the bibliography that showed a huge number of papers dealing with the experimental success of single molecule trial, suggesting a further clinical application.

The study of Marqui *et al.*[10] demonstrated that pentoxifylline has preventive effects and therapeutic potential in I/R-induced lung injuries, but the same authors are carefull to suggest a direct application while the clinical relevance of that manuscript referred to the use of pentoxifylline in situations requiring procedures of ischemia with reperfusion to reduce or prevent distant organs damage. The study of Seifi *et al.*[18] showed that PTX and N- Acetilcysteine (NAC) administered intraperitoneally protected kidney tissue against oxidative damage caused by experimental induced hepatic I/R. Kalay *et al*.[19] and Lloris Carsi *et al*.[1] demonstrated the beneficial effects of intraperitoneally administered pentoxyphilline on intestinal injury after ischemic and reperfusion event in an *in vivo* animal model.

The study of Salehi *et al*.[20] demonstrate that a treatment with a amino acid-based (AA) solution could reduce neutrophil infiltration in tissues reperfused after 30 min of ischemic event, indicating the possibility of lower graft immunogenicity over this time frame. Presumably, reduced neutrophil localization implies a decrease of chemotactic factors and influencing reduction of neutrophils attraction to the site of injury. Although diminished neutrophil recruitment supports the link between I/R injury and inflammatory response, additional adjunctive therapies involving anti-inflammatory agents seem to be required to fully protect the injured mucosa during inflammatory attack following I/R. The study of Berger *et al*.[13] demonstrated that systemic administration of lidocaine arrest transmigration of neutrophils through endothelial cells and the results obtained by Elhakim *et al*.[21] demonstrated that intraperitoneal lidocaine enhance bowel recovery after surgery.

First in 1988, Kelner *et al*.[22], reviewing the mechanisms of action of methylene blue, observed that it could be used to inhibit the production of superoxides in ischemic tissues. For the same reason this molecule was chosen in other experiments[23-26] and in the present study. On the other side, the study of Miranda *et al*.[27] demonstrated that MB infusion had not beneficial effect on the healing of intestinal anastomoses in an experimental I/R model in rats.

Seen these previous works we hypothesized that a combination of these molecules could have a synergic effect on different aspects of I/R injuries in intestines and lungs. The scarce efficacy of the mixture could be due to different causes.

First, the dosage of each component in the mixture has been extrapolated from previous literature. By testing other doses of each components, results could be different.

Second, the intraperitoneal administration may be effective for some of the components, but not for others. Lidocaine, for instance, has been extensively tested for intravenous administration, and very little for intraperitoneal administration. Although intraperitoneal absorption can be comparable to intravenous absorption for some drugs, this could be not the case for lidocaine.

Third, I/R injury involves complex pathways and many of these could not be targeted by the intraperitoneal route. Last possibility is that instead of having a synergic effect, the combination of these molecules has a counter effective effect on the efficacy of all or some of them.

Further studies should be conducted to evaluate these limits, considering different dosages and different routes of administration.

Conclusion

The combination of lidocaine, methylene blue and pentoxyphylline administered intraperitoneally at the dose hereby presented, did not significantly decreased histological lesion scores and biochemical markers levels in intestinal ischemia/reperfusion injury.

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Received: Jan 22, 2016
Review: Mar 19, 2016
Accepted: Apr 18, 2016
Conflict of interest: none
Financial source: none

[1]Research performed at Department of Veterinary Sciences, University of Turin, Italy.