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Effects of Doxycycline on Intestinal Ischemia Reperfusion Injury Induced by Abdominal Compartment Syndrome in a Rat Model

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ABSTRACT

BACKGROUND: Abdominal compartment syndrome (ACS) refers to organ dysfunction and ischemia resulting from intra-abdominal hypertension (IAH). Ischemia of the gut results in the triggering of a systemic inflammatory response by releasing cytokines which, in turn, causes capillary leakage leading to bowel edema, further increasing intra-abdominal pressure and resulting in a morbid cycle of ischemia and edema.

OBJECTIVE: The aim of this study was to determine the effects of doxycycline on intestinal ischemia reperfusion (I/R) injury in a rat model of ACS.

METHODS: Sprague-Dawley rats were divided into 5 equal groups. In groups 1 and 2, saline (1 cc IP) was administered during induction of ACS and intestinal samples were removed at 1 and 24 hours, respectively, after decompression. In groups 3 and 4, doxycycline (10 mg/kg IP) was injected during induction of ACS and, similarly, intestinal samples were removed at 1 and 24 hours after decompression. In the control group (group 5), intestinal samples were collected without induction of ACS. Malondialdehyde (MDA), interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, matrix metalloproteinase-2 (MMP-2), and tissue inhibitor of metalloproteinase-1 were studied and the apoptotic cells were enumerated histopathologically. Apoptosis and β-cell lymphoma 2 (βcl-2) expression were assessed immunohistochemically.

RESULTS: Thirty-five rats were evenly divided into 5 groups of 7 rats each. MDA, IL-1β, IL-6, TNF-α, and MMP-2 levels were significantly higher in group 1 one hour after the reperfusion period compared with the control group ($P < 0.001$, $P < 0.001$, $P < 0.05$, $P < 0.001$, and $P < 0.01$, respectively). The same parameters were significantly lower in group 3, in which doxycycline was administered, than in group 1 ($P < 0.001$, $P < 0.05$, $P < 0.05$, $P < 0.001$, and $P < 0.01$, respectively). However, there

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was no significant difference between groups 2 and 4 in the 24th hour (all, $P > 0.05$). The mean (SD) number of apoptotic cells and the expression of βcl-2 was highest in group 2 at 24 hours after the reperfusion period (92.5 [11.4] and 35.9 [5.0], respectively) and significantly greater than that in group 4 ($P < 0.001$ and $P < 0.05$, respectively).

CONCLUSION: Doxycycline was associated with protective effects against I/R injury through decreasing apoptosis via attenuating the response of proinflammatory cytokines and inhibiting the activity of MMP-2 in this rat model. (*Curr Ther Res Clin Exp*. 2010;71:186–198) © 2010 Excerpta Medica Inc.

KEY WORDS: abdominal compartment syndrome, intestinal ischemia-reperfusion injury, doxycycline, apoptosis.

INTRODUCTION

The pressure within the abdominal cavity is normally atmospheric or subatmospheric and intra-abdominal hypertension (IAH) affects each organ system separately at different levels of pressure.[1] Abdominal compartment syndrome (ACS) refers to organ dysfunction and ischemia resulting from IAH. The gut is the organ most sensitive to IAH and Diebel at al[2] found that mesenteric and intestinal mucosal flow was first reduced at 20 mm Hg before the renal, pulmonary, and cardiovascular systems were impaired.

Ischemia of the gut results in the triggering of a systemic inflammatory response by releasing cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6, which, in turn, causes capillary leakage leading to bowel edema, further increasing intra-abdominal pressure (IAP), resulting in a morbid cycle of ischemia and edema.[3]

Increased IAP reduces blood flow to intra-abdominal organs and decompression may cause other serious problems including ischemia/reperfusion (I/R) injury.[4–6] It is known that reperfusion of the ischemic tissue may promote the generation of various reactive oxygen metabolites (eg, superoxide radicals, hydrogen peroxide, and hydroxyl free radicals) which have deleterious effects on cell membranes by mediating lipid peroxidation.[5,7] Besides their direct-damaging effects on tissues, free radicals may trigger the accumulation of leukocytes in the tissue involved.[4,5] It has been found that activated neutrophils secrete enzymes (eg, myeloperoxidase, elastase, proteases) and liberate even more oxygen radicals further promoting inflammation and increasing levels of TNF-α, IL-1, IL-6, and malondialdehyde (MDA), an index of lipid peroxidation.[4,5] All these processes lead to apoptosis, a crucial mechanism of I/R injury.[8]

Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade the extracellular matrix and play important roles in inflammation, and neoplastic invasion and metastasis.[9] MMPs are activated in I/R injury in the lung, heart, brain,[9] kidney,[10] and intestine.[11,12] Ubiquitous tissue inhibitor of metalloproteinases (TIMPs) can interfere with MMP proteolytic activation and enzymatic activity.[9] MMP expression via immune cells is modulated by inflammatory mediators, such as TNF-α and IL-1β,[13] and the precursors of these cytokines may be processed into biologically active forms by MMPs.[14,15]

Doxycycline belongs to the family of tetracycline antibiotics and inhibits bacterial protein synthesis.[16] Doxycycline induces the degradation of the pro-MMP zymogen or inhibits the transcription of MMP mRNAs[16] and may also reduce apoptosis associated with β-cell lymphoma 2 (βcl-2) by inhibiting MMP-2 activity.[17] Doxycycline has anti-inflammatory effects through lowering the expression of the genes encoding the proinflammatory cytokines, TNF-α and IL-1β.[18] It may also up-regulate TIMP-1 expression.[19] All of these features of doxycycline may have protective roles in I/R injury.

This study was designed to determine whether doxycycline has protective effects on the ACS, the mechanisms by which it facilitates these effects, and whether it has any possible relationship with apoptosis.

MATERIALS AND METHODS

Sprague-Dawley rats of either sex, weighing 225 to 250 g, were used in the present study. The rats were kept in individual cages and acclimated for ≥7 days before study initiation. All animals were kept in a temperature-controlled room (22±2°C) with a 12-hour light-dark cycle with free access to water and standard laboratory chow. To induce ACS, the rats were anesthetized with ketamine (ketamine IM) and a catheter connected to a laparoscopic insufflator (Storz, Tuttlingen, Germany) was inserted intraperitoneally. IAP of 20 mm Hg was maintained by insufflating with carbon dioxide gas for 60 minutes.

GROUPS

Rats were divided evenly into 5 groups. In groups 1 and 2, saline (1 cc IP) was administered during induction of ACS and intestinal samples were removed 1 and 24 hours, respectively, after decompression. In groups 3 and 4, doxycycline (10 mg/kg IP) was injected during induction of ACS and, similarly, all tissue samples were collected 1 and 24 hours after decompression. At the end of the reperfusion period, all animals were euthanized via decapitation prior to excision. Starting 2 cm distal to the ligament of Treitz, 5 cm of jejunum were excised and were homogenized (Ultra Turrax T18 basic homogenizer, IKA Works Inc., Wilmington, North Carolina) in 0.1 M phosphate buffer (pH = 7.4). The homogenates were centrifuged at 8000 rpm at 4°C for 10 minutes and the supernatants were removed to measure TNF-α, IL-1β, IL-6, MMP-2, and TIMP-1 levels using a commercially available ELISA kit (R&D Systems Inc., Minneapolis, Minnesota) and to analyze MDA levels via HPLC (Agilent 1100 Series, Agilent Technologies, Palo Alto, California) using commercial kits (Chromsystems Diagnostics, Munich, Germany). The results were expressed as nmol/mg protein. Protein concentrations of samples were spectrophotometrically estimated with commercial kits purchased from Fluka (Buchs, Switzerland). In the control group (group 5), rats were anesthetized similarly but the inserted catheters were not inflated and intestinal samples were collected 60 minutes after the induction of anesthesia.

Tissue specimens were fixed in 10% neutral buffered formalin for 18 to 24 hours according to routine procedures and embedded in paraffin. Slides were prepared from 4- to 5-μm tissue sections according to standard surgical pathology procedures. The tissue sections were stained immunohistochemically with anti–βcl-2 and the apoptotic

cells were identified using a commercially available apoptosis detection kit (Apoptag Peroxidase In Situ Apoptosis Detection Kit, Chemicon, California). The counting of apoptotic and βcl-2$^+$ cells on 6 different villi in each microscopic slide was performed by the same investigator blinded to animal group, under conventional light microscopy at a magnification of ×40.

All experimental protocols were approved by the Eskisehir Osmangazi University School of Medicine Animal Care and Use Committee.

Statistical Analysis

Statistical analyses were performed using SPSS 10.0 for Windows (SPSS, Inc., Chicago, Illinois). The results were analyzed by Kruskal-Wallis 1-way ANOVA with Tukey test for TNF-α, IL-1β, IL-6 and 1-way ANOVA with the Fisher least significant difference method for MDA, MMP-2, and TIMP-1. The histology data were analyzed by 1-way ANOVA with post hoc Scheffé tests. $P < 0.05$ was considered statistically significant.

RESULTS

TNF-α, IL-1β, and IL-6 levels were significantly higher in group 1 one hour after the reperfusion period following ischemia compared with the control group (TNF-α [$P < 0.001$], IL-1β [$P < 0.001$], and IL-6 [$P < 0.05$]) (**Table I**); and were significantly lower in group 3, in which doxycycline was administered, than in group 1 ($P < 0.001$, $P < 0.05$, and $P < 0.05$, respectively). On the other hand, 24 hours after reperfusion, no significant difference in cytokine levels was observed between groups 2 and 4 (**Figure 1**). The MDA levels were significantly higher 1 hour after the reperfusion period in group 1 compared with the control group ($P < 0.001$). MDA levels in group 3, in which doxycycline was administered, were significantly lower than that in group 1 ($P < 0.001$). The difference between the MDA levels at 1 hour after reperfusion was not observed at 24 hours (**Figure 2**).

MMP-2 levels were significantly higher in group 1 one hour after the reperfusion period compared with the control group ($P < 0.01$). In group 3, the MMP-2 level was significantly lower than that in group 1 ($P < 0.01$). At 24 hours after reperfusion, there was no significant difference between the groups administered saline (group 2) and doxycycline (group 4) ($P > 0.05$). However, the TIMP-1 levels were significantly higher in the groups administered doxycycline (groups 3 and 4) than the other groups (all, $P < 0.05$) (**Figure 2**).

Under the microscope, the immunohistochemically stained tissue samples revealed that there were only a few βcl-2$^+$ cells in the control group, whereas a statistically increased number of βcl-2$^+$ cells were noted in groups 1 and 3. The highest number of βcl-2$^+$ cells was observed in group 2 ($P < 0.01$). In group 4, there remained only a small number of βcl-2$^+$ cells and the difference between groups 2 and 4 was statistically significant ($P < 0.05$) (**Figure 3A–3D**, **Table II**). Similarly, the number of apoptotic cells observed in group 5 was significantly less than all of the other groups (groups 1, 3, 4 [$P < 0.05$]; and group 2 [$P < 0.001$]). The number of apoptotic cells was significantly higher in group 2 than in all of the other groups ($P < 0.001$) (**Figure 4A–4D**, **Table III**).

Table I. Cytokine levels at 1 and 24 hours in rats with ischemia/reperfusion injury induced by abdominal compartment syndrome (ACS) (N = 35).

Group	TNF-α, ng/g protein	IL-1β, ng/g protein	IL-6, ng/g protein	MDA, nmol/g protein	MMP-2, nmol/g protein	TIMP-1, nmol/g protein
1*	31,461 (3951)	43,229 (4939)	43,217 (9173)	2,585,771 (192,443)	2,169,266 (257,164)	568,121 (82,627)
2†	10,883 (1786)	35,933 (7409)	20,556 (4518)	1,888,933 (228,605)	1,375,253 (89,891)	670,484 (95,458)
3‡	15,704 (3359)	23,511 (6009)	10,592 (2709)	1,341,834 (157,615)	1,406,769 (143,503)	1,072,904 (185,011)
4§	9536 (1723)	20,654 (4970)	11,518 (4,236)	1,437,021 (188,479)	1,226,639 (159,843)	1,279,233 (283,456)
5‖	9156 (1742)	7135 (884)	9804 (3708)	1,330,364 (115,597)	1,006,842 (135,493)	459,633 (100,096)

TNF = tumor necrosis factor; IL = interleukin; MDA = malondialdehyde; MMP = matrix metalloproteinases; TIMP = tissue inhibitor of metalloproteinase.

*Saline (1 cc IP) administered during induction of ACS; intestinal samples removed at 1 hour after reperfusion.

† Saline (1 cc IP) administered during induction of ACS; intestinal samples removed at 24 hours after reperfusion.

‡ Doxycycline (10 mg/kg) administered during induction of ACS; intestinal samples removed at 1 hour after reperfusion.

§ Doxycycline (10 mg/kg) administered during induction of ACS; intestinal samples removed at 24 hours after reperfusion.

‖ The inserted catheters were not inflated and nothing was administered; intestinal samples were collected 60 minutes after induction of anesthesia.

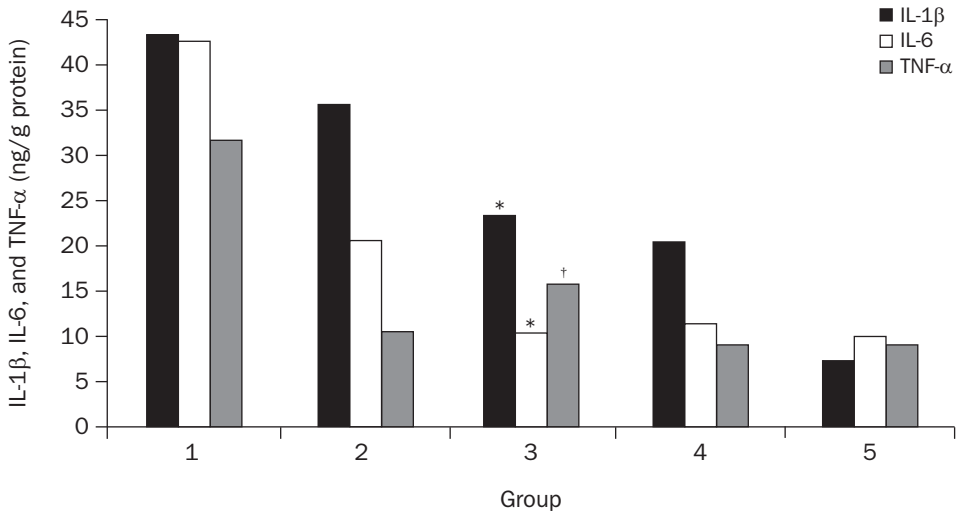

Figure 1. The effect of doxycycline on intestinal tissue interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α levels at 24 hours after ischemia/reperfusion injury due to abdominal compartment syndrome in a rat model. *$P < 0.05$ versus group 1; †$P < 0.001$ versus group 1.

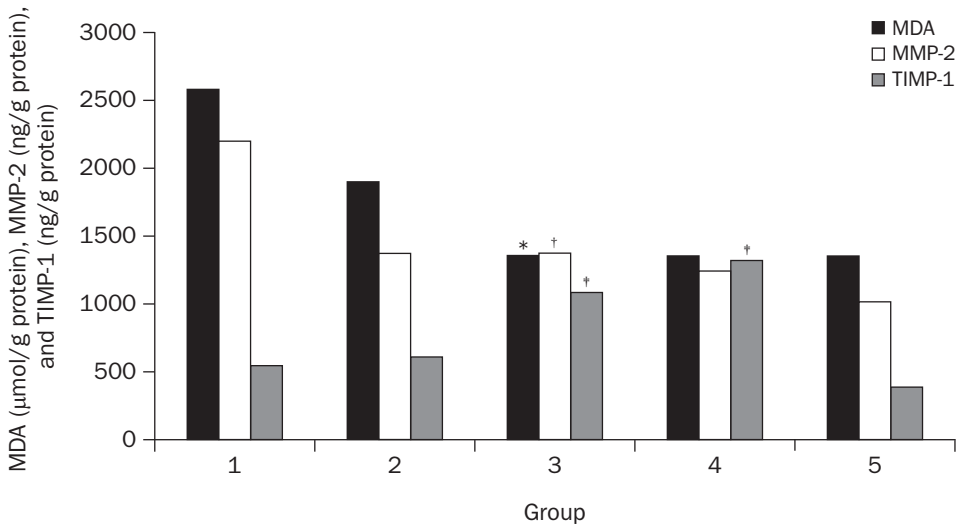

Figure 2. The effect of doxycycline on intestinal tissue malondialdehyde (MDA), matrix metalloproteinase-2 (MMP-2), and tissue inhibitor of metalloproteinase-1 (TIMP-1) levels at 1 hour after ischemia/reperfusion injury due to abdominal compartment syndrome in a rat model. *$P < 0.001$ versus group 1; †$P < 0.01$ versus group 1; ‡$P < 0.05$ versus groups 1, 2, and 5.

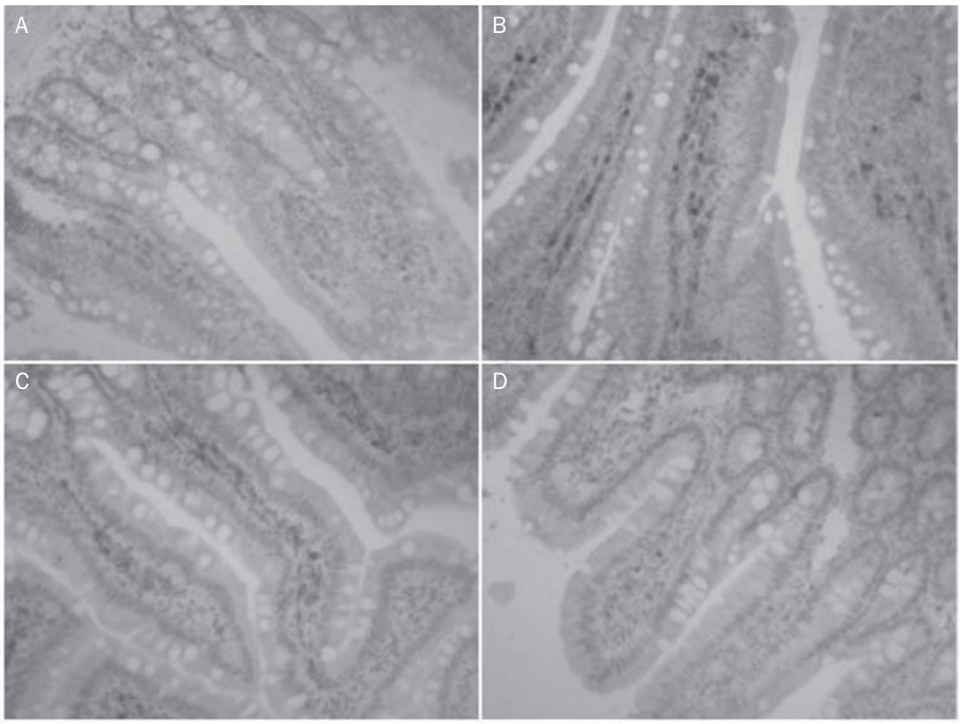

Figure 3. β-cell lymphoma 2 positive cells in rats in (A) group 1 (1 cc of saline administered IP during induction of abdominal compartment syndrome [ACS] and intestinal tissue removed 1 hour after decompression); (B) group 2 (1 cc of saline administered IP during induction of ACS and intestinal tissue removed 24 hours after decompression); (C) group 3 (10 mg/kg of doxycycline administered IP during the induction of ACS and intestinal tissue removed 1 hour after decompression); (D) group 4 (10 mg/kg of doxycycline injected IP during induction of ACS and intestinal tissue removed 24 hours after decompression).

DISCUSSION

The protective effects of doxycycline and its derivative, minocycline, against I/R injury and oxidative stress have been previously studied on different types of organs[20–23] including intestine.[24] However, in the present study, it was observed that doxycycline was associated with protective effects on I/R injuries of intestine induced by ACS. Several possible mechanisms of action were found including attenuation of the response of proinflammatory cytokines and MMPs, decreased βcl-2 expression, and inhibition of apoptosis.

In the present study, IAP was maintained at 20 mm Hg for 1- and 24-hour reperfusion periods to maintain the I/R injury as Diebel et al manifested.[2]

Tissue MDA levels are assayed for products of lipid peroxidation as an indirect index of reactive oxygen metabolites and a biochemical marker of tissue I/R injury.[5,7]

Table II. The number of β-cell lymphoma 2 positive cells in rats with intestinalischemia/reperfusion injury induced by abdominal compartment syndrome (ACS) (N = 35).

Group (n = 7 each)	Mean (SD)	Range
1*	25.23 (4.86)	18–33
2†	35.88 (5.02)	28–43
3‡	24.25 (3.95)	20–29
4§	14.88 (2.97)	9–20
5‖	8.63 (2.73)	4–11

*Saline (1 cc IP) administered during induction of ACS; intestinal samples removed at 1 hour after reperfusion.
† Saline (1 cc IP) administered during induction of ACS; intestinal samples removed at 24 hours after reperfusion.
‡ Doxycycline (1 cc IP) administered during induction of ACS; intestinal samples removed at 1 hour after reperfusion.
§ Doxycycline (1 cc IP) administered during induction of ACS; intestinal samples removed at 24 hours after reperfusion.
‖ The inserted catheters were not inflated and nothing was administered; intestinal samples were collected 60 minutes after induction of anesthesia.

In the present study, the MDA levels were significantly higher after the 1-hour reperfusion period in group 1 compared with the control group, whereas it was lower in group 3, in which doxycycline was administered. In groups 2 and 4, the MDA levels were significantly lower compared with group 1, but there was no significant difference between these 2 groups. Previous studies[6,25] also found increased intestine MDA levels in rodent models of ACS, in which IAP was maintained at 20 mm Hg for 1 hour; in the present study doxycycline appeared to block this tendency in group 3. However, we found that the MDA levels decreased spontaneously following 24 hours of reperfusion and there remained no difference between the groups administered saline and doxycycline.

Tissue I/R injury activates cascades which up-regulate cytokines (TNF-α, IL-1β, and IL-6), and has an important role in the initiation of systemic inflammatory reaction. Spanos et al[26] reported that the content of cytokines, including TNF-α, IL-1β, and IL-6, were increased significantly after 2 hours of ischemia and 2 hours of reperfusion in the intestine of male pigs (all, $P < 0.05$). Similarly, Di Paola et al[27] reported that the tissue expression of TNF-α and IL-1β was increased after 45 minutes of ischemia and 1 hour of reperfusion in the intestine of rats ($P < 0.01$). In the present study, to better understand the possible effects of doxycycline on I/R injury, the TNF-α, IL-1β, and IL-6 levels were measured. The levels were all significantly higher in group 1 compared with the control group. However, the levels were significantly lower in group 3 than in group 1. It has been reported that doxycycline exhibits anti-inflammatory effects in different tissues by inhibiting proinflammatory cytokines.[28]

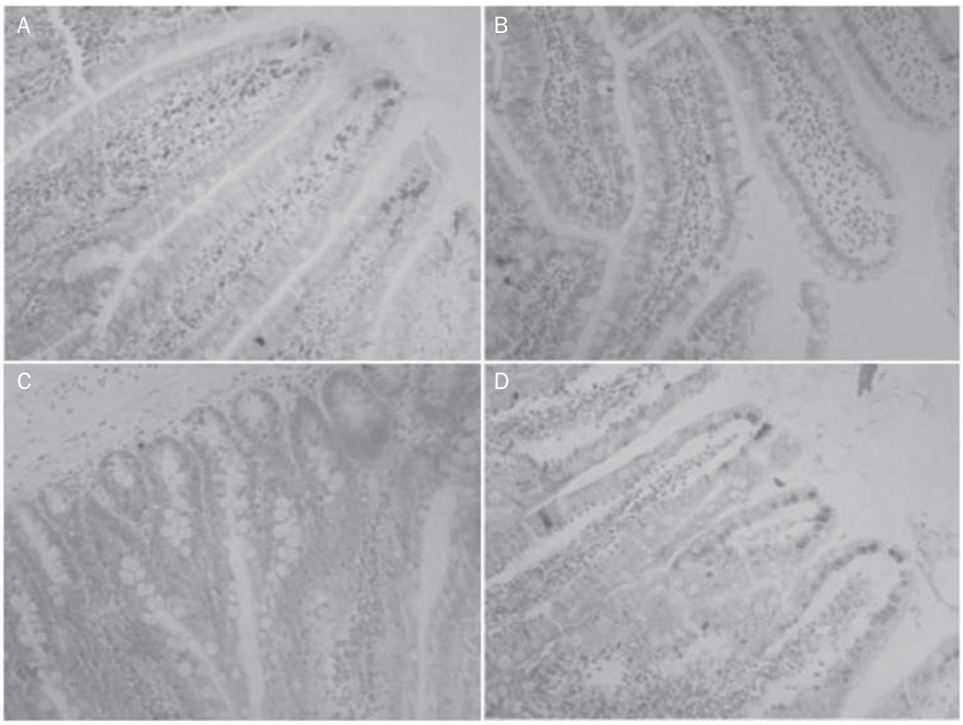

Figure 4. Apoptotic cells in rats in (A) group 1 (1 cc of saline administered IP during induction of abdominal compartment syndrome [ACS] and intestinal tissue removed 1 hour after decompression); (B) group 2 (1 cc of saline administered IP during induction of ACS and intestinal tissue removed 24 hours after decompression); (C) group 3 (10 mg/kg of doxycycline administered IP during induction of ACS and intestinal tissue removed 1 hour after decompression); (D) group 4 (10 mg/kg of doxycycline injected IP during induction of ACS and intestinal tissue removed 24 hours after decompression).

According to the experimental study of De Paiva et al,[29] doxycycline suppressed inflammatory cytokines (IL-1β, TNF-α) in the corneal epithelium in dry eye in mice ($P < 0.05$ and $P < 0.01$, respectively). Kirkwood et al[30] reported that doxycycline had potentially beneficial therapeutic effects in the prevention and treatment of metabolic bone diseases by modulating osteoblast and osteoclast activities through the inhibition of IL-1β–induced IL-6 secretion.

Doxycycline was associated with improved recovery from I/R injury through attenuating the response of cytokines after a 1-hour reperfusion period; however, it did not appear to be associated with the spontaneous decrease observed after 24 hours.

MMPs are proteolytic enzymes that belong to a family of zinc-dependent enzymes that share a zinc-binding catalytic domain and are classified as collagenases (MMP-11, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), and stromelysins (MMP-3) on the basis of the substrate specificity of individual MMPs. TIMPs can interfere with

Table III. The descriptives and the numbers of apoptotic cells in rats with intestinal ischemia/reperfusion injury induced by abdominal compartment syndrome (ACS) (N = 35).

Group (n = 7 each)	Mean (SD)	Range
1*	32.56 (7.86)	24–45
2†	92.50 (11.35)	83–118
3‡	27.22 (5.72)	19–35
4§	33.54 (6.98)	23–47
5‖	15.90 (4.01)	10–21

*Saline (1 cc IP) administered during induction of ACS; intestinal samples removed at 1 hour after reperfusion.

† Saline (1 cc IP) administered during induction of ACS; intestinal samples removed at 24 hours after reperfusion.

‡ Doxycycline (1 cc IP) administered during induction of ACS; intestinal samples removed at 1 hour after reperfusion.

§ Doxycycline (1 cc IP) administered during induction of ACS; intestinal samples removed at 24 hours after reperfusion.

‖ The inserted catheters were not inflated and nothing was administered; intestinal samples were collected 60 minutes after induction of anesthesia.

MMP proteolytic activation and enzymatic activity. MMPs are activated in I/R injury in the lung, heart, brain,[9] kidneys,[10] and intestine.[11,12,31] They may also play a role in inflammation by affecting cytokines, such as TNF-α and IL-1β.[6] Rosário et al[11] found that infiltrating neutrophils in the postischemic intestine correlated positively with the densitometric measurement of MMP-9 monomers ($r = 0.56$; $P = 0.02$) and MMP-9 dimers ($r = 0.68$; $P = 0.004$) in the zymogram and suggested that this gelatinase activity which was derived from MMP-9 was generated by infiltrating neutrophils. Similarly, Souza et al[12] and Robinson et al[31] suggested that there were significant increases in the postischemic intestinal levels of MMP-2, MMP-3, and MMP-9.

In the present study, we observed that the MMP-2 level was significantly higher in group 1 than in the control group 1 hour after the reperfusion period. Doxycycline was associated with significantly lower MMP-2 levels at the first hour compared with the control group and there remained no difference between group 3 and the control group. Twenty-four hours after reperfusion, there was no significant difference between groups 2 and 4.

TIMP-1 levels in groups 1, 2, and 5 were not significantly different. TIMP-1 was highest in group 4, but there was no significant difference compared with group 3. Even if TIMP-1 levels appeared to be numerically higher in the groups administered doxycycline, it is unlikely that there was a correlation between inhibition of MMPs by TIMP-1 and the recovery of the I/R injury observed in the first hour of reperfusion; whereas TIMP-1 appeared to have inhibitory effects on MMP-2 primarily at 24 hours after the ischemia.

The protective effects of doxycycline were also studied immunohistochemically. For this purpose, the number of apoptotic cells and βcl-2 expression were evaluated in

ischemic intestinal tissue.[21] As it is known, βcl-2 is a proliferative gene and suppresses apoptosis.[21] It can be detected in normal and hyperplastic tissues, such as psoriasis and gingival hypertrophy, or malignant tissues.[32] However, if a tissue is exposed to I/R injury, the cells in that tissue have died to protect tissue from abnormal cell formation. It has been shown that βcl-2 overexpression protects enterocytes from I/R and subsequent injury by inhibiting apoptosis.[33,34]

In the present study, we found that the number of the βcl-2$^+$ cells and apoptotic cells was highest in group 3. These results suggest that, to control increased apoptosis, the cells in the intestinal tissues express the βcl-2 gene for self-protection. On the other hand, the reduced apoptosis and βcl-2 expression in group 4, in which doxycycline was administered, suggests that doxycycline may be involved in the protection of intestinal cells from I/R injury. Because the number of apoptotic cells was lower in intestinal tissue, it can be assumed that βcl-2 expression was diminished.

Smith and Gabler found that pretreatment with doxycycline (10 mg/kg; 2 hours prior to occlusion) significantly increased the survival time (up to 30%) ($P < 0.05$) in rats, in which the splanchnic artery was occluded for 1 hour and reperfusion was allowed for 2 hours. They proposed that this protective effect of doxycycline against I/R injury was due to suppression of leukocyte adherence to endothelial cells of the gastrointestinal tract and inhibition of the migration of adherent polymorphonuclear neutrophils.[24] However, they could not explain how it was accomplished. It is our opinion that it produces functional protective effects by attenuating the response of proinflammatory interleukins in the acute phase following 1 hour of reperfusion while it reverses histopathologic changes by inhibiting the apoptotic mechanism at the 24th hour of reperfusion. We also believe that down-regulation of MMPs contributes to doxycycline's protective effects against intestinal I/R injury.

CONCLUSIONS

Doxycycline, a tetracycline analogue, was associated with functional and histopathologic protective effects against intestinal I/R injury caused by ACS in this rat model. These results need to be verified by larger animal studies and appropriate clinical studies.

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