ORIGINAL ARTICLE

The Effect of Doxycycline on Matrix Metalloproteinase-9 Levels in the Colon of Wistar Rats with Obstructive Ileus

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ABSTRACT

INTRODUCTION: Obstructive ileus after colonic anastomosis resection can increase leakage up to 2.5-fold. One possibility could be the extracellular matrix degradation, where matrix metalloproteinase-9 (MMP-9) negatively influences anastomotic healing. This study aimed to determine the effect of doxycycline on MMP-9 levels in the colon of experimental rats with obstructive ileus. **MATERIALS AND METHODS**: In this study, 28 male Wistar rats were selected randomly in a post-test control-group design comprising: Group A - control group, Group B - group without obstructive ileus given doxycycline, Group C - group with obstructive ileus not given doxycycline, and Group D - group with obstructive ileus given doxycycline. Doxycycline was given at a dose of 30 mg/kg. Groups C and D underwent anastomotic resection 24 hours following obstructive ileus procedure. Groups A and B underwent anastomotic resection at the same time as Groups C and D. Colonic tissue was collected to examine MMP-9 levels using an enzyme-linked immunosorbent assay (ELISA). Data were compared using Shapiro–Wilk tests, ANOVA, and Mann–Whitney U tests, and $p<0.05$ was considered significant. **RESULTS**: The results showed that the average MMP-9 levels increased with doxycycline treatment, [Group C (4.294 ± 0.226)], with significantly higher MMP-9 levels than Group A (1.017 ± 1.569, $p<0.001$). Group B did not differ significantly from Group A ($p=0.848$). MMP-9 levels differed significantly between Groups B and D ($p<0.001$), and between Groups C and D ($p<0.001$). **CONCLUSION**: Oral administration of doxycycline can attenuate increasing MMP-9 levels in Wistar rats with obstructive ileus.

Keywords
obstructive ileus; doxycycline; matrix metalloproteinase-9.

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INTRODUCTION

Obstructive ileus and anastomotic leakage (AL) are the most frequent complications of colorectal cancer, manifesting in 7%-39% of cases. AL and perforation are fatal complications of colorectal surgery involving anastomosis. Obstructive ileus after colonic anastomosis resection can increase leakage by up to 2.5-fold. AL is 2.5 times more common in patients with peritonitis than in patients without peritonitis.[1] The prevalence of AL is lower in the right colon (1.35%) than in the left colon (5.20%) in patients who underwent colonic anastomosis.[2] More than 30% of patients with colorectal cancer present with obstructive ileus, of which 10% require urgent surgery, which is associated with an increased risk of AL.[3,4]

One potential factor involved in AL is the degradation of the extracellular matrix (ECM), which provides structure and integrity to healthy intestinal tissues. Experimental studies investigating the relationship between matrix metalloproteinases (MMPs) and AL have concluded that MMPs negatively affect anastomotic healing. Moreover, MMP inhibitors (MMPIs) have been shown to increase the breaking strength of colonic anastomosis during the early post-operative phase. In particular, MMP-9, a zinc-dependent enzyme, plays a role in the degradation mainly of collagen, which comprises the ECM.[5]

Stumpf et al.[6] found significantly higher MMP-9 levels in

the submucosa of patients with AL than without AL. Another study found higher MMP-9 activity in tissues extracted from mice with AL than from mice with well-healed anastomotic tissue.[7] Therefore, MMP-9 inhibition is an important target in healing intestinal anastomosis. Doxycycline has been shown *in vitro* to inhibit the regulation of MMP-2 and MMP-9, reduce cancer cell invasion and migration, induce anti-inflammatory effects in normal tissues, and support better anastomotic healing.[8–10]

In this study, we used doxycycline as an MMP inhibitor (MMPI) to examine whether it can reduce MMP-9 levels in the colon of Wistar rats with obstructive ileus.

MATERIALS AND METHODS

This study was conducted over one month (December 2022) in the Laboratory of Animal Microbiology, Faculty of Medicine, Hasanuddin University, according to the recommendations in the research ethics approval from the Health Research Ethics Commission, Faculty of Medicine, Hasanuddin University (approval number: 464/UN.4.6.4.5.31/PP.36/2022).

The sample size was calculated using the Federer formula. This experimental study randomly selected 28 male Wistar rats and used a post-test control-group design comprising Group A - normal rats with no obstructed ileus and no doxycycline (control group), Group B - normal rats with without obstructive ileus given doxycycline, Group C - rats with obstructive ileus not given doxycycline, and Group D - rats with obstructive ileus given doxycycline. Doxycycline was given at a dose of 30 mg/kg. Groups C and D (rats with obstructive ileus) underwent anastomotic resection 24 hours after the obstructive ileus procedure. Groups A and B (rats without obstructive ileus) underwent anastomotic resection simultaneously as Groups C and D. Colonic tissue was collected to examine MMP-9 levels using an enzyme-linked immunosorbent assay (ELISA). Statistical analyses were performed using SPSS (version 27.0; Armonk, NY: IBM Corp.).

The research procedure was assisted by veterinarians from beginning to end. This study used 28 healthy male Wistar rats aged 3–4 months and weighing 300–400 grams. All rats were kept in separate cages and acclimatized for 14 days. Before surgery, each rat was fasted for at least 12 hours, and access to water was restricted for at least 2 hours. Then, the rats were premedicated with xylazine-ketamine-induced anaesthesia [dose: 20 mg/kg, intramuscular (IM) for xylazine + 40 mg/kg, IM for ketamine).

Surgical Procedure

The rats received intramuscular 40 mg/kg ketamine. Their body weight, oxygen saturation, heart rate, and respiratory rate were monitored. Next, the rats were fixed to the operating table, their abdominal skin was shaved and cleaned with 10% povidone-iodine and infiltrated with lidocaine in the median area (Figure 1A). The aseptic technique was used according to the surgical procedure. A 3-cm median laparotomy incision penetrated the peritoneum to reveal the colon. The obstructive ileus condition was created by tying the colon 5 cm from the rectum with a non-absorbable 3.0 thread (Figure 1B), according to Despoudi models.[4]

After 24 hours, a re-laparotomy was performed, an anastomotic resection was performed in the obstructed area (Figure 1C), and the rat colon was sutured in eight directions with a non-absorbable 6.0 thread; one surgeon performed all procedures (Figure 1D). The laparotomy wound was closed again (Figure 1E).

Doxycycline Administration

An oral dose of 30 mg/kg doxycycline was administered to rats in Group D (rats with obstructive ileus given doxycycline) immediately after the procedure via their drinking water. MMP-9 levels were assessed in resected colonic tissue using an MMP-9 ELISA kit, purchased from MyBioSource with Catalogue No. MBS2702012 (San Diego, CA, USA).

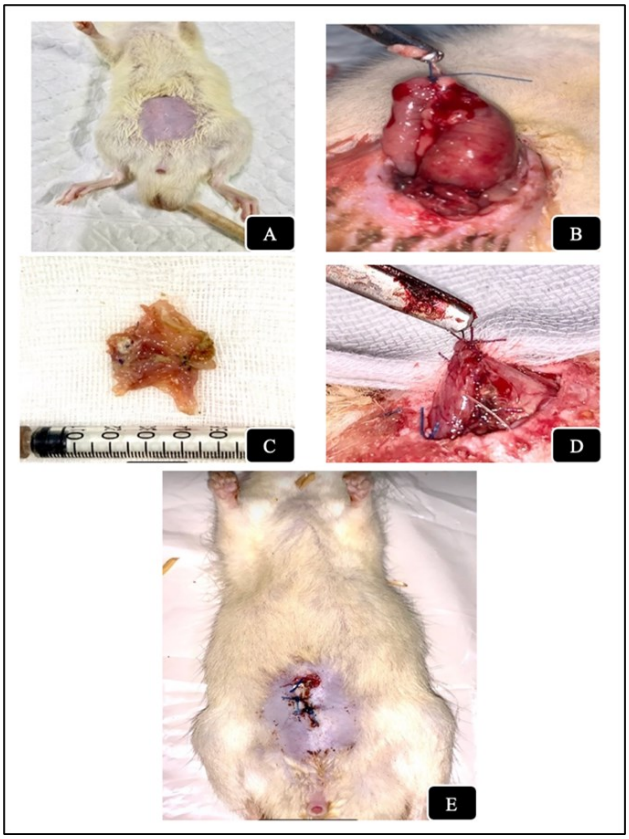

Figure 1. Experimental clinical research photos. (A) A rat shaved after anaesthetised. (B) The colon condition 24 hours after obstruction. (C) Colon tissue resected for examination of MMP-9 levels. (D) The colon condition after anastomosis. (E) The postoperative wound.

Statistical Analysis

Data was statistically analysed using SPSS software (version 27; IBM Corp., Armonk, NY, USA) based on predetermined variables. Data were compared using Shapiro–Wilk tests, ANOVA, and Mann–Whitney U tests, and $p<0.05$ was considered significant.

RESULTS

Table I shows that the average MMP-9 levels increament with doxycycline treatment. The Shapiro–Wilk normality test shows normal distribution for all groups. Group C (rats with obstructive ileus without doxycycline) had the highest MMP-9 levels (mean = 4.2941 ng/mL).

Table I. MMP-9 levels in all groups

Group (n=7)	MMP-9 levels (ng/mL)			*p*-value
	Average (SD)	Median	Min–Max	
A	1.017 (1.569)	0.846	0.461–2.278	<0.001
B	1.079 (0.562)	1.075	0.420–1.885	
C	4.294 (0.226)	4.212	4.100–4.758	
D	3.366 (0.142)	3.366	3.175–3.614	

Group A - control group (normal rats: no obstruction, no doxycycline), Group B - rats without obstructive ileus given doxycycline, Group C - rats with obstructive ileus not given doxycycline, and Group D - rats with obstructive ileus given doxycycline. Note: data are normally distributed and were compared by ANOVA; SD, standard deviation.

The statistical analysis using interquartile range (IQR) show a significant difference in MMP-9 levels (ng/mL) between Groups A (normal rats) and C (rats with obstructive ileus not given doxycycline) ($p<0.001$). However, there was no significant difference in MMP-9 levels (ng/mL) between Groups A (normal rats) and B (rats without obstructive ileus given doxycycline) ($p=0.848$; Table II). The statistical analysis using standard deviation (SD) show a significant difference in MMP-9 levels (ng/mL) between Groups C (rats with obstructive ileus not given doxycycline) and D (rats with obstructive ileus given doxycycline) ($p<0.001$; Table II). There was significant difference in MMP-9 levels (ng/mL) between Groups B (rats without obstructive ileus given doxycycline) and D (rats with obstructive ileus given doxycycline) ($p<0.001$; Table II).

Table II. Comparing MMP-9 levels between groups

Group (n=7)	Average	SD	*p*-value
A	1.017	1.569	0.848^
B	1.079	0.562	
A	1.017	1.569	<0.001^
C	4.294	0.142	
B	1.079	0.562	<0.001*
D	3.366	0.142	
C	4.294	0.226	<0.001*
D	3.366	0.142	

Group A-control group (normal rats: no obstruction, no doxycycline), Group B-rats without obstructive ileus given doxycycline, Group C - rats with obstructive ileus not given doxycycline, and Group D - rats with obstructive ileus given doxycycline. Note: ^IQR, interquartile range, *SD, standard deviation

DISCUSSION

MMP-9 is unique as its protein expression and activity is undetectable in most healthy intestinal tissue but is highly expressed in various inflammatory states, including inflammatory bowel disease (IBD).[11] MMP-9 levels increase after being induced by inflammation, oxidative stress, apoptosis, and excitotoxicity due to ischemic reperfusion in secondary tissue injury. MMP-9 appears to be triggered by inflammatory cells in the early inflammatory stage of tissue healing. Excessive MMP-9 activation could upset the balance during anastomotic healing such that collagen degradation resulting in leakage instead of healing.[12] Numerous assay methods have also shown increased MMP levels and activity in anastomotic wounds, including *in situ* hybridization, immunohistochemistry, zymography, and functional activity tests. MMP-9 was found to be more elevated in the presence of local infection and obstructive ileus. The amount of colonic collagen degraded by active MMPs in anastomosis is at least 10-fold higher in the anastomotic incision's sutured area than in the neighbouring non-sutured area.[13]

Another theory by Jacobson and colleagues has elucidated to the molecular mechanisms by which commensal microbes that survive antibiotic decontamination protocols, in addition to their direct collagenolytic activity on the anastomotic wound, such as *E. faecalis* can convert MMP-9 from its preform to its active form resulting in an excess of tissue protease activity and, thus failure to heal the anastomotic wound.[14] Sparreboom and colleagues were the first to investigate the diagnostic value of peritoneal inflammatory factors such as cytokines and matrix metalloproteinase with serum C-reactive protein (CRP) to predict anastomotic leakage (AL) in European patients undergoing rectal surgery. Their results showed that peritoneal MMPs improved the diagnostic value in detecting AL over serum CRP alone.[15]

An experimental study on colonic obstruction in rats and reported decreased collagen concentration with stenosis.[16] This finding suggests that collagen degradation exceeds collagen synthesis near colonic stenosis. Their study also showed that colonic obstruction for four days increased collagen synthesis, which was positively correlated with obstruction time and significantly higher (more than 2-fold) in the obstructed than in the non-obstructed left colon wall. This finding is consistent with Hayashi et al.[17] and Perdana et al. [18], who showed that MMP-9 levels increased significantly at 24 and 48 hours after treatment of traumatic brain injury in experimental rats.

Table II shows that the Group A (normal rats) had an average MMP-9 level of 1.017 ng/mL, significantly lower than the Group C (rats with obstructive ileus not given doxycycline), which had an average MMP-9 level of 4.294 ng/mL ($p<0.001$). During inflammatory events, MMP-9 transcription is induced by proinflammatory mediators (the metabolic products of integrins, arachidonic acid, plasminogen, tumour necrosis factor-alpha [TNF-α], interleukin [IL]-1β, transforming growth factor-beta [TGF -β], and nitric oxide). Many mediators have been implicated in cellular inflammation, forming the basis for the pathology associated with post-operative ileus (POI).[19] Moore et al. [19] examined MMP-9 gene expression in the small intestine of rats and MMP-9 levels in the colon muscle following laparotomy and bowel manipulation, finding that MMP-9 plays a crucial role in both. MMP-9 gene activation followed a time course that closely followed the beginning of the recognized time for leukocyte migration and when maximal numbers of leukocytes actively migrate to the muscles of the inflamed gut, starting three hours after surgery and peaking at 12–24 hours. This study also observed increased MMP-9 protein levels in the colonic mucosa of rats with POI, indicating that MMP-9 may be beneficial in inflammatory diseases affecting the intestinal mucosa.

Doxycycline, a tetracycline family member, is a broad-spectrum antibiotic that works well against Gram-negative and Gram-positive microorganisms.[20] In recent years, doxycycline has also been extensively studied in humans and animals due to its association with high levels of pro-inflammatory mediators and protease activity.[21–23] Previous studies have examined the effect on MMPs of doxycycline administered at an oral dose of 30 mg/kg. Studies have previously shown this dose to block MMP activity *in vivo*.[24] Meli et al.[25] used doxycycline (30 mg/kg) to examine its effects on reducing mortality and brain and

cochlear injury in pneumococcal meningitis. Robinson et al.[26] used doxycycline (20 mg/kg given intravenously) to examine its ability to inhibit MMP-9 and reduce microvascular hyperpermeability after traumatic brain injury.

Based on the finding shown in Table II, there is a significant difference in MMP-9 levels (ng/mL) between rats with obstructive ileus not given doxycycline and rats with obstructive ileus given doxycycline (p<0.001). This finding shows that administering doxycycline to rats with obstructive ileus is beneficial.

It is believed that doxycycline therapy, which has anti-inflammatory and antimicrobial properties, is beneficial.[27,28] This action may be direct or occur by modulating chemokines and cytokines.[27,29,30] It may also change the expression of MMPs-proteases known to control tissue remodelling, a crucial phase in tissue repair.[31,32]

In a study of 58 colorectal anastomotic patients, peritoneal levels of MMPs-1, 2, 3, 8, and 9 and tissue inhibitors of metalloproteinases (TIMP) 1 and 2 were assessed for 8 days following surgery. Differential MMP and TIMP levels were assessed each day, along with total MMP activity. Their levels were shown to vary depending on the operation type and duration, the amount of bleeding, and the occurrence of post-operative complications. Only MMP-2 and MMP-9 levels positively correlated with the development of post-operative complications, whereas TIMP-1 and TIMP-2 levels demonstrated a negative correlation. The authors suggested that peritoneal MMP and TIMP may be biomarkers of intestinal wound healing and surgical outcomes.[33]

Healing of an intestinal anastomosis follows the basic principle of wound healing. Four overlapping phases *viz* i) haemostasis, ii) inflammation, iii) proliferation, and iv) remodelling, these characterize the process of uncomplicated healing.[34] Physiologically, MMP secretion functions in regeneration, tissue remodelling, embryogenesis, angiogenesis, and wound healing.[35] These functions are followed by a balancing of the tissue inhibitors of metalloproteinases (TIMPs). TIMPs can inhibit MMPs by binding to the hemopexin domain and inhibiting the pro-MMP chain conversion into MMP. MMP and TIMP concentrations must reach equilibrium to perform the appropriate tissue remodelling function. Excess or deficiency of one of these functions can damage the network. Following tissue injury, well-characterized repair components emerge, including early MMP activation followed by neovascularization and scar-tissue formation.[16,36]

A study examining normal non-inflamed intestine, showed that doxycycline administration did not produce statistically significant results (p>0.001).[12,37] This finding can be explained by the assumption that MMP activity causes the degradation of relatively small amounts of collagen located around the sutures of the submucosa. The hydroxyproline test of the tissue segment containing non-inflamed bowel adjacent to the anastomotic area is not very sensitive to such small differences. Alternatively, collagen may be damaged structurally by the action of MMPs but only around the injured intestinal area.

The transcription of MMP peptide coding genes is regulated by growth factors, cytokines, hormones, UV light, and cell-matrix interactions. MMP inducers are also called extracellular MMP inducers, such as cluster of differentiation 147 (CD147). In addition, inflammatory mediators such as TNF-α, IL-6, and IL-1 can increase MMP expression. Here, MMP functions to degrade collagen as the main ECM component. Increases in these mediators will increase MMP levels in the tissue. In contrast, TIMP expression does not increase, and as a result, there is an increase in ECM degeneration activity. This imbalance causes inflamed tissue to be easily damaged.[38]

Lamparter et al.[38] investigated whether oral doxycycline influences angiogenesis and collagen turnover and whether, in turn, this influences the turnover of fibrous tissue. They found that doxycycline reduced neovascularization and prevented the early development of fibrous tissue in rat subcutaneous pocket tissue induced by croton oil. Additionally, inhibiting MMP activity, which can be detected noninvasively in serum, reduced pocket-tissue regression. These results indicate that doxycycline

inhibits angiogenesis and the molecular and cellular elements of tissue repair, including the development and resorption of fibrous tissue. Tetracyclines and their chemically altered congeners may provide pharmacological methods for altering tissue healing in various disease conditions, such as during the progression of dilated cardiomyopathy when MMP activity is elevated. Investigations are being conducted on several recently developed synthetic MMPIs with more targeted MMP inhibition; further research is required to examine their potential for pharmacologically altering tissue repair.

In another experimental study, Kaitu'u et al.[39] examined rats with endometrial damage and increased MMP production, finding that treatment with doxycycline reduced endometrial MMPs without affecting endometrial breakdown or repair. Pasternak et al.[40] showed that the strength of the anastomosis was at its lowest three days after surgery. Of the three rat groups observed, oral administration of 10 mg doxycycline/day resulted in a 36% increase in the average bursting pressure in the large intestine and a 100% average increase in the breaking strength of the ileum. When doxycycline was administered subcutaneously (2x5 mg/day), colonic bursting pressure and colon/ileal breaking strength were significantly higher than in the control group. Therefore, doxycycline increases the strength of the anastomotic wound, and histological examination shows a normal wound healing pattern.

Further experimental and pharmacodynamic studies are needed to identify the optimal MMPIs and dosing regimens to achieve maximal healing of intestinal anastomosis.[41] Preventing the complications of colorectal surgery is a promising indication for therapy with MMPI. In the future, researchers should explore the therapeutic value of MMPIs in colorectal patients. Experimental studies have shown that ileus increases the activity of metalloproteinases, which reduce the deposition of new collagen in the anastomosis and increase the degradation of the old. Their greater concentration and consequent more significant collagen degradation are observed near the anastomotic line. Newer studies demonstrated that selective MMP inhibition increased anastomotic breaking strength and improved colonic anastomoses' outcomes.[42,43]

This experimental which studied on rats with obstructive ileus had several limitations. First, MMPs are thought to be responsible for collagenolysis around surgical sutures in many tissues. Groups and specific MMPs can degrade all ECM and epithelial basement membrane components. Among all MMP types discovered to date, the gelatinase type (in this case, MMP-9) is the main enzyme degrading collagen types IV, V, VII, X, XI, and XIV, as well as elastin, gelatine, proteoglycan core protein, fibronectin, fibrillin-1, myelin basic protein, IL-1b, and TNF-α precursors. MMP-9 can break down type-I collagen, which has been shown to increase due to inflammation (in this study, inflammation was treated as an obstructive ileus condition). Second, doxycycline is thought to be a specific MMP-9 inhibitor, which was successfully used in this experimental study. Third, this experimental research is still being conducted on experimental animals and has yet to be clinically tested on humans. Therefore, while doxycycline is thought to play a role in MMP-9 inhibition in obstructive ileus conditions in experimental animals, it is unknown whether it plays a role in obstructive-ileus conditions in humans.

CONCLUSIONS

Oral doxycycline administration can attenuate increased MMP-9 levels in obstructive ileus in Wistar rats. We observed a significant difference in MMP-9 levels between rats with obstructive ileus that were given doxycycline and rats with obstructive ileus that were not given doxycycline. Further research is needed on factors other than MMP-9 that influence the occurrence of obstructive ileus. Further experimental and pharmacodynamic studies are needed to identify the optimal MMPI and dosage regimen to achieve maximal healing of intestinal anastomosis and assess its effects in humans.

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