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Doxycycline-coated sutures improve mechanical strength of intestinal anastomoses

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Abstract

Background and aims: After resection and repair of the intestines, tissue degradation leads to weakening of the repair site and a risk of postoperative leakage. Matrix metalloproteinases (MMPs) are thought to be responsible for collagenolysis in the direct vicinity of surgical sutures in many tissues. Several experimental studies show that MMP-inhibitors administered systemically alleviate postoperative weakening of intestinal anastomoses. We hypothesised that local delivery of MMP-inhibitors would achieve a similar effect.

Methods: Implementing a novel method for the coating of biomaterials, we coated sutures with a crosslinked fibrinogen film and bound the MMP-inhibitor doxycycline into this film. The sutures were then used in a standard rat model for evaluating mechanical properties of colonic anastomoses 3 days after surgery.

Results: The breaking strength of the anastomoses on the critical third day after operation was 17 % higher with doxycycline-coated sutures compared to controls (P=0.026). Energy uptake at failure was enhanced by 20 % (P=0.047).

Conclusion: Drug delivery by means of MMP-inhibitor-coated sutures appears to improve tissue integrity during anastomotic repair and may reduce postoperative complications.

Key words: colon; matrix metalloproteinase (MMP); wound dehiscence; anastomotic leakage; suture.

Introduction

Anastomotic leakage is a major and unresolved problem in patients undergoing colonic or rectal resection [1]. Under experimental conditions, the strength of intestinal anastomoses diminishes postoperatively reaching a minimum on the third postoperative day [2,3]. Increased matrix metalloproteinase (MMP) activity is thought to mediate the loss of anastomotic strength by causing local matrix degradation in the tissue surrounding the sutures [4]. Several MMPs, e.g. collagenase 2 (MMP-8), gelatinase B (MMP-9) and stromelysin 1 (MMP-3), are upregulated in the direct vicinity of the anastomotic suture line [4,5]. Anastomotic MMP activity is yet higher in concurrent bacterial peritonitis, generating further deterioration of anastomotic strength [2]. Furthermore, a recent study showed that patients with higher preoperative levels of collagenase 1 (MMP-1) and gelatinase A (MMP-2) in the mucosal layers, and of MMP-2 and MMP-9 in the submucosal layers of the large bowel wall had an increased rate of anastomotic leakage [6]. This demonstrates the critical roles of MMPs as important mediators of a decreased suture holding capacity and indicates that MMPs are potential drug targets to improve anastomotic integrity. Accordingly, several experimental studies have shown beneficial effects of treatment with systemic MMP-inhibitors [7], e.g. doxycycline [8], most notably on the critical third postoperative day [3,7,8].

Potent MMP-inhibitors administered systemically can cause joint stiffness and swelling [9] and possibly other toxic reactions [10]. Additionally, there are concerns about detrimental effects of broad-spectrum hydroxamate MMP-inhibitors on secondary healing of cutaneous wounds [11] although these types of MMP-inhibitors can increase tensile strength of primary skin wounds [12]. The less potent MMP-inhibitor doxycycline does not appear to delay wound closure [13]. Because of adverse systemic effects, local delivery of an MMP-inhibitor in humans would be advantageous over systemic administration.

In this study, we used a new method for coating sutures with the MMP-inhibitor doxycycline and tested the hypothesis that this treatment would improve intestinal anastomotic strength on the critical third postoperative day.

Materials and methods

Animals

Eighty-five male Sprague Dawley rats (Taconic M&B, Ry, Denmark) weighing 240-345 g were housed in standard type III cages with aspen wood-chip bedding that was changed twice weekly. Cages were kept at 20-24°C and relative humidity 50-60%. Light cycle was 12-hour light (6:00 am-6:00 pm)/dark. Rats were given pellets (Altromin 1314) and tap water ad libitum, and were acclimatized for 7 days before being operated.

Design and surgical procedure

Two separate experiments, A and B, were carried out. In experiment A, 45 rats were randomised to four groups. To evaluate the effect of doxycycline treatment, 15 rats were allocated to doxycycline-coated sutures and 15 rats to carrier-coated sutures. These 30 rats underwent biomechanical evaluation three days postoperatively. Additionally, the biomechanical properties were evaluated directly after the operation (immediate day 0 controls) of anastomoses constructed using carrier-coated sutures in 10 rats. The fourth group comprised five rats that were not operated on and used to determine the biomechanical properties of uninjured colon. In experiment B, uncoated sutures were compared with carrier-coated sutures to investigate any possible effect of the fibrinogen carrier coating. Forty rats were randomized to two groups, 20 rats allocated to uncoated sutures and 20 rats to carrier-coated sutures. Biomechanics of the anastomoses were determined three days postoperatively. The same surgeon, blinded for treatment throughout the entire study period, performed all operations and resections in experiment A (L. A.) and B (M. R.), respectively.

Anaesthesia was induced by a subcutaneous injection of a mixture of fentanyl citrate (0.16 mg/kg), droperidol (11.1 mg/kg) and midazolam (0.13 mg/kg). After laparotomy, a standardised 10 mm segment of the colon was resected 6 cm proximally to the anal orifice [4]. An end-to-end anastomosis was constructed using 8 interrupted sutures placed approximately 2 mm from the resection margin. The abdomen was closed with continuous polyglactin suture in the musculofascial layer and metal clips in the skin.

The animals were given carprofen (5 mg/kg s.c.) for analgesia, and allowed immediate mobilisation and free access to water and diet.

This study was approved by the Danish National Experimental Animal Inspectorate and followed the established guidelines.

Mechanical testing

After sacrifice, the abdomen was opened and the colon carefully freed from adhesions. A 4 cm segment with the anastomosis was resected and gently cleaned of faecal contents. A corresponding segment was resected in the unoperated rats. The segment was mounted in a materials testing machine (LF Plus; Lloyds Instruments, Fareham, UK) equipped with a 10 N loadcell (XLC-0010-A1; Lloyds Instruments) with 10 mm between the clamps. The colon segments were stretched at a constant deformation rate of 10 mm/min until rupture. The maximal load (breaking strength) and the area under the curve to the breaking point (energy uptake at failure) were derived from the load-strain curve calculated by the software (Nexygen; Lloyds Instruments). The breaking point was defined by the maximum force value. These measurements were carried out by an individual unaware of treatment.

Suture coating

Sterile 6-0 polybutester monofilament sutures (Novafil, Tyco Healthcare, Schaffhausen, Switzerland) were activated during 20 seconds in a radio frequency plasma chamber (Plasmaprep 100; Nanotech, Sweden). The activated sutures were incubated for 30 minutes in 6 % glutaraldehyde in phosphate-buffered saline (PBS), pH 9. The surfaces were extensively rinsed in PBS, pH 9. Ten layers of fibrinogen (Hyphen BioMed, Neuville-sur-Oise, France; molecular weight: 340 kDa, clotability 98%) were prepared as follows [14]: the glutaraldehyde coated sutures were incubated for 30 minutes in 1 mg/ml fibrinogen dissolved in PBS at pH 7.4. The sutures were extensively rinsed in PBS followed by incubation during 30 minutes in PBS, pH 5.5, containing 0.2 M N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC; Sigma-Aldrich, St. Louis, MO, USA) and 0.05 M N-hydroxy-succinimide (NHS; Sigma-Aldrich). Then a new 1 mg/ml fibrinogen solution was prepared in PBS buffer, pH 5.5, and the sutures incubated for

30 minutes in this, rinsed in PBS buffer, and again incubated in the EDC/NHS solution. As the EDC solution is unstable at room conditions, new solutions were prepared every second hour. This procedure was repeated until ten fibrinogen layers were immobilised. The crosslinked fibrinogen surface was subsequently incubated in EDC/NHS as above, and for 3 hours in a 1 mg/ml solution doxycycline hyclate (Sigma-Aldrich) or for 3 hours in PBS (control sutures), and finally rinsed in distilled water.

Thicknesses of the fibrinogen and doxycycline layers on the sutures were measured by null ellipsometry (Auto-Ell III; Rudolph Research, Flanders, NJ, USA) in air, calculated according to the McCrackin evaluation algorithm [15] and converted into an approximate adsorbed amount per unit area by de Feijter´s formula [16]. The assumed refractive index of the protein and immobilised doxycycline film was $n_f = 1.465$ [17]. During the measurements, 1 nm of adsorbed proteins equalled approximately 120 ng/cm^2 [18].

The so aseptically prepared sutures for experiment A were stored at room temperature in dark in a 0.5 mg/ml doxycycline PBS solution, pH 5.5, until use within 6 days. Fibrinogen-coated control sutures were stored in PBS, pH 5.5, under identical conditions. In experiment B, the fibrinogen-coated sutures were dried using nitrogen gas, stored at room temperature in sterile bags and used within 14 days after production. Uncoated sutures were packaged aseptically in identical sterile bags. The sutures were indistinguishable by visual inspection and physical handling such as elasticity and pliability.

Statistics

The main hypothesis was that there would be a difference at 3 days postoperatively. Therefore, a sequential analysis of data was employed, as predetermined, to first evaluate the effect of doxycycline treatment at three days. Subsequently, the early decrease of anastomosis breaking strength was analysed, comparing immediate and day 3 carrier-coated controls. Thereafter, uncoated and carrier-coated controls (experiment B) were compared. Student´s t-test (two-tailed) was used. $P<0.05$ was considered statistically significant. Results are presented as mean±standard deviation (SD) or 95% confidence interval (CI) for differences between means.

Results

All rats gained body weight preoperatively. In experiment A, three rats in the control group and one rat in the doxycycline group died immediately after the operation, probably due to the anaesthesia. The remaining operated rats lost body weight but this did not differ between rats treated with doxycycline-coated sutures and rats treated with carrier-coated sutures. One rat in the carrier-coated suture group of experiment B was excluded from analysis due to anastomotic rupture during killing. The number of animals and their body weights before and after operation are given in Table 1.

Table 1. Body weights of the rats included in the two separate experiments (A and B) of the study

	Day of	
Group	Operation	biomechanical testing
Experiment A		
Doxycycline sutures day 3 (*n*=14)	331±17 g	321±21 g
Carrier sutures day 3 (*n*=12)	333±18 g	326±21 g
Carrier sutures day 0 control (*n*=10)	NA	326±32 g
Non-operated (*n*=5)	NA	287± 7 g
Experiment B		
Carrier sutures day 3 (n=19)	289±25 g	284±27 g
Uncoated sutures day 3 (n=20)	288±24 g	285±30 g

Mean±SD. NA: not applicable.

Suture coating

By ellipsometry it was estimated that the total fibrinogen layer thickness was approximately 30 nm while the doxycycline layer was 2 nm. Thus, the immobilised amount of doxycycline was approximately 240 ng/cm^2. The diameter of the uncoated suture threads was 0.095 mm. Therefore it could be calculated that 1 cm of the doxycycline-coated sutures carried about 7 ng of doxycycline. In experiment B the total fibrinogen layer thickness was approximately 50 nm.

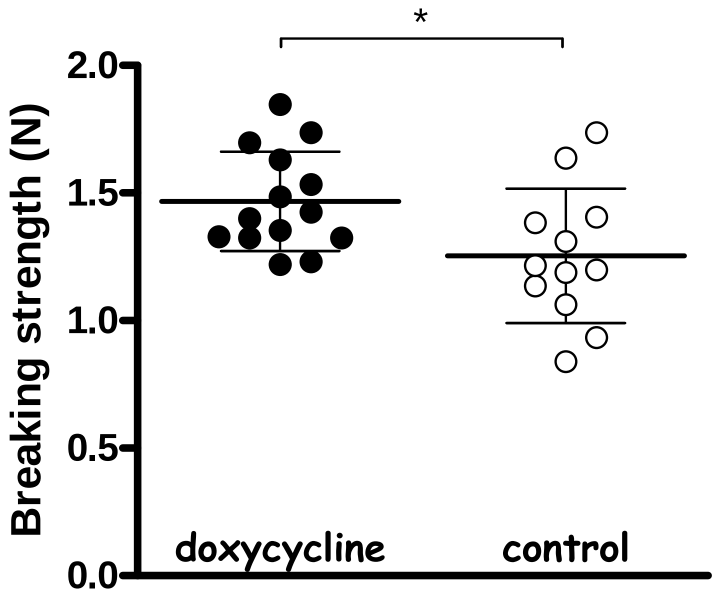
Mechanical evaluation

Anastomosis rupture occurred within the suture line in all animals. Breaking strength (Fig. 1a) on the third postoperative day was significantly (*P*=0.026) higher in the doxycycline-coated sutures group (1.47±0.20 N) compared with the carrier-coated control group (1.25±0.26 N). The difference was 17% (95% CI: 2 to 32%). Energy uptake (Fig. 1b) was also significantly (*P*=0.047) higher in the doxycycline-coated sutures group (12.1±2.2 Nmm) compared with the carrier-coated control group (10.1±2.7 Nmm). The difference was 20% (95% CI: 0 to 40 %).

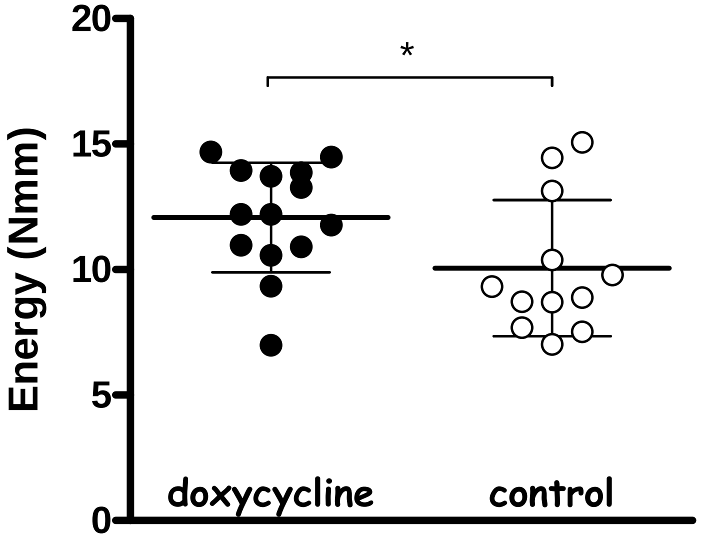
The breaking strength in the immediate day 0 control group was 1.53±0.44 N. Thus, the breaking strength of the carrier-coated control group on the third day was 18% (95% CI: -2 to 39%) lower than the immediate day 0 control group although this was not a statistically significant difference (*P*=0.08). Local doxycycline treatment aborted roughly three quarters of this decrease at 3 days. The energy uptake in the immediate day 0 control group was 11.4±3.1 Nmm. Thus, the energy uptake of the carrier-coated control group at three days did not differ from immediate controls (mean difference 12%; 95% CI: -11 to 34%; *P*=0.30).

In experiment B, breaking strength (Fig. 2a) on the third postoperative day did not differ significantly (*P*=0.64) between uncoated sutures (1.21±0.37 N) and carrier-coated sutures (1.16±0.36 N; mean difference 5%; 95% CI -15 to 24%). There was no significant (*P*=0.78) difference in energy uptake (Fig. 2b) either between uncoated sutures (9.5±4.2 Nmm) and carrier-coated sutures (9.2±4.0 Nmm; mean difference 4%; 95% CI -24 to 32%;).

For uninjured colon in rats not subjected to surgery, the breaking strength was 2.93±1.07 and energy uptake 18.9±4.5 Nmm.

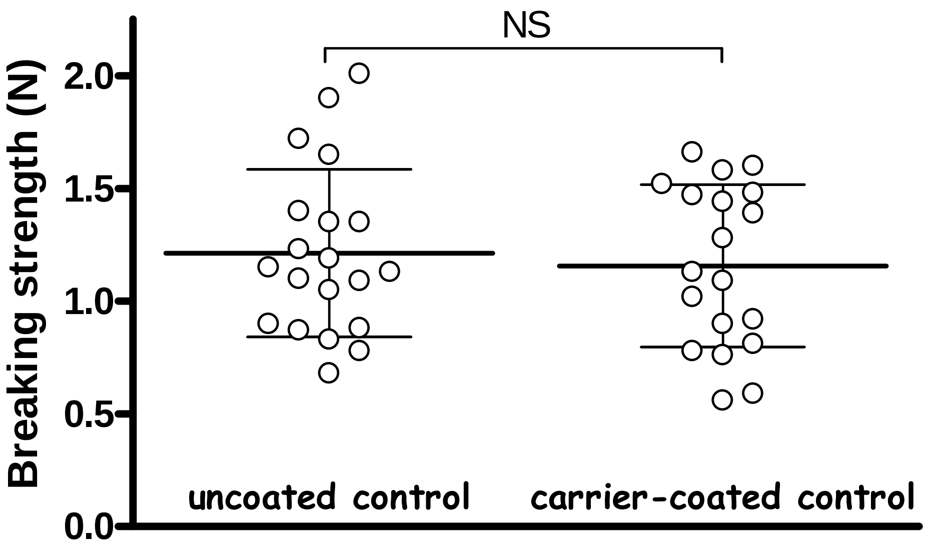


a



b

*Fig. 1 Anastomotic strength of the rat colon on the third postoperative day in experiment A. Doxycycline-coated sutures increased the breaking strength (a) by 17 % (P=0.026) and the energy uptake at failure (b) by 20 % (P=0.047) compared with carrier-coated sutures. Data are shown as mean (thick horizontal line) and SD interval. Filled circles, doxycycline-coated sutures; open circles, carrier-coated sutures. *P<0.05.*



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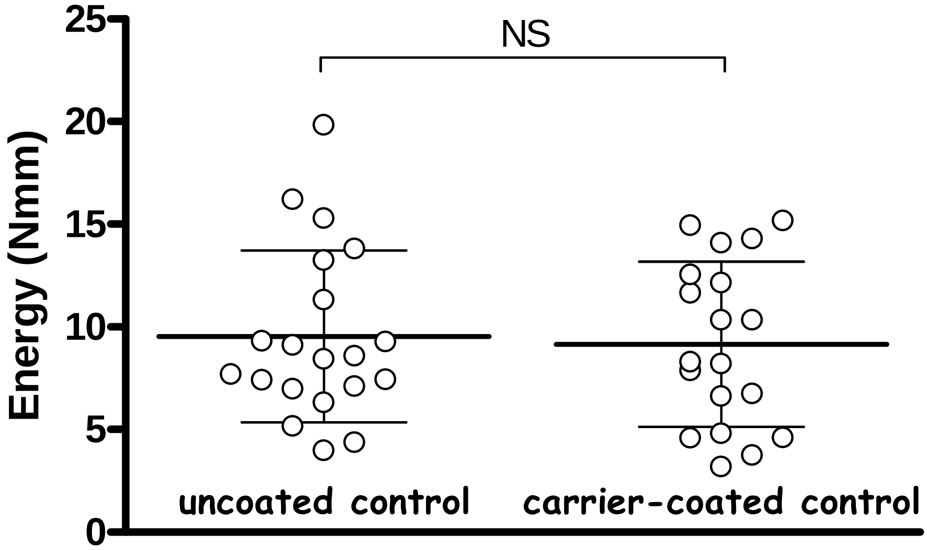

b

Fig. 2. Anastomotic strength of the rat colon on the third postoperative day in experiment B. Uncoated and carrier-coated sutures did not differ in breaking strength (P=0.64) or energy uptake at failure (P=0.78). Data are shown as mean (thick horizontal line) and SD interval. NS; non significant.

Discussion

The unwanted decrease in anastomotic breaking strength of the colon is amenable to pharmacologic manipulation with systemically administered MMP-inhibitors [3,7,8]. The findings of the present study indicate that an MMP-inhibitor can be delivered locally from the surface of the suture thread in extremely small doses and achieve similar results to systemic MMP-inhibitor treatment. Colon anastomosis breaking strength and energy uptake were enhanced by local treatment with doxycycline, inhibiting the unwanted decrease in mechanical strength.

One cm of doxycycline-coated 6-0 suture carries approximately 7 ng of doxycycline to the anastomotic area. This dose seems sufficient to result in significant effects on anastomotic strength, supporting the notion that MMP-mediated degradation of colon matrix is a localised phenomenon restricted to the immediate vicinity of the sutures [4].

In the present study doxycycline was immobilised onto a relatively thick crosslinked fibrinogen matrix. This protein can be replaced by other suitable carriers, such as other proteins, proteoglycans and synthetic polymers. Fibrinogen is advantageous due its low immunogenicity and ease of EDC/NHS activation of its terminal –COOH groups [14].

The postoperative decline in breaking strength in this study was not as profound as reported in previous communications with this model [3,7]. One explanation could be that the polybutester suture material used in the present study had different mechanical properties [19] or elicited a milder tissue reaction [20] compared to the polyamide sutures used in previous studies [3,7]. The possibility that the fibrinogen film coating might render the sutures more tissue-compatible is unlikely in view of the results of experiment B which demonstrated that the biomechanical properties did not differ significantly between anastomoses constructed with fibrinogen-coated and uncoated sutures.

We have no direct evidence that the effect of doxycycline was exerted via MMP inhibition. However, it is well established that doxycycline functions as an MMP-inhibitor both by inhibiting MMP activity as well as proenzyme synthesis [21]. Studies also show that doxycycline administered systemically enhances intestinal anastomotic repair via inhibition of MMPs [8]. Nonetheless, further studies are needed to establish the exact mechanisms of action and the most efficacious drug to be delivered locally into the anastomotic area.

We used doxycycline as the experimental MMP inhibitor to study the novel principle of local drug delivery. Any type of MMP-inhibitor can potentially be immobilised onto the suture, and by using more potent MMP-inhibitors one can expect even further improvement of anastomosis biomechanics. In this experimental model of colonic anastomosis, GM 6001 (Galardin or Ilomastat) is the most efficacious MMP-inhibitor tested by the systemic route to date [7]. With our method of local drug delivery, problems associated with systemic administration and harmful effects may be avoided. It is unclear if the dose delivered on sutures is enough to inhibit the extra elevation of MMPs observed in compromising conditions, e.g. peritonitis [2], or suffices to inhibit the relatively greater amounts of MMPs in larger animals and humans. This should be the focus of future studies.

We chose to focus our study on the third postoperative day because the anastomosis is weakest at this time point [2,3]. This is probably because MMP induction and activation after resection and suturing occur immediately, while de novo matrix synthesis does not begin until the third-fourth postoperative day [22,23]. Thus, early suture holding capacity is dependent on the pre-existing collagen-rich matrix of the submucosa, which is rapidly degraded by MMPs, while later the anastomosis is supported by newly deposited extracellular matrix. In effect, therefore, intervention with systemic MMP-inhibitors is only efficient up to about the fourth postoperative day in the rat [3,7,8,24,25].

Local MMP-inhibitor drug delivery via the suture surface appears to improve colon anastomotic strength in this experimental model. This route of drug delivery is appealing since doses are minimal. This knowledge might inspire further studies involving pharmacological manipulation of intestinal healing by local drug delivery.

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