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Preoperative irradiation with 5×5 Gy in a murine isolated colon loop model does not cause anastomotic weakening after colon resection

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Abstract

Introduction There are conflicting studies on the influence of fractionated preoperative 5 days of 5 Gy irradiation on tissue oxygenation and subsequent colonic anastomotic strength. To elucidate the effect of preoperative irradiation on anastomotic strength, an isolated colon loop model was developed.

Methods Male Wistar rats ($n=164$) were randomly divided into three groups. One group remained untreated (control). In the other two groups, a loop of descending colon was exteriorized to create a hernia of the abdominal wall. After 4 weeks' recovery, this loop was locally irradiated with 5× 5 Gy of γ-rays or sham irradiated. One week after (sham-) irradiation, an anastomosis was performed in all groups. Tissue oxygenation (StO_2) was determined with visible light spectroscopy. The animals were sacrificed 3 or 7 days after the operation and the anastomosis was tested for bursting pressure and breaking strength.

Results Irradiated rats showed significantly more weight loss (90% SD 4.3 of initial body weight vs. 96% SD 2.8, $p \leq 0.05$) and enteritis (18% vs. 5%, $p=0.013$) compared to sham and control animals. StO_2 was not influenced by irradiation and was not predictive for anastomotic strength. The control group showed significantly lower bursting pressure and breaking strength compared to (sham-) irradiated animals.

Conclusion We developed a new isolated loop model for intermittent irradiation of the colon. Preoperative irradiation of the distal part of a colon anastomosis was successfully administered with acceptable side effects and did not cause reduced tissue oxygenation nor clinical signs of anastomotic weakening, nor objective reduction in bursting pressure and breaking strength.

Keywords Colorectal anastomosis ·
Preoperative irradiation · Tissue · Oxygenation ·
Visible light spectroscopy · Fractionated irradiation model

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Introduction

Leakage of anastomosis after colorectal resection is a serious problem in gastrointestinal surgery [1]. After the introduction of preoperative irradiation, conflicting results have been reported on the incidence of anastomotic leakage [2–5]. In several animal [6–11] and clinical [4, 5, 12] studies, decreased anastomotic strength or higher incidence of anastomotic leakage after irradiation was demonstrated. Nevertheless, in most of the animal studies, the irradiation protocols were not comparable or even completely different

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from the clinical situation. In studies simulating clinical protocols with respect to fractionation of preoperative radiation doses [11, 13, 14] and irradiation of one or both limbs of the anastomosis [15], the irradiation technique appeared to be a major challenge. The murine colon is difficult to irradiate without considerable damage to surrounding tissue. In these studies, the colon was irradiated in situ by placing radiopaque markers and dosimeters during a laparotomy [13, 15] or by irradiation of the entire pelvis which resulted in high mortality [11, 14].

The detrimental effects on normal tissue in the acute phase after irradiation are thought to result mainly from loss of intestinal crypts and an acute inflammatory reaction, which might result in reduced blood flow [16]. This reduction in blood flow may play an important role in anastomotic weakening as shown by a study investigating microvascular blood flow after irradiation measured by laser Doppler in relationship to anastomotic strength of the colon [6]. A clear decrease in blood flow 4 weeks after irradiation with 5× 8.5 Gy in combination with diminished anastomotic strength was found. As in capillary beds such as colon mucosa pulsatile blood flow required for laser Doppler velocimetry is absent, we used a technique for measurement of microperfusion and thus tissue oxygenation based on visible light spectroscopy. The system uses the principle of absorbance and scattering of light in tissues, not requiring pulsatile blood flow or tissue contact. In previous studies [17–21], visible light spectroscopy has been validated to be useful for the detection of tissue ischemia in human gastric and colonic mucosa.

The aim of this study was twofold. First, to develop a novel murine model for localized fractionated irradiation of the descending colon based on a technique previously described for fractionated small bowel irradiation [22, 23] to simulate the clinical relevant context in colorectal cancer more closely with respect to fractionation of irradiation, irradiation damage of surrounding tissue, and generalized irradiation effects. Second, to evaluate tissue oxygenation and anastomotic strength after preoperative irradiation using an irradiation scheme.

Materials and methods

Overview of study design

An overview of the design of the study is shown in Fig. 1. After inclusion, animals were randomly assigned per cage to receive an abdominal wall hernia of the sigmoid colon (irradiation and sham group) or to serve as control group. Four weeks after the hernia operation, the surviving animals were divided into an irradiation group and a sham group. At each operative procedure, tissue oxygenation of the colon segments forming the future anastomosis was evaluated with visible light spectroscopy (VLS). In the week following irradiation of the herniating exteriorized colon segment with 5×5 Gy or sham irradiation, an anastomosis within this colon segment was constructed. The animals were terminated on the third or seventh postoperative day. Signs of anastomotic leakage, such as abscess or dehiscence, and adhesion formation were noted. Anastomotic strength was quantified by measurements of bursting pressure and breaking strength.

A power analysis based on the results of a pilot study revealed that a sample size of 23 animals per group was needed to detect a difference of 15 mmHg in bursting pressure between the irradiation and the sham group on the third postoperative day ($\alpha=0.05$, $\beta=0.95$). Twenty-six additional animals were included to compensate for anticipated mortality of 30% after the hernia operation

Fig. 1 Overview of the study design. The study groups are shown on the *left*, the interventions for each group are shown on a time scale in weeks. *Arrows* indicate irradiation, † indicates termination
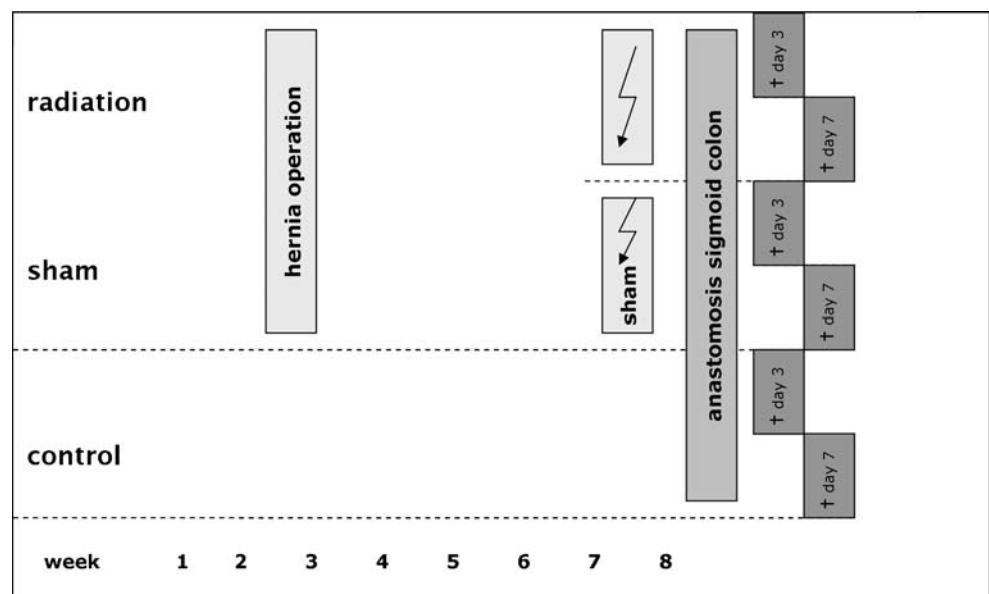
(based on unpublished data from a pilot study conducted at our center). The study protocol was approved by the Animal Ethics Committee of the University of Groningen.

Animals

Male Wistar rats ($n=164$, mean weight 344 g; Harlan BV, Horst, The Netherlands) were allowed to adapt to laboratory conditions for 1 week before experimental use. The animals were housed five per cage under specified pathogen-free conditions and had free access to water and standard rodent food (AB diets, Woerden, The Netherlands). After each laparotomy, the first 24 h 0.03 mg/kg buprenorphine s.c. was administered twice daily. To compensate for fluid loss, 10 ml 0.9% NaCl was administered subcutaneously. The animals were housed in separate cages until passage of stool. Based on the findings in a pilot study (data not shown), the animals were euthanized on the fourth postoperative day if no passage of stool was observed. After an operation or (sham) irradiation, they were weighed daily and inspected for signs of illness.

Hernia operation

Rats in the irradiation and sham groups were anesthetized with a mixture of isoflurane 2.5% and oxygen and received 0.03 mg/kg buprenorphine s.c. after induction of anesthesia. The abdomen was shaved in the midline and disinfected. During the operation, body temperature was maintained at 38°C using a heating pad. The operation was performed under semi-sterile conditions.

The steps carried out to create an abdominal wall hernia containing descending colon approximately 1.5 cm from the peritoneal reflection are shown and described in Fig. 2. The skin was closed with an intracutaneous resorbable running suture (Monocryl™ 5-0, Ethicon, Amersfoort, The Netherlands).

Visible light spectroscopy measurements of StO_2 were performed at the herniating sigmoid colon and at the cecum before and after construction of the hernia. A system consisting of a VLS oximeter (T-Stat®, model 303 Ischemia Detection System; Spectros Corp., Portola Valley, CA, USA) was used. Details and descriptions of this system have been described previously [18, 19]. In short, the oximeter emits white light from a probe (5 mm diameter) placed on or near the tissue, collecting any light returning to the probe from the tissue. VLS uses the principle of absorbance and scattering of light in biological tissues. Each type of molecule has a characteristic absorbance spectrum. As oxygenated and deoxygenated hemoglobin have a different absorption spectrum and hemoglobin is considered a critical derivative of perfusion, tissue microperfusion can be assessed using a technique that detects these differences (Fig. 3). To measure StO_2, the probe was held a few millimeters above the gauze-cleaned serosal surface until values stabilized within a 5% margin..

Technique of irradiation

After 4 weeks, the irradiation group was anesthetized with 40 mg/kg ketamine and 0.1 mg/kg medetomidine s.c. and placed in lateral position on a 2-cm lead shield with a 2× 1.5-cm opening for the hernia (Fig. 4). On five consecutive days, 5 Gy gamma (Cs-137) irradiation (CIS bio international, IBL 303) with a focus subject distance of 25 cm was administered with exposure to scatter radiation of <5%. The sham group was treated similarly to the irradiation group, but no irradiation was applied. In case of diarrhea, the animals were rehydrated with 16 ml 0.9% NaCl s.c. in two doses daily until weight gain and cessation of diarrhea. Otherwise, no side effects were noticed.

Anastomosis operation

Three to 7 days after irradiation, all animals were anesthetized and prepared for surgery as described previously (see the section on 'Hernia operation'). The anastomosis operation was performed using an operation microscope. The skin was opened and the herniating colon loop was carefully freed of adhesions from fascia and omentum. StO_2 of the herniated bowel was measured as soon as serosa became visible. Next, the abdomen was opened and a 1-cm segment of the descending colon was resected ensuring that only the distal part of the anastomosis consisted of irradiated sigmoid. Bowel continuity was restored by an end-to-end anastomosis using 10–12 inverting interrupted non-absorbable monofilament sutures (Dafilon® 8-0, Braun, Tuttlingen, Germany). After construction of the anastomosis, StO_2 values of the colon proximal and distal to the anastomosis were measured. The abdomen was closed using a continuous resorbable suture (Polysorb™ 4-0, Tyco Healthcare, Gosport, UK) for the musculofascial layer and the skin was closed with an intracutaneous running resorbable suture (Monocryl™ 5-0, Ethicon). In the control group, the same procedure was followed.

Termination

Either 3 or 7 days after anastomosis construction, the animals were anesthetized. After relaparotomy, StO_2 of the colon proximal and distal to the anastomosis was measured. The abdominal cavity was explored for signs of ileus and dehiscence of the anastomosis. The animal was subsequently terminated by intracardiac pentobarbital injection.
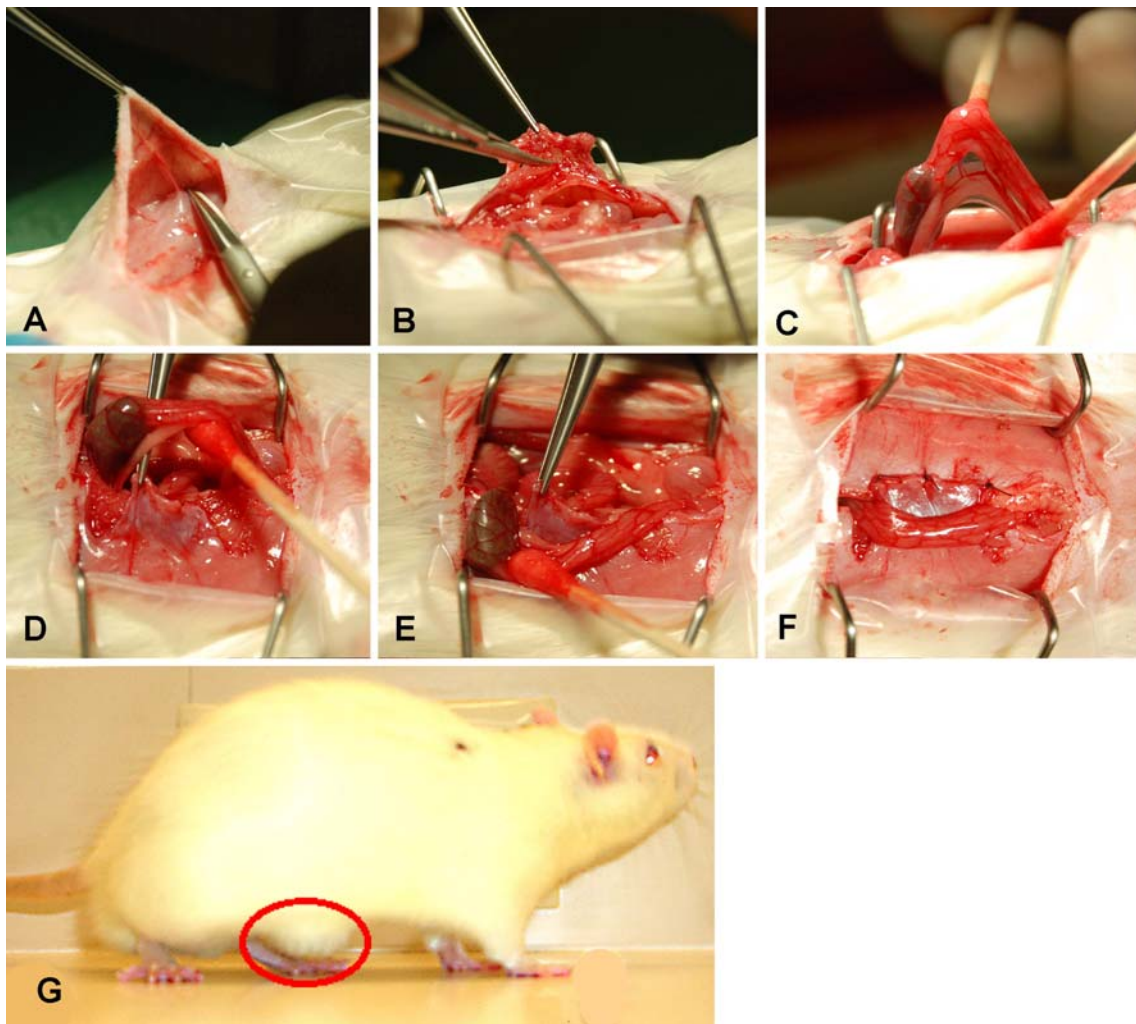
Fig. 2 Creation of an abdominal wall hernia. First a subcutaneous pocket was created (**a**). After laparotomy, a musculofascial flap from the rectus abdominis muscle was mobilized by sharp dissection (**b**). The mesentery of the descending colon was opened and the descending colon and its vasculature was lifted from the abdominal cavity (**c**). Subsequently, the musculofascial flap was pulled through the opening in the mesentery (**d**, **e**) and sutured to the contralateral fascia of the rectus abdominis muscle (**f**). The skin was closed covering the herniating colon loop. A rat with completed abdominal wall hernia (*encircled in red*) is shown (**g**)
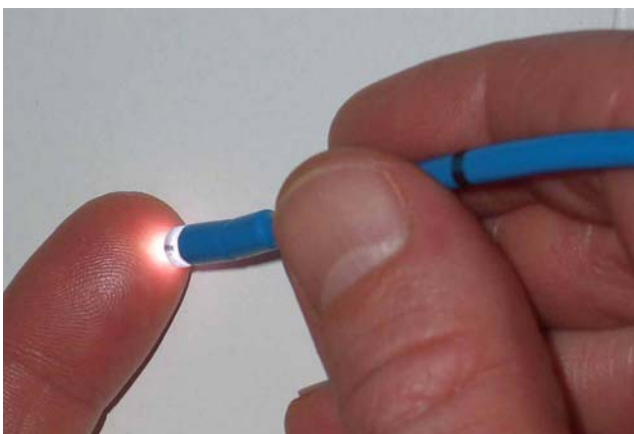

Fig. 3 Visible light spectroscopy system: handheld probe used in this study (T-Stat® 303 Ischemia Detection System; Spectros Corp., Portola Valley, CA, USA)
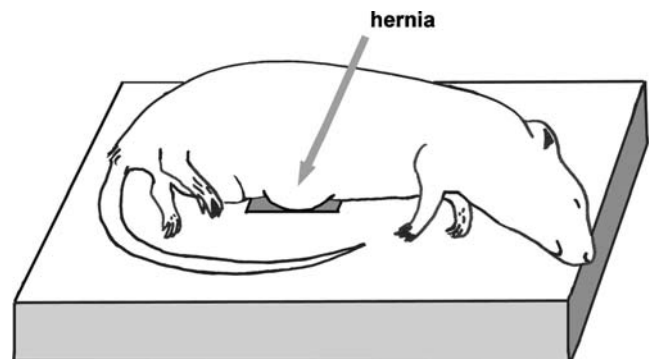

Fig. 4 Rat positioned on lead shielding for irradiation. To achieve isolated irradiation and to minimize systemic effects on the abdomen, the hernia containing the descending colon (*arrow*) is placed above an opening of 2×1.5 cm in the lead shielding

Anastomotic strength

Anastomotic strength was evaluated on the third and seventh postoperative day by measuring resistance to intraluminal pressure (bursting pressure) and to longitudinal forces (breaking strength) by using well-described methods [24–28].

After termination, the anastomosis was resected together with adhesions and adhesive structures. The severity of adhesions was noted as none, mild, moderate, and severe as described by van der Ham et al. [29]. Intraluminal feces were removed.

Subsequently, all colon segments were tied off at the distal end. A cannula was inserted in the proximal end and the colon was tied around it. The cannula was connected to an infusion pump and a manometer and infused at a rate of 1 ml/min with methylene blue dissolved in saline. The rising pressure was recorded graphically and the bursting pressure (in mmHg) was determined and defined as the maximal pressure the anastomosis or colon segment resisted. The site of leakage was recorded.

Thereafter, the segment was placed in a tensiometer system (Mecmesin AFG 10, Horsham, UK). A constantly increasing force was applied and the linear breaking strength was recorded. This procedure has been previously described and validated [28].

Statistical analysis

Data were analyzed with Statistical Package for the Social Sciences software (SPSS 15-0, SPSS, Chicago, IL, USA). Statistical differences between groups were calculated using one-way analysis of variance (ANOVA), Student t-test, and chi-squared test when appropriate. Correlations between variables were analyzed by Pearson or Spearman correlation or multiple logistic regression analysis when appropriate. Power analysis was carried out by using Sample Power 2.0 (SPSS). Statistical significance was accepted at the 5% level.

Results

General outcome

An overview of the mortality of the hernia operation, irradiation procedure, and anastomosis operation, respectively, is shown in Fig. 5.

Hernia operation

All animals suffered transient weight loss after the hernia operation (mean weight loss 5% SD 5.4%). The mean

Fig. 5 Flow chart of included animals. *Control*: control group. *Hernia*: first operation in which an abdominal wall hernia was constructed. † indicate mortality and causes. *Sham*: sham group. *5× 5 Gy*: irradiation group, irradiated with 5×5 Gy. *Anastomosis*: second operation during which an anastomosis was constructed. *Day 3/7*: postoperative day when termination took place. *Bold numbers* represent numbers included
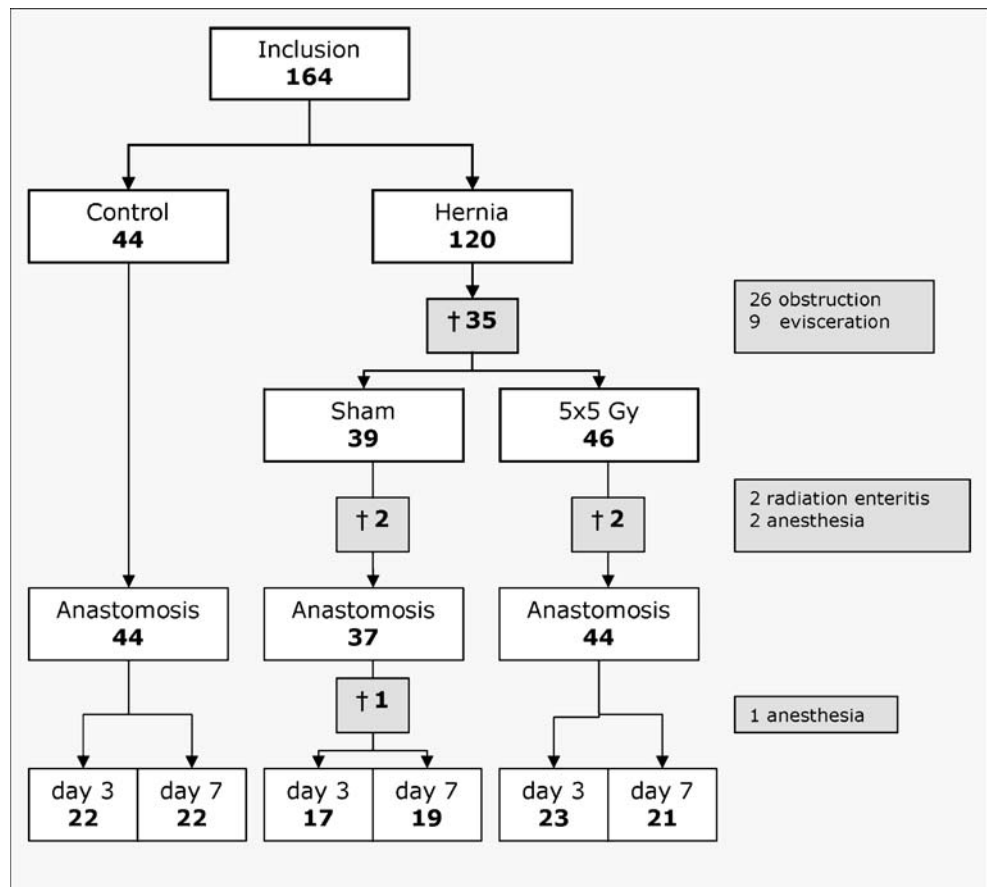
period until passage of stool was 0.8 (SD 1.3) days. Twenty-six (22%) animals showed persistent bowel obstruction at the fourth postoperative day and were terminated according to protocol. On termination, obstruction of the colon at the site of the hernia was found in all animals, and in one animal an abscess due to a perforation at the level of the fascia was seen. In nine (7%) animals, evisceration after wound dehiscence due to gnawing had occurred and these animals were euthanized the first day after hernia operation. The overall mortality of the hernia operation was 29% (35 animals).

Irradiation

During irradiation, animals in both the irradiation and the sham group lost weight; this was significantly more pronounced in the irradiation group, as shown in Fig. 6 (90% SD 4.3 of initial body weight vs. 96% SD 2.8).

In 19 (22%) of the animals, enteritis was observed, 15 of them after irradiation (sham vs. irradiation, $p=0.013$) as shown in Table 1, indicating successful irradiation of the bowel. Diarrhea typically started on the last day of the irradiation scheme and resolved after 1–3 days. On the last irradiation day, three animals were considered too sick for anesthesia and transport. Therefore, in three cases, the last irradiation took place 2 days later than scheduled. These three animals survived and were analyzed in the irradiation group and separate analyses showed no difference.
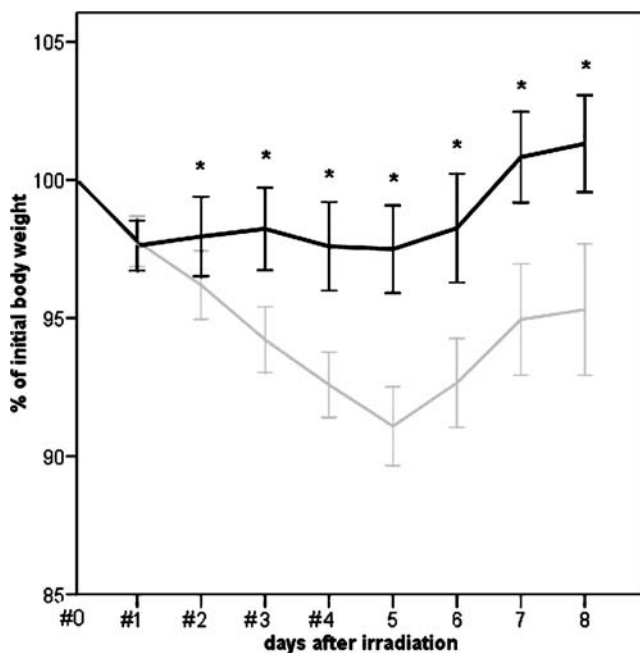

Fig. 6 Percentage of body weight change during and after 5×5 Gy irradiation of the isolated colon segment. * indicate significant difference ($p \leq 0.05$, *t*-test) between irradiation and sham group. *Light grey* indicates radiation group, *black line* indicates sham group. *Error bars* show 95% confidence interval

Table 1 Enteritis after irradiation

	no enteritis	enteritis	p
radiation	31 (36%)	15 (18%)	0.013
sham	35 (41%)	4 (5%)	
total	66 (78%)	19 (22%)	

Between brackets percentages of the total number of animals are indicated

During irradiation, four animals died. Two animals in the sham group died during anesthesia. At obduction, no clear cause of death could be identified. Two animals of the irradiation group showed severe enteritis with persistent weight loss and progressive abdominal distension and were euthanized. On post-mortem examination, severe paralytic ileus was seen.

Anastomosis operation

In most animals of the irradiation group, mild to moderate edema of the herniating colon loop was present at laparotomy. During the postoperative period, all animals lost weight, which was most pronounced in the sham group. At anastomosis operation, the control group (384 g SD 29) was heavier compared to the radiation group (354 g SD 31, $p \leq 0.05$) and comparable to the sham group (377 g SD 25, $p=0.83$). During or after the anastomosis operation, one animal in the sham group died during anesthesia. At termination, no clear cause was identified, but the animal had suffered considerable weight loss due to enteritis.

The mean period until passage of stool after the operation was 0.4 (SD 0.9) days. None of the animals was killed earlier than scheduled due to ileus. At termination, three animals (2%, two sham and one control) showed signs of obstruction at the anastomosis without clear evidence for anastomotic leakage.

At termination, adhesions were absent in four (3%) animals, mild in 36 (29%), moderate in 44 (35%), and severe in 40 (32%) animals. There were no significant differences between irradiation, sham, and control group nor between early (day 3) and late (day 7) termination.

Colon oxygenation

The changes in StO_2 as measured by VLS are shown in Fig. 7. There were no significant changes in StO_2 in the colon proximal or distal to the anastomosis. At univariate regression analysis, there was no significant correlation between StO_2 and anastomotic bursting pressure nor breaking strength. There were no correlations between StO_2 and clinical outcome parameters (i.e., adhesions, ileus, enteritis, and mortality).
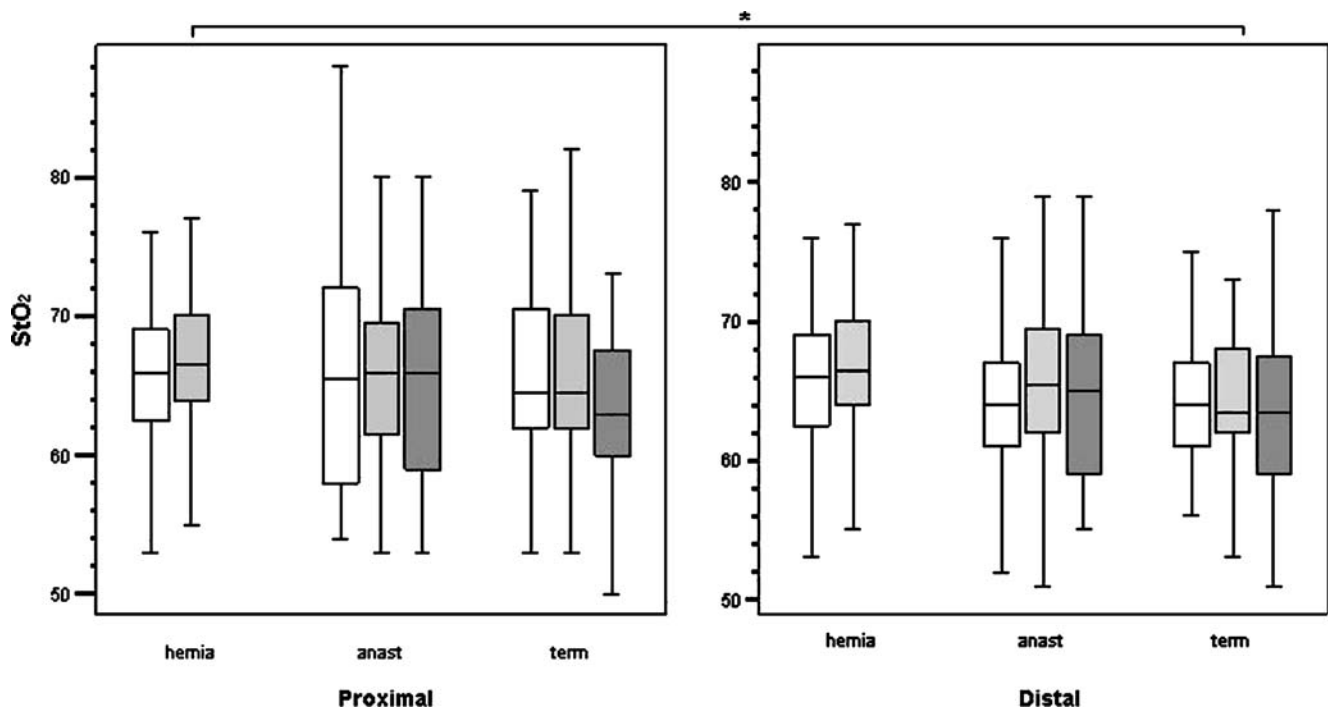
Fig. 7 StO$_2$ at proximal and distal part of the anastomosis. The irradiation group is shown in *white bars*, the sham group in *light grey*, and the control group in *dark grey*. 'hernia' designates StO$_2$ measured during the hernia operation, '*anast*' the anastomosis operation and '*term*' prior to termination. *Boxes* represent first and third quartiles with the median as a *central line*. The *whiskers* represent 5th and 95th percentiles. * indicates significant differences ($p<0.05$)

Anastomotic strength

Anastomotic strength expressed as bursting pressure and breaking strength increased significantly between the third and seventh postoperative day in all groups, as shown in Fig. 8.

On the third and seventh postoperative day, bursting pressures were significantly higher in both the irradiation (159 mmHg SD 32/196 mmHg SD 39) and sham (167 mmHg SD 25/209 mmHg SD 40) group when compared to the control group (105 mmHg SD 56/ 175 mmHg SD 22). In breaking strength measurements, a similar pattern was seen on day 3 with significantly higher resistance to longitudinal forces in both the irradiation (244 g SD 37) and sham (235 g SD 57) group compared to the control group (174 g SD 62). On day 7, the difference in breaking strength was only significant between the sham and the control group (irradiation 323 g SD 65, sham 364 g SD 103, control 303 g SD 82).

There was no significant difference in bursting pressure or breaking strength between the irradiation and the sham group on the third or seventh postoperative day. In Table 2, the percentage of anastomoses bursting within the anastomotic line are shown.

Discussion

In this study, we describe a new animal model with an isolated colon loop to investigate the effects of fractionated preoperative radiotherapy on colon anastomosis closely resembling the clinical situation.

In most earlier conducted animal studies, intraoperative single-dose irradiation of both limbs of the anastomosis [6, 9, 10, 30] was applied. This resulted in weakening of the anastomosis in some of the studies [6, 9, 10], while others did not detect this effect [13, 30]. In these studies, all evaluating anastomotic strength after irradiation, high irradiation doses without fractionation were applied, but these were not representative for the clinical situation [6–10]. In the clinical situation, usually CT-planned irradiation with 5×5 Gy is applied on the rectum with minimal involvement of the adjacent tissue. After colorectal resection, the remaining, previously irradiated rectal stump forms the distal limb of the anastomosis, whilst the proximal part consists of non-irradiated descending colon, thus creating a partially irradiated anastomosis. Several animal studies were conducted evaluating clinically relevant fractionated preoperative irradiation schemes. Two studies describe conflicting results using fractionated
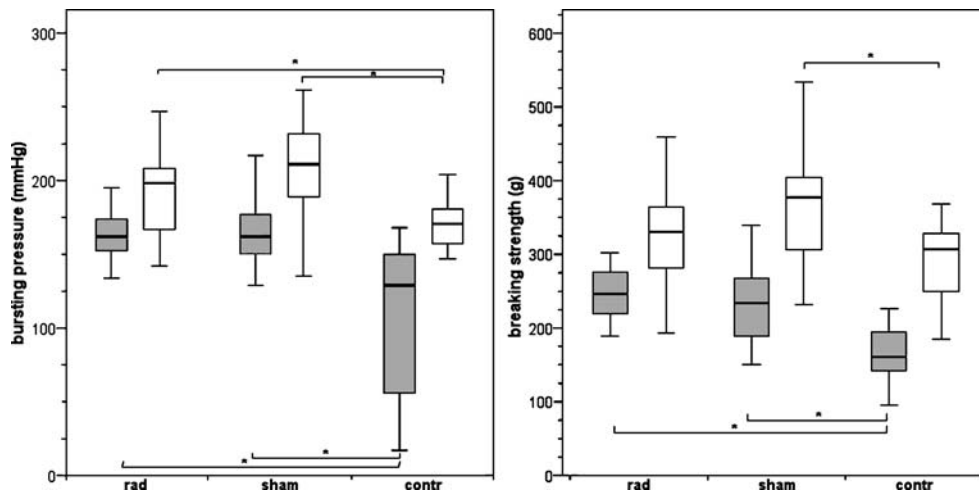
Fig. 8 Bursting pressure and breaking strength 3 (grey bars) and 7 (white bars) days after colon anastomosis. rad represents irradiation group, sham sham-irradiated group, contr control group. Boxes represent first and third quartile with the median central. The whiskers represent 5th and 95th percentiles. * marks significant difference (p=0.05)

irradiation of the colon in situ after previous implantation of radiopaque markers and dosimeters [13, 15]. First, De Meerleer et al. found weakened anastomoses after cumulative doses higher than 80 Gy [15], which are never used clinically. Indeed, Ceelen et al. describes no diminished anastomotic strength after total doses up to 80 Gy [13]. Moreover, De Meerleer et al. [15] studied irradiation of one limb of the anastomosis vs. irradiation of both limbs and found diminished bursting pressure after irradiation of both limbs of the anastomosis, whereas no effect was seen after irradiation of only one limb. Kuzu et al. applied 5×5 Gy irradiation on the pelvis in rats and found diminished bursting pressure and hydroxyproline content of the anastomosis, but this study did not resemble the clinical situation as both limbs of the anastomosis were irradiated [11]. Seifert et al. [6] describe similar results although in the early phases of healing all anastomoses were weaker after irradiation, whereas at 7 days postoperatively only anastomoses after irradiation of both limbs showed diminished bursting pressure. In this study, however, an irrelevant

irradiation scheme as single-dose intraoperative irradiation with 25 Gy was applied.

Apart from studies describing clinically irrelevant irradiation schemes, previously applied methods for preoperative fractionated irradiation of colon anastomoses have a high mortality. Kuzu et al. reports a 27% mortality after pelvic irradiation [11] and De Meerleer et al. found a 26% mortality due to implantation of dosimeters [15]. Apart from a lower mortality (22%), the hernia technique presented in this paper is the only technique in which an isolated colon loop can be irradiated with relatively low mortality of the irradiation itself, making it more suitable for evaluation of higher irradiation doses in the current and future studies in order to elucidate the effect of prolonged irradiation as given in the human situation for locally advanced malignancies. The model could also be suitable to evaluate radioprotective compounds for protection of the large bowel, such as 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (Tempol), a nitroxide radioprotector [31].

This study confirms the results from several other studies evaluating anastomotic strength after irradiation with 5× 5 Gy, being that no signs of anastomotic weakening by low dose preoperative irradiation of the colon could be detected. Surprisingly, in our study, both the irradiated and sham-irradiated colon showed even higher bursting pressure and breaking strength when compared to control animals. As this increased strength after irradiation has not been described previously, this finding might be explained by the observed adhesions caused by the hernia operation, but based on our data no clear conclusions can be drawn so far. There were no differences between the irradiation and the

Table 2 Percentage of rupture within the anastomotic line in bursting pressure measurements

	% rupture within anastomosis	
	day 3	day7
irradiation	48	0
sham	35	
control	82	14

*p=0.008, #p=0.057 between sham and irradiation vs. control group

sham group with respect to anastomotic strength. Contrary, significant effects of irradiation were observed with regard to weight loss and enteritis in the radiation group compared to the sham group, as might be expected.

In conclusion, we successfully developed a new animal model suitable for fractionated irradiation of an isolated colon loop by surgically placing a colon loop in an abdominal wall hernia. Partially irradiated anastomoses in the sigmoid colon, irradiated with 5×5 Gy, did not show diminished anastomotic strength. The model for intermittent irradiation of a short, isolated colon segment is a useful model to study the effect of irradiation dosage and fractionation in future studies either for surgical studies or for evaluation of compounds to diminish irradiation effects of the colon.

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