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Published in:
Journal of Gastrointestinal Surgery

DOI:
10.1016/j.gassur.2024.08.008

Publication date:
2024

Document version:
Final published version

Journal of Gastrointestinal Surgery 28 (2024) 1777–1782

Contents lists available at ScienceDirect

Journal of Gastrointestinal Surgery

journal homepage: www.jogs.org

Original Article

Short cycles of remote ischemic preconditioning had no effect on tensile strength in small intestinal anastomoses: an experimental animal study

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ARTICLE INFO

Article history:
Received 15 June 2024
Received in revised form 19 July 2024
Accepted 6 August 2024
Available online 9 August 2024

Keywords:
Anastomotic healing
Maximal anastomotic tensile strength
Remote ischemic preconditioning
Small intestine

ABSTRACT

Purpose: This study aimed to investigate the effect of remote ischemic preconditioning (RIPC) on the healing of small intestinal anastomoses, evaluated by tensile strength and histologic wound healing on postoperative day 5.

Methods: A total of 22 female pigs were randomized 1:1 into either an intervention or control group. The intervention group received 5 cycles of 3-minute ischemia followed by 3-minute reperfusion on the right forelimb. Two end-to-end anastomoses, a distal and a proximal, were created in the small intestine 30 and 60 min after RIPC, respectively. On postoperative day 5, the anastomoses were harvested and underwent a maximal anastomotic tensile strength (MATS) test (MATS 1–3) followed by histologic analyses.

Results: MATS 1, when a tear became visible in the serosa, was significantly increased in the proximal anastomoses of the RIPC group compared with the control group (4.91 N vs 3.83 N; P = .005). No other significant differences were found when comparing these 2 groups.

Conclusion: Our study showed no convincing results of RIPC on intestinal anastomotic healing to recommend its use in a general clinical setting. Further animal studies on RIPC's effect after relative or absolute intestinal ischemia may be recommended.

Introduction

Anastomotic leakage (AL) is a severe and potentially life-threatening complication to intestinal resection. The AL rates vary from 1.8% to 19.2% depending on the location in the gastrointestinal tract, with a higher risk in colorectal anastomoses [1,2]. After intestinal resection, there is a risk of ischemia and reperfusion injury (IRI), which might impair wound healing with an increased risk of AL [3].

Remote ischemic preconditioning (RIPC), which consists of short episodes of induced ischemia; for example, with a tourniquet on the forearm, has been shown to protect against IRI in several organs, although with conflicting results [4,5]. The physiologic mechanisms behind RIPC have not been fully understood, but are believed to occur through humoral, neural, and immunologic pathways [6].

Several studies have investigated various protocols with regard to the number and duration of ischemic cycles to obtain the optimal benefit of RIPC, but no definite protocol has been established [7–9].

From a clinical point of view, the preoperative application of short cycles of RIPC to improve anastomotic healing may be relevant and interesting to study.

This randomized controlled study on pigs aimed to investigate the effect of RIPC, before creating small intestinal anastomoses. The effect was measured by maximal anastomotic tensile strength (MATS) and histologic healing on postoperative day 5 and compared with a matched nonpreconditioned group. Finally, we aimed to investigate whether the time from RIPC to the creation of the anastomoses had an influence on MATS and/or histologic healing.

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https://doi.org/10.1016/j.gassur.2024.08.008

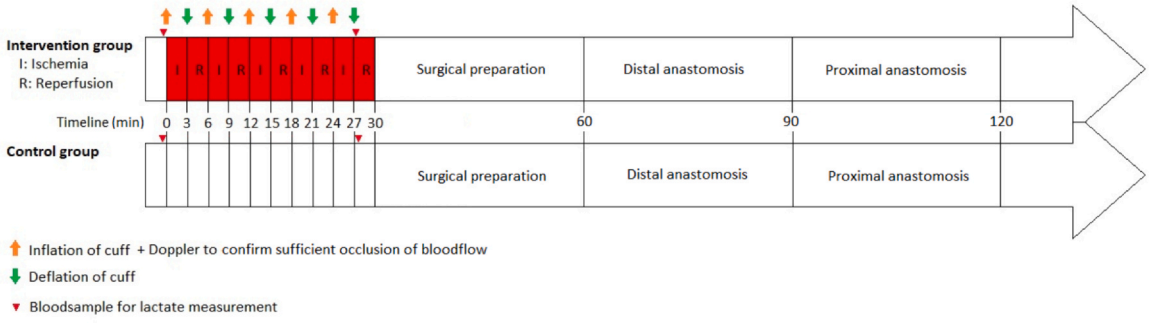

Figure. Study design.

Materials and methods

Study design

In this randomized controlled study, pigs were randomized (www.randomization.com) 1:1 into a control group or an intervention (RIPC) group. In the intervention group, a blood flow restriction band (BFR bands, San Diego, USA) was placed on the right forelimb and inflated to more than 300 mm Hg. Sufficient occlusion of the arterial blood was controlled by Doppler ultrasound (Ultrasound Vascular Doppler, The Occlusion Cuff Ltd). The inflation lasted for 3 min followed by 3 min of reperfusion. The procedure was repeated for a total of 5 cycles. Before the first and after the last cycle of ischemia, a blood sample was taken from one of the hindlimbs to measure the systemic lactate levels (Epoc, Epocal Inc). Afterward, the surgical procedure was initiated and included the creation of 2 small intestinal anastomoses, respectively 30 (distal) and 60 min (proximal) after RIPC. In the control group, there was a 30-minute waiting period after induced anesthesia, equivalent to the RIPC duration, before the start of the surgical procedure (Fig.).

Postoperatively the pigs were observed for pain, evaluated by inactivity, reduced food intake, shivering, piloerection, and hyperventilation. Untreatable pain or failure to thrive would result in premature euthanasia after evaluation by an independent veterinarian. A record of all administered medications and observations was kept for each animal.

On postoperative day 5, the pigs were anesthetized and underwent a relaparotomy, in which the anastomoses were harvested for analysis. Subsequently the pigs were euthanized while still under anesthesia.

Sample size

A previous study has shown a mean MATS 3 (maximum load before the load strain curve decreased) of 12.9 N in small intestinal anastomoses in pigs on postoperative day 5 with an SD of 2.4 N [10]. An increase in MATS of at least 25% was considered clinically relevant, and with a power of 80% and a significance level of 0.05, this study would require 10 pigs in each group to show statistical significance. With an expected mortality of 10%, a total of 22 pigs were included.

Animals

Weaned female LY-pigs (Danish Landrace × Yorkshire) of mean weight 31.0 kg (SD ± 3.08) were included in the study. The pigs arrived 1 week before the first operation day and were acclimatized at the biomedical laboratory.

Premedication, anesthesia, and postmedication

The pigs were premedicated with an intramuscular injection of medetomidine (0.03 mg/kg) (CP-Pharma Handelsges mbH), midazolam (0.25 mg/kg) (Hameln Pharma GmbH), ketamine (5 mg/kg) (Intervet International B.v.) and butorphanol (0.2 mg/kg) (Richter Pharma AG). General anesthesia was induced with propofol (10 mg/kg) (Orion Cooperation) before the pigs were intubated with a cuffed orotracheal tube.

Prophylactic antibiotics consisted of metronidazole (20 mg/kg) (B. Braun Melsungen AG) given intravenously, combined with an intramuscular injection of amoxicillin (15 mg/kg) (Univet Ltd) upon arrival to the operating room approximately 45 min before skin incision.

General anesthesia was maintained by infusion of propofol (15 mg/kg/h) (Orion Cooperation) and fentanyl (50 µg/kg/h) (Hameln Pharma GmbH). The pigs were mechanically ventilated with a tidal volume of 7 to 10 mL/kg and a respiratory frequency of 16 to 20 per minute.

During surgery, the pigs were placed on a heating pad set to 40 °C to prevent hypothermia. They received Ringer acetate (Fresenius Kabi AB) intravenously at a rate of 4 to 10 mL/kg/h. Noninvasive blood pressure, electrocardiogram, heart rate, oxygen saturation, and capnography were monitored continuously and recorded every 30 min.

For postoperative pain treatment, a transdermal fentanyl patch (2 µg/kg/h) (Takeda Pharma A/S; Lavipharm S.A.) was placed behind the ear at the start of surgery. To ensure sufficient painkilling, repeated doses of buprenorphine (0.03 mg/kg) (Richter Pharma AG) were given intramuscularly every 4 to 6 h during the first postoperative day.

Surgical procedure

Through a 10 cm long lower midline laparotomy, the ileocecal ligament was identified. At 1 and 3 m proximal to the ileocecal ligament, the small intestine was transected and 2 end-to-end anastomoses with a continuous seromuscular Monocryl Plus 4–0 suture (Ethicon, Johnson & Johnson International) were performed.

The abdominal fascia was closed with a continuous PDS Plus 2–0 suture (Ethicon, Johnson & Johnson International). The skin was closed intracutaneously with a continuous Monocryl Plus 3–0 suture (Ethicon, Johnson & Johnson International) and sealed with a liquid bandage (OPSITE spray, Smith & Nephew Medical Limited) [9].

On postoperative day 5, the pigs were anesthetized as described earlier. A relaparotomy was performed; the anastomoses were identified and examined for macroscopic findings such as adhesions, pseudodiverticula, abscess, visible leakage, signs of ileus, and stenosis. Adhesions were graded according to the modified Leach grading score of adhesion [11] (Supplementary Material).

Table 1
MATS values in the intervention (RIPC) and control groups.

Groups	Intervention (RIPC)			Control		
	All anastomoses, n = 22	Distal anastomosis, n = 11	Proximal anastomosis, n = 11	All anastomoses, n = 22	Distal anastomosis, n = 11	Proximal anastomosis, n = 11
MATS 1 (N)	5.76 (±1.68)	6.60 (±1.93)[b]	4.91 (±0.82)[a,b]	4.83 (±1.79)	5.84 (±1.97)	3.83 (±0.78)[a]
MATS 2 (N)	12.7 (±4.00)	12.6 (±4.53)	12.9 (±3.60)	12.7 (±3.26)	13.5 (±3.34)	11.8 (±3.10)
MATS 3 (N)	13.5 (±3.76)	13.2 (±4.30)	13.7 (±3.33)	13.2 (±3.03)	13.8 (±3.12)	12.5 (±2.94)

MATS, maximal anastomotic tensile strength; MATS 1, when a tear became visible in the serosa; MATS 2, when a transmural rupture appeared; MATS 3, a simultaneous drop in the load strain curve calculated by the software; N, number of anastomoses; RIPC, remote ischemic preconditioning.
All MATS data are presented as mean values with SD in parenthesis.
[a] $P < .05$ when comparing anastomosis in the intervention group with the one in the control group.
[b] $P < .05$ when comparing anastomoses within the same group.

After the anastomoses were freed from adhesions, small intestinal segments of 10 cm with the anastomoses in the center were excised and were subjected to the tensile strength test within 5 min to avoid the influence of cold ischemia.

Finally, the pigs were euthanized with an intravenous overdose of pentobarbital (140 mg/kg) (Richter Pharma AG).

MATS test

The tensile strength test was performed with Lloyd LF Plus (Lloyds Instruments) equipped with an XLC 100 N loadcell (Lloyds Instruments). The resected small intestinal segments were individually mounted to the testing machine that had been preset with a distance of 60 mm between the clamps. The segments were stretched at a constant deformation rate of 15 mm/min until rupture occurred.

The force applied was noted at different points during the examination: when a tear became visible in the serosa (MATS 1), when a transmural rupture appeared (MATS 2), and when a simultaneous drop in the load strain curve occurred as calculated by the software (NexygenPlus, Materials Testing Software) (MATS 3). Finally, it was noted whether the rupture was located within or outside the anastomotic line.

Histologic analysis

After the tensile strength test, tissue samples, chosen at random, but including the anastomotic line were fixated by being submerged in a 4% formaldehyde solution for at least 48 h. The samples were embedded in paraffin and sliced in 3 µm thick slices. The slices were stained with hematoxylin and eosin (HE), alpha smooth muscle actin (α-SMA), desmin, and sirius red.

HE was used to identify epithelium coverage and inflammatory reaction, which was used to assess the anastomotic healing by the Verhofstad score [12] (Supplementary Material) and to evaluate the ischemic changes both in- and outside of the anastomotic line by the Chiu score [13] (Supplementary Material). α-SMA and desmin staining were used to identify growth of smooth muscle cells. Sirius red was used for evaluating collagen content by estimating the amount of collagen compared with the total surface area of an anastomotic region as defined by Verhofstad [12].

All tissue samples were examined under a light microscope at ×10 to ×400 magnification. The histologic examinations were performed by an experienced pathologist blinded for the randomization.

Statistical analysis

Numerical outcomes were compared with linear regression between groups. In analyses including both proximal and distal measurements from the same pig, a linear mixed model with random intercept for each pig was applied to take into account dependence between measurements from the same pig. Ordinal outcomes were compared by ordinal logistic regression, applying an ordinal logistic mixed model in cases in which 2 measurements from each pig were included. Analyses were repeated stratified by distal and proximal anastomosis. P values < .05 were considered statistically significant and estimates were reported with 95% CIs. All analyses were performed in Stata 17 (StataCorp LLC).

Ethical considerations

This study was approved by the Danish Animal Experiments Inspectorate (J. nr. 2022–15-0201–01109) and was conducted in accordance with the Danish legislation on animal experiments and the ARRIVE guidelines [14].

Results

All pigs survived and completed the study with no signs of pain or other complications. All anastomoses were included in the statistical analysis. The mean weight of the pigs on the day of the first operation was 30.9 kg (SD ± 3.63 kg) and 31.0 kg (SD ± 2.60 kg) in the RIPC and control groups, respectively. The mean weight gain on postoperative day 5 was 2.26 kg (SD ± 1.06 kg) in the RIPC group and 2.59 kg (SD ± 0.99 kg) in the control group ($P = .46$).

There was no significant difference in systemic lactate concentration between the 2 groups ($P = .95$).

There were no signs of visible leakage, ileus, pseudodiverticulosis, intra-abdominal abscess formation, or anastomotic stenosis in any of the pigs. The highest modified Leach score [11] for degree of adhesions was 1, and there was no significant difference between the RIPC and control groups.

MATS: RIPC group vs control group

For the proximal anastomoses, the MATS 1 value was significantly higher in the RIPC group than the control group, with a mean value of 4.91 N and 3.83 N, respectively (Table 1) and with a nonadjusted intergroup difference of 1.08 (95% CI, 0.37–1.79) ($P = .005$). When adjusted for degree of adhesions, weight on the day of the first operation, and the site of rupture (inside vs outside the anastomotic line), the results remained statistically significant. There were no other statistically significant differences in MATS results when comparing the 2 groups, also when adjusted for the abovementioned factors (adhesions, weight, and site of rupture). The number of ruptures inside the anastomotic line was 13 and 8 in the RIPC and control groups, respectively. The difference was not significant.

Histologic parameters: RIPC group vs control group

There were no significant differences in any of the histologic parameters between the RIPC and control groups (Table 2).

Table 2
Histologic parameters based on Verhofstad [12] and Chiu [13] scores evaluated in intervention (RIPC) and control groups.

Groups	Intervention (RIPC)			Control		
	All anastomoses, n = 22	Distal anastomosis, n = 11	Proximal anastomosis, n = 11	All anastomoses, n = 22	Distal anastomosis, n = 11	Proximal anastomosis, n = 11
Collagen, %	20.7 (±8.35)	21.8 (±8.74)	19.5 (±8.20)	21.6 (±9.43)	22.7 (±12.3)	20.5 (±5.68)
Verhofstad score						
Necrosis	1.41 (±0.73)	1.36 (±0.81)	1.45 (±0.69)	1.55 (±0.96)	1.45 (±0.93)	1.64 (±1.03)
PMNs	1.86 (±0.64)	1.82 (±0.60)	1.91 (±0.70)	1.82 (±0.66)	1.82 (±0.75)	1.82 (±0.60)
Lymphocytes	1.27 (±0.46)	1.36 (±0.50)	1.18 (±0.40)	1.41 (±0.80)	1.73 (±0.65)	1.09 (±0.83)
Macrophages	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)
Edema	1.00 (±0.69)	0.82 (±0.75)	1.18 (±0.60)	1.05 (±0.72)	0.82 (±0.60)	1.27 (±0.79)
Mucosal epithelium	2.27 (±1.03)	2.27 (±1.01)	2.27 (±1.10)	2.41 (±0.96)	2.09 (±1.14)	2.72 (±0.65)
Submucosal muscular layer	1.45 (±0.60)	1.45 (±0.69)	1.45 (±0.52)	1.27 (±0.55)	1.18 (±0.40)	1.36 (±0.67)
Chiu score						
In anastomotic line	3.91 (±2.07)	4.09 (±2.02)	3.73 (±2.20)	3.86 (±2.14)	3.18 (±2.52)	4.55 (±1.51)
Outside anastomotic line	0.14 (±0.35)	0.18 (±0.04)	0.09 (±0.30)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)

PMNs, polymorphonuclear cells; RIPC, remote ischemic preconditioning.
All histologic parameters are stated as mean values with SD in parenthesis.

MATS in RIPC group: distal anastomoses vs proximal anastomoses

In the RIPC group, the mean value of MATS 1 in the proximal anastomoses was significantly lower than for the distal anastomoses—with a nonadjusted difference of −1.69 (95% CI, −2.70 to −0.69) ($P = .001$). When adjusted for degree of adhesions and weight on the day of the first operation, the results remained significant. There were no other significant results when comparing the distal and proximal anastomoses in the RIPC group (Table 1).

Discussion

This randomized controlled animal study in pigs found that 5 cycles of RIPC on the forelimb and with a duration of 3 min resulted in a statistically significant increase in only 1 of 3 parameters (MATS 1) of the anastomotic tensile strength tests on postoperative day 5 compared with the control group. It is worth noting that the only significant differences found in the study were for the proximal anastomoses between the 2 groups and between the proximal and distal anastomoses in the RIPC group. The 2 other parameters (MATS 2 and MATS 3) showed a nonsignificant tendency toward increased values in the RIPC compared with the control group. There were no significant differences in the anastomotic healing as evaluated by histology.

Both local and RIPC have shown to have a protective effect against reperfusion injury after induced ischemia in various organs, including heart, liver, kidney, brain, and intestines. This study was designed to explore the impact of RIPC on anastomotic healing, acknowledging the unclear physiologic mechanisms underlying its effects. Future research aimed at elucidating these mechanisms should consider incorporating serologic analysis of recognized humoral mediators [6].

Different protocols for RIPC have been reported but the optimal design has not been determined [7–9,15,16]. RIPC's cardioprotective effect after 25 min of global no-flow ischemia has been studied in a mouse model, in which 2, 4, 6, or 8 cycles of RIPC consisting of 5-minute ischemia followed by 5-minute reperfusion were performed. The results showed a significant effect after 4, 6, and 8 cycles, but not after 2 cycles [8]. In a study similar to ours, 2 cycles of RIPC, each of a duration of 15 min, had no effect on the anastomotic strength [9].

The duration of ischemia within each RIPC cycle could also be an important factor. A study on mice showed that 6 RIPC cycles of 2- and 5-minute ischemia had a cardioprotective effect compared with the control group and with no significant difference between the 2- and 5-minute groups. When investigating cycles with a duration of 10-minute ischemia, there was no significant effect [8]. This might suggest that RIPC should consist of shorter cycles of ischemia, potentially keeping each cycle more than 2 min, but less than 10 min. When it comes to histologic changes in the intestine, Hummitzsch et al. [15] found significantly reduced signs of IRI after 3 cycles of RIPC with a duration of 5 min in rats. Similar findings have been reported after 2 cycles of RIPC each of 10-minute ischemia [16] and after 1 cycle of either 5 min or 10 min [7].

Our research was designed as a continuation of a previous study [9], conducted by our research unit, with an exclusive emphasis on preconditioning because they found that postconditioning showed a detrimental effect on anastomotic healing. The previous study, which was based on a rat study that investigated the effect of RIPC on stabilizing intestinal anastomoses, performed 2 cycles of each 15-minute preconditioning [9]. In our current study, we expanded our focus to explore RIPC's application across various organs, adapting our protocol from research examining its impact on different tissues [7–9,15,16]. From this, we chose a study design of 5 cycles of 3-minute ischemia, given that most studies tend to favor multiple cycles and a shorter duration of each cycle, but it is possible that longer cycles up to 10 min would have been more effective. Another factor for choosing our design was that it could easily be transferred into a clinical setting. However, further studies are needed to find the optimal period of RIPC and the number of cycles.

The timeframe from RIPC to induced ischemia has also been studied. In the study by Johnsen et al. [8], after performing 6 cycles of 5-minute RIPC in mice, they waited 0.5, 1.5, 2.0, or 2.5 h before inducing prolonged ischemia to the heart. A significant reduction in infarct size was seen after 0.5 and 1.5 h, but no effect after 2.0 and 2.5 h. The authors concluded that an early window of protection could last up to 1.5 to 2.0 h after RIPC. Based on this, the design of our study included the creation of the anastomoses at 30 and 60 min after the completion of RIPC. Our findings of a significant increase in MATS 1 suggested that there is an early window of protection, which last at least until 60 min after RIPC. To further examine the protective window, we also compared the anastomotic strengths between the distal and proximal anastomoses in the RIPC group. Here we found that the distal anastomoses (created after 30 min) had a significantly higher tensile strength in 1 parameter (MATS 1) than the proximal anastomoses (created after 60 min). Given that this contradicted our expectations, we performed a similar analysis on the control group and found matching results, thereby suggesting that the difference between the distal and proximal anastomoses may be caused by other factors than the time from RIPC to creation of anastomosis; for example, a physiologic or anatomic variation in the strength of the small intestine depending on the location. This has

also previously been suggested after finding a decrease in small intestinal anastomotic strength with a more oral location [10].

It is difficult to compare different studies, given that they use different animal models, study designs, types of injury, and outcome measurements. A consensus report concluded that pigs are considered the most appropriate model for clinical translation to a human setting [17]. For organ injury, most of the animal studies have chosen to include a prolonged period of ischemia [3, 8, 15, 16]. To mimic the clinical situation and to examine whether RIPC could be used for elective procedures, the only trauma subjected to the intestine in our study was a transection followed by an anastomosis, because it has been suggested that this would to some extent lead to IRI [7]. However, given that our results only reached a statistical significance in 1 parameter for anastomotic strength, it could mean that a transection was too minor of a trauma to show a definitive effect of RIPC. The application of a more serious ischemic injury by clamping the marginal arteries and measuring blood flow by indocyanine arteriography as demonstrated by Gosvig et al. [10] could be an interesting model for future studies.

In an ideal setting, AL should be the primary outcome. This would require a large sample size to reach statistical power, which may not be considered ethically acceptable. Anastomotic tensile strength and anastomotic healing were chosen as outcome measurements because they are suitable surrogate markers for AL [17]. In this study, we chose tensile strength at postoperative day 5, given that this may reflect the clinical situation in which AL in most cases is seen on postoperative days 4 to 7. Furthermore, early anastomotic strength is dependent on the staple or suture holding capacity of existing collagen until fibroblasts and smooth muscle cells can synthesize more. Therefore, a postoperative time span of 5 days, before measuring endpoints, is suitable given that differences in MATS will be more evident in a tissue not completely healed to avoid tears outside of the anastomotic site. Bursting pressure could also have been an outcome measurement, but it seems more suitable for the investigation of the inflammatory phase of wound healing at postoperative days 2 to 3 [18,19].

A limitation could be the measuring of lactate levels systemically instead of locally. These did not show significant changes, which could be explained by clearance between RIPC cycles. Lactate levels had initially been thought to be used as a marker for ischemia. However, we had decided to use a Doppler as an additional tool to ensure sufficient compression during preconditioning. Our insignificant lactate changes could also be explained by our ischemic cycles of 3 min, which may not have been enough to induce complete ischemia in the peripheral tissue, but instead merely a state of low oxygen saturation. It is also important to note that the amount of muscle (metabolically active) tissue is sparse in both the forelimb and hindlimb of pigs compared with human extremities; as such the response of RIPC may not have been sufficient to have a protective effect on the intestinal anastomoses.

Other limitations could be our histologic evaluation. Regarding the collagen content, our analysis only consisted of a subjective estimation of the amount. A qualitative analysis on collagen content, collagen subtypes and maturation may have been more relevant. Furthermore, the use of the Verhofstad score [12], which focuses on the presence of inflammatory cells, may not have been sufficient, given that an inflammatory reaction is a physiologic process in wound healing. For future studies, it could be relevant to examine factors such as the restoration of the layers in the intestinal wall.

Finally, for standardization purposes, future studies may benefit from creating anastomoses mechanically (by means of staplers) as opposed to manually (use of sutures) as done in this study.

Conclusion

Our study showed no convincing results of RIPC on anastomotic healing to recommend its use in a general clinical setting. Further animal studies on the effect after relative or absolute intestinal ischemia may be recommended.

Funding

This study was supported by Odense University Hospital Fund for Free Research (No. R122-A5097) and A.P. Møller Doctors Foundation (No. L-2021-00171).

Author contributions

The idea for the project and its initiation were conducted by MZ, PTD, MBE, and NQ. All necessary approvals, fundraising, the execution of the experimental study, and data collection were carried out by MZ and PTD. GIM performed pathologic analysis of the samples. SM guided the statistical analysis. MBE, MDK, and NQ provided guidance throughout the study. MZ and PTD drafted the manuscript. All authors have actively contributed to and approved the final manuscript.

Data availability

The raw data of this study are available from the corresponding author, MZ, upon request.

Declaration of competing interest

The authors declare no competing interests.

Acknowledgments

The authors are grateful to the animal caretakers and the veterinarian at the Biomedical Laboratory at University of Southern Denmark for their help and assistance throughout the study and to Odense University Hospital Fund for Free Research (No. R122-A5097) and A.P. Møller Doctors Foundation (No. L-2021-00171) for funding this project.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.gassur.2024.08.008.

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