



Persistent effects of everolimus on strength of experimental wounds in intestine and fascia

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ABSTRACT

The introduction of mTOR-inhibitors in transplantation surgery has been associated with an increase in wound complications. We have previously reported a massive negative effect of everolimus on anastomotic strength in rat intestine at 7 days postoperatively. Because it is clinically important to know if this effect persists and occurs generally, repair in both intestine and abdominal wall has been investigated over a period of 4 weeks. Wistar rats received a daily dose of 1 or 2 mg/kg everolimus orally, from the operation day onwards. Controls received saline. In each rat a resection of ileum and colon was performed, and end-to-end anastomoses were constructed. On day 7, 14, and 28 the animals were killed and anastomoses and abdominal wall wounds were analyzed, wound strength being the primary parameter. Breaking strength of ileum, colon, and fascia was consistently and significantly reduced in the experimental groups at all time points. Anastomotic bursting pressures followed the same pattern. Loss of strength was accompanied by a decrease in hydroxyproline content after 7 days. Thus, the negative effect of everolimus on wound repair persists for at least 4 weeks after operation in this rodent model. This protracted effect may have clinical consequences and cause surgical morbidity.

The number of patients receiving solid organ transplantation is ever increasing. As a consequence, there is a growing number of people who depend on immunosuppressive drugs for transplant survival, not only immediately after operation but also in the long term. Therefore, efforts are aimed at improving immunosuppressive drugs and establish optimal regimens for both purposes.

Sirolimus is a lipophilic macrocyclic lactone antibiotic produced from *Streptomyces hygroscopicus*,¹ which binds to the mammalian target of rapamycin (mTOR) and blocks its function. Inhibition of mTOR-mediated pathways results in arrest of the cell cycle at the G1 phase in various cell types, including T- and B-lymphocytes, and thus constitutes a potent immunosuppressive tool. Everolimus, a more recently developed mTOR inhibitor, is very similar to sirolimus in its pharmacodynamic effects.²

Although rapamycin (sirolimus) was already discovered in the 1970s, interest in its use in transplantation surgery originated only a decade ago. The purpose of using mTOR inhibitors was minimizing cyclosporine exposure and avoidance of steroids, because of the relatively strong side effects of the latter drugs. Addition of an mTOR inhibitor to the polydrug posttransplantation regimen seemed promising, but now that sufficient clinical experience has been gathered, they appear to induce their own specific adverse effects. The most important clinical side effect recognized so far is a negative influence of mTOR inhibitors on wound repair. For instance, wound healing disorders were seen in 7–53% of renal transplantation patients and rapamycin derivatives (e.g., sirolimus) seem to play an important role.^{3–7} The same phenomenon is noted in cardiac transplant recipients.^{8,9} For the transplant recipient, sub-

optimal wound healing can lead to serious complications and therefore an understanding of the contribution of the individual drugs in this respect is essential and can only be gained from experimental studies.

Sound preclinical data on the effects of sirolimus on wound repair are only sparsely available, while nothing is known of everolimus, the mTOR inhibitor that has been the last in line to become available. Recently, we have described a strong, dose-dependent negative effect of everolimus on the healing of intestinal anastomoses in a rodent model. The negative effect on wound strength became apparent after the third postoperative day and was followed to the seventh day, at which point wound strength was still decreasing compared with the control group.¹⁰ This observation raises the clinically very relevant question if the disturbance of wound healing by everolimus is an early and transient effect or if it extends over a longer period of time, thereby presumably increasing the chances for wound complications even further. Here, we have followed wound repair for 4 weeks after operation. Apart from the anastomoses in ileum and colon, laparotomy wounds in the abdominal fascia have also been analyzed, in order to demonstrate that the effect is not limited to the intestine but found universally.

METHODS

Animals

Male Wistar rats (body weight 240–260 g; Harlan, Horst, the Netherlands) were housed two per cage and allowed to

become accustomed to laboratory conditions for 1 week before the start of the experiment. All animals had free access to water and standard rodent chow (Sniff Specialdiäten GmbH, Soest, the Netherlands). The study was approved by the institutional animals ethics review committee of the Radboud University Nijmegen.

Study design and surgery

One hundred forty-four rats were randomly divided into three groups of 48 animals each. Two groups received everolimus (Certican, a gift from Novartis Pharma BV, Arnhem, the Netherlands) orally by gavage in daily dosages of 1.0 (group E-1) and 2.0 (group E-2) mg/kg, respectively, starting 4 hours before the operation until termination of the experiment. A control group (group C) received the same volume of saline daily. All rats were operated on day 0 and 16 animals from each group were killed on postoperative days 7, 14, and 28, respectively, for analysis of wound healing. From each group of 16 animals, 10 were destined for biomechanical and biochemical analysis and six for histology.

On day 0, the rats were anesthetized using isoflurane 3%, in a 1 : 1 mixture of O₂ and N₂O. After a midline laparotomy, a resection of 1 cm of both ileum and colon was performed at 15 cm proximal to the ileocecal junction and 3 cm proximal to the rectal peritoneal reflection, respectively. End-to-end anastomoses were constructed with eight single-layer, inverting, interrupted 8-0 Ethilon (Ethicon, Somerville, NJ) sutures. The abdominal wall was closed with an absorbable, polygalactin 3-0 suture, the skin was closed with staples. A heating pad was used to maintain body temperature at 38 °C. The intestines were covered with gauze pads soaked with 0.9% NaCl to minimize desiccation. To compensate for fluid loss, 10 mL of 0.9% NaCl was administered subcutaneously during the operation. Postoperative analgesia was performed with buprenorphine, 0.02 mg/kg subcutaneously, twice daily for 2 days. The animals were weighed daily and observed for signs of illness. All operative procedures were performed by the same investigator (M.C.M.W.).

Biomechanical analysis

At the designated times the animals were killed by intracardiac puncture and hemorrhage under general anesthesia. Relaparotomy was performed by excision of a part of the abdominal wall of approximately 4 by 4 cm, including the suture line of the fascia. Anastomotic intestinal segments, approximately 4 cm in length with the suture line in the middle, were carefully resected, including surrounding tissues and adhesions, and washed in saline. Each anastomotic segment was connected to an infusion pump on one side and to a manometer on the opposite side. The pressure was then raised by infusion of methylene blue dissolved in saline at a rate of 2 mL/min. Bursting pressure was defined as the maximum intraluminal pressure the segment resisted. Thereafter, the breaking strength was measured in the same segment with traction in a tensiometer. This procedure has been validated previously.¹¹ The breaking strength was defined as the peak force necessary to induce disruption of the suture line. From each segment of the abdominal wall, two separate strips of 1 by 2 cm were cut

out, with the fascia suture line in the middle, and the breaking strength was measured in both. After biomechanical analysis, segments were cleaned from adhering tissue and standard sized samples containing the suture line were frozen in liquid nitrogen and stored at -80 °C until further processing.

Collagen content

After lyophilization, tissue samples were weighed, pulverized, and lyophilized again. The hydroxyproline content, as a measure of the collagen content, was measured by high-performance liquid chromatography after hydrolysis with 6-N-hydrochloric acid and derivatization with dabsyl chloride.

Histology

Intestinal samples of approximately 1-cm length and containing the anastomosis in the middle were fixed in 4% (v/v) phosphate-buffered formaldehyde, pH 7.3, immediately after harvesting. Each anastomosis was divided into two or three longitudinal strips. Specimens were dehydrated and embedded in paraffin. Sections of 4 mm in thickness were stained with hematoxylin and eosin and a semiquantitative analysis of epithelial damage, wound area surface, degree of necrosis, edema, bridging submucosal-muscular layer, and presence of neutrophils and macrophages was carried out.

In addition, another set of sections was stained with picosirius red to identify collagen fibers. Collagen measurements in the wound area were performed by digital image analysis.¹² Images were recorded by an RGB camera (Sony DXC-950 P, Sony Corporation, Tokyo, Japan) mounted on a conventional light microscope (Zeiss Axioskop 2 plus, Carl Zeiss BV, Sliedrecht, the Netherlands), using a ×5 objective. Image acquisition and analysis were performed using a complimentary software program (Zeiss KS 400[®]). Microscopic images were digitized and the area positive for picosirius red staining was recognized by segmentation in RGB using fixed threshold values. The ratio of picosirius red positive and the total amount of pixels yielded the percentual area of anastomotic collagen.

Statistical analysis

To analyze the statistical differences of the postoperative weight between all groups, a one-way analysis of variance was performed followed by a Tukey-Kramer multiple comparisons test. For comparisons of the healing parameters the differences between each of the two experimental groups and the control group were analyzed with a one-sided unpaired *t*-test with Welch correction. Differences with *p*-value < 0.05 were considered statistically significant.

RESULTS

Seven rats died prematurely, four in group E-2, two in group of E-1, and one in the control group. The latter animal died immediately after operation due to hypothermia. The other rats died between the second and 13th

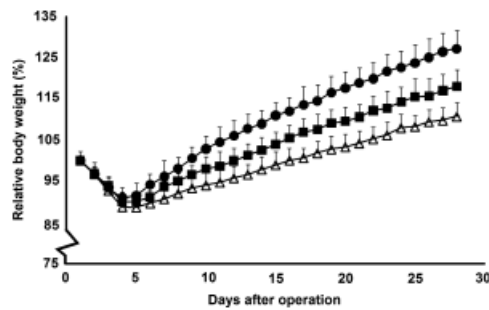


Figure 1. Course of body weight. Data represent mean and standard deviation ($n=10$) of relative weights (vs. value before surgery) during 4 weeks after surgery: ●: control group; ■: E-1 group; △: E-2 group.

postoperative day. A postmortem examination revealed no signs of anastomotic dehiscence and no apparent cause of death.

The remaining rats recovered quickly and resumed normal behavior within 48 hours. All animals experienced a transient weight loss of approximately 10% of their body weight. From day 4 onwards all animals regained weight. However, the weight gain in animals receiving everolimus was less than the control group. This effect was consistent up to 28 days postoperatively, dose-related and significant ($p < 0.001$) after 28 days (Figure 1). The difference was caused by a prolonged period of malaise, noted by a longer period of loss of appetite and piloerection in the experimental groups. At relaparotomy there were no signs of peritonitis or anastomotic dehiscence and filmy adhesions were common.

Wound strength

Wound breaking strength of fascia, ileum, and colon is summarized in Figure 2. The mean breaking strength of fascia after 7 days was 62 ± 19 (SD) g, which was significantly higher than in the experimental groups. At 14 and 28 days the mean breaking strength of the control group had increased further. The breaking strength of the experimental groups did increase as well, but far less so than in the control group, resulting in a significantly lower strength than in the control group at all measuring points. Average breaking strength of the control ileum anastomoses at day 7 was 93 ± 32 g and increased during the entire experimental period. Again, the experimental groups showed a slower development of wound strength. After 28 days, the reduction was most pronounced, the E-1 and E-2 groups displaying a 44% and 53% lower breaking strength, respectively, than the control group. The breaking strength of the colon anastomoses after 7 days was 251 ± 35 g in the control group and significantly lower in the experimental groups (control vs. E-1 as well as vs. E-2: $p < 0.0001$). At 14 days this pattern was the same but after 28 days only the strength in the E-1 group was significantly lower than in the control group.

The individual bursting pressure data for ileum and colon anastomoses are summarized in Figure 3. The mean bursting pressure of the ileum after 7 days in the control group (240 ± 81 mm Hg) did not significantly differ from

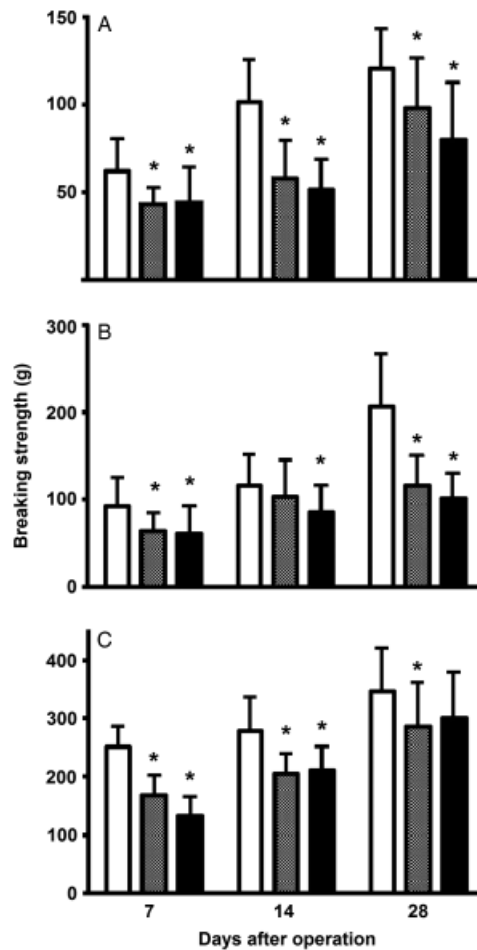


Figure 2. Wound breaking strength. Data represent mean and standard deviation ($n=10$) for fascia wounds (A), ileal anastomoses (B), and colonic anastomoses (C). White bars: control groups; gray bars: E-1 groups; black bars: E-2 groups. * $p < 0.05$ vs. control group.

that in the E-1 group, but was significantly ($p=0.006$) higher than that in the E-2 group. No significant differences were measured at 14 days, but a very strong effect was measured after 28 days (Control vs. E1 and E2 groups: $p=0.0003$ and 0.0025 , respectively). In the colonic anastomoses maximum differences between control and experimental groups were found at 14 days (Control vs. E1 and E2 groups: $p=0.0008$ and 0.0002), while after 4 weeks bursting pressure was similar in all groups.

Figure 3 shows that, in a number of cases, the bursting site was outside the true wound area. The anastomosis has grown stronger than the surrounding tissue and bursting pressure value thus represents a minimal value. The fact that anastomoses were weaker in the everolimus groups is also illustrated by the fact that the percentage of anastomoses that ruptured outside the suture line was always lower than in the controls. For instance, after 7 days in the colon, these percentages were 100 for controls, 60 for the E-1 group and 33 ($p=0.003$ vs. controls, Fisher's exact test) for the E-2 group (Table 1).

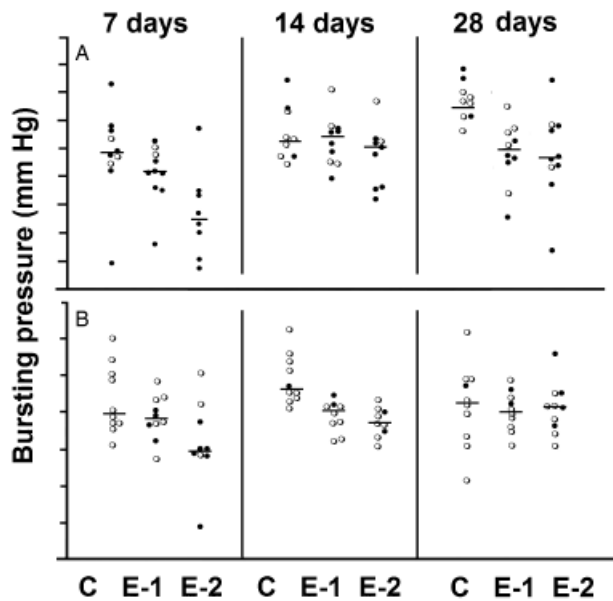


Figure 3. Anastomotic bursting pressure. Individual values and medians (horizontal lines) in ileum (A) and colon (B) for controls (C) and groups receiving everolimus (E-1 and E-2). Open symbols denote rupture outside the suture line and closed symbols rupture inside the suture line.

Collagen and histology

The hydroxyproline content ($\mu\text{g}/5\text{ mm}$) is used as a measure for total collagen. In the ileum the anastomotic hydroxyproline content in the experimental groups was significantly lower than in the controls after 7 and after 28 days (Figure 4). After 7 days, the same pattern was seen in the wound samples from fascia and colon. However, such a decrease in hydroxyproline content was absent in fascia or colon at 28 days.

Quantitative morphology of collagen fibers in the histological slides shows the same trend, although less explicitly so (Figure 5). After 7 days, the average percentage of

Table 1. Anastomotic bursting site

	Days		
	7	14	28
Ileum			
Control	3/10	7/10	7/10
E-1	2/10	4/10	5/10
E-2	0/8	2/9	2/10
Colon			
Control	10/10	9/10	9/10
E-1	6/10	7/10	8/10
E-2	3/9	7/9	6/10

Numbers represent the number of times that rupture occurred outside the suture line vs. the total number of observations.

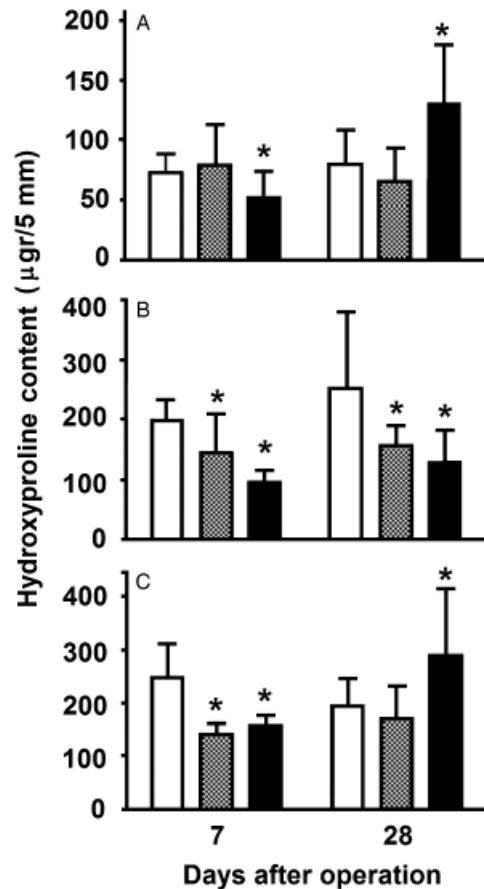


Figure 4. Wound hydroxyproline content. Data represent mean and standard deviation ($n=10$) in fascia wounds (A), ileal anastomoses (B), and colonic anastomoses (C). White bars: control groups; gray bars: E-1 groups; black bars: E-2 groups. $*p < 0.05$ vs. control group.

wound collagen in fascia and ileum was reduced in the experimental groups, though not significantly. After 28 days postoperatively, no decrease in wound collagen was observed. Figure 6 shows examples of the picrosirius red coloring of fascia tissue at 7 days and colon anastomotic tissue at 28 days.

A comprehensive histological examination of sections obtained from rats from each group did not reveal the existence of any obvious architectural differences between the groups in either intestine or fascia at any of the time points.

DISCUSSION

The current data show the existence of a considerable and protracted impairment of wound healing induced by the administration of the mTOR inhibitor everolimus. The effect on the development of wound strength persists for at least 4 weeks after operation and is present in both tissues investigated, intestine and the abdominal fascia.

Previously, we have reported the compromised restoration of strength in healing intestinal anastomoses of the rat

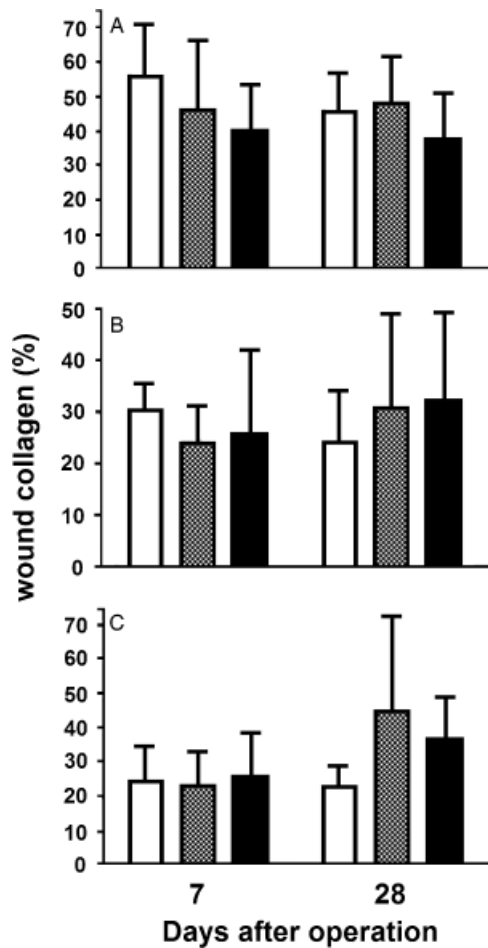


Figure 5. Wound collagen content. Data represent mean and standard deviation for the histological evaluation of wound collagen in fascia wounds (A), ileal anastomoses (B), and colonic anastomoses (C). White bars: control groups ($n=5$ at day 7 and $n=6$ at day 28); gray bars: E-1 groups ($n=4$ at day 7 and $n=6$ at day 28); black bars: E-2 groups ($n=6$).

as measured at day 7 after operation.¹⁰ Both breaking strength and bursting pressure are measures for anastomotic strength in the intestine, yielding essentially different information. They represent the ability of the suture line to withstand longitudinal and intraluminal forces, respectively. It should be noted that here both parameters show similar trends, thereby fortifying the general picture of impaired strength. We have now included an analysis of the breaking strength of the suture-line in the anterior fascia of the abdominal wall as well. One reason to include these measurements is that a systemic effect of an immunosuppressant drug probably would affect multiple tissues and that addition of such data allows more generalized conclusions. Also, healing disorders of the abdominal wall are responsible for a large body of surgical complications observed in kidney transplantation patients. The effects of everolimus on fascia and bowel wall strength are similar, which strongly suggests that everolimus is responsible for a

generalized negative effect on wound repair. In addition, the data support the concept that this model indeed reflects the clinical increase in wound healing complications such as seroma or herniae, commonly found in patients after kidney transplantation who are treated with immunosuppressant drugs, in particular mTOR inhibitors.

The doses of everolimus we have chosen are based on the dose range used in our previous study¹⁰ and similar to those used to elicit immunosuppressive effects in rats.^{13–15} Daily oral doses of 1 or 2.5 mg/kg are expected to result in trough levels approximating 6 and 8 ng/mL, respectively.^{16,17} In patient studies, target trough levels are usually between 3 and 15 ng/mL,¹⁸ although an upper therapeutic concentration limit is likely to exceed 15 ng/mL.^{19,20} Thus, the doses used are clinically relevant in terms of the human therapeutic range.

Others have investigated the effects of mTOR inhibitors on wound repair,^{21–24} though not those of everolimus. Published data concern other tissues and analysis of repair is mostly restricted to one time point only. Very recently, Wagner and coworkers have concluded that sirolimus does not impair the healing of bowel anastomoses, in seeming contradiction to data reported earlier by us for everolimus.¹⁰ However, these authors based their conclusion on an experiment with colon anastomoses that lasted only 4 days.²⁵ Proliferation inhibitors such as sirolimus and everolimus are logically thought to affect the proliferative phase of wound healing, which starts around the third postoperative day. It is therefore very likely that their effects only become apparent somewhat later, for instance at day 7. Indeed, in our previous study no effects were observed at day 3, and severe effects at day 7, emphasizing the dangers of drawing generalized conclusions on the basis of measuring at one time point only.

The maximum duration of preclinical studies of mTOR inhibitors is 15 days.^{22,26} Transplant patients receive a life-long immunosuppressive drug regimen from the day of their operation onwards and a prolonged disturbance of wound repair may have clinical consequences and cause surgical morbidity. For this reason we have extended our observations to 28 days, in order to determine if at that time point initial suppression of wound repair would be compensated for, even during continued use of everolimus. However, the data prove a lasting negative effect on wound strength that, in the ileum and fascia, even outlasts the duration of the experiment.

Wound strength depends on the extracellular matrix, more specifically on the structural protein collagen. Several mechanisms of action can, exclusively or in combination, be responsible for the negative effects of everolimus. A prolonged proliferation phase, deposition of insufficient collagen or collagen of inferior structural quality, and even disturbance of the remodeling phase may play a role. After 7 days, the wound hydroxyproline content in the experimental groups is less than that in the control group. This is a consistent finding¹⁰ and probably constitutes the main reason for a weaker wound long after operation. However, after 28 days the hydroxyproline content in colonic anastomoses and fascia in the everolimus groups is restored to normal levels, or even exceeds them, while at the same time wound strength in all tissues still lags behind the control group. Although the results of the histological determination of collagen does not yield significant differences

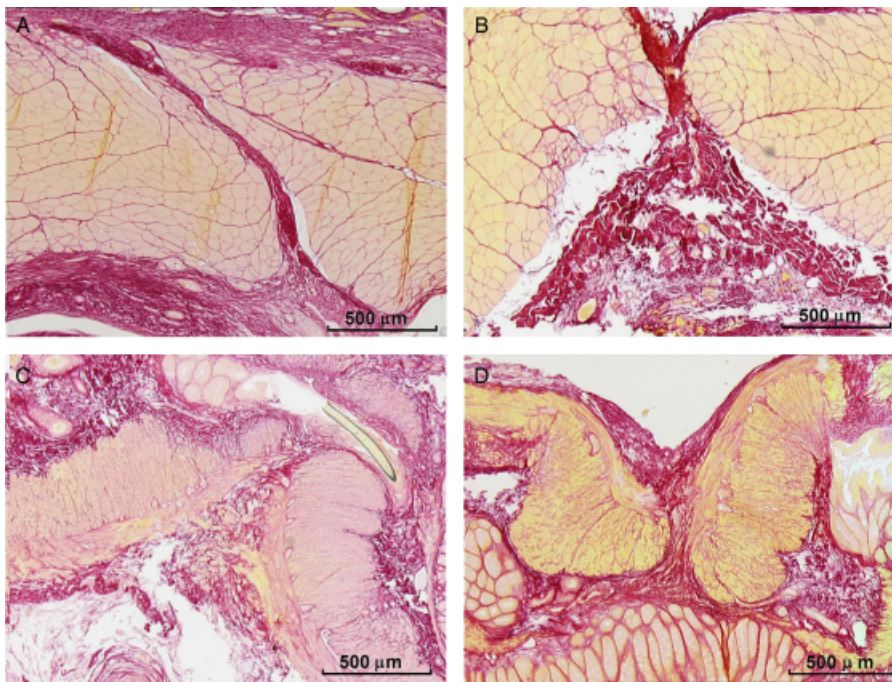


Figure 6. Picrosirius red coloring. A=fascia control group at 7 days, B=fascia E2 group at 7 days, C=colon anastomosis control group at 28 days (with a piece of suture in the sample), D=colon anastomosis E2 group at 28 days.

between control and experimental groups, which might be due to the limited number of observations, the qualitative pattern appears the same. One should realize that the data on wound collagen, obtained by histomorphometry, represent the true wound area, while those obtained by hydroxyproline measurements represent a tissue sample of 5 mm length with the wound in the middle. The latter thus contains some degree of adjacent tissue and this may explain the apparent difference in outcome between the two separate methods. Therefore, the conclusion that diminished strength is caused exclusively by a decrease in quantity of collagen is not justified. In other experimental studies that have addressed the mechanism of action of mTOR inhibitors, reduced inflammatory cell numbers are evident, while hydroxyproline levels are not consistently decreased.^{24,27} Also, sirolimus treatment leads to a decreased expression of vascular endothelial growth factor and nitric oxide, mediators of angiogenesis and immune function, in skin wounds.²⁴ More research is thus needed to determine the exact mechanisms of everolimus on wound healing.

In conclusion, the potentially negative effects of mTOR inhibitors on wound healing gradually have become evident over the last couple of years and should be well recognized. Still, mTOR inhibitors may possess certain benefits over other immunosuppressive agents such as calcineurin inhibitors, not in the least because of the absence of nephrotoxicity in transplant patients. With a delay in administration of mTOR inhibitors, until wound healing is completed or the development of wound strength well under way, we might be able to combine advantages and disadvantages. Such an approach may optimize posttransplant medication regimens and further reduce surgical complications thereby improving transplant and patient survival.

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