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ORIGINAL ARTICLE

Sirolimus and intraoperative hyperthermic peritoneal chemoperfusion with mitomycin-C do not impair healing of bowel anastomoses

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Summary

Surgeons will increasingly have to address the development of gastrointestinal disease in transplant patients or deal with extended bowel resection and bowel anastomosis in advanced cancer patients. Immunosuppressants as well as intraoperative hyperthermic peritoneal chemoperfusion (IHPC) may alter intestinal anastomotic healing. We evaluated the effects of the immunosuppressant sirolimus and of IHPC on healing and stability of bowel anastomoses in pigs. Twenty-four pigs were divided into four groups (SIR: sirolimus was administered orally; IHPC: animals received IHPC with mitomycin-C; COMP: combination of sirolimus and IHPC was administered; CON: sham-treated control group). Animals underwent hand-sutured small bowel and left colon anastomoses and were killed on postoperative day 4. Anastomoses were evaluated by morphometric analysis and immunohistochemistry (IHC) and by measuring the bursting pressure (BP). In all experimental groups (SIR, IHPC, COMP), anastomotic BPs remained unaltered and were not statistically different compared with control (CON). In addition, ileum villous height and colonic crypt depth analysis revealed no significant difference in mucosal thickness, and IHC showed no difference among groups in proliferation, as assessed by the number of KI-67- and bromodeoxyuridine-labeled cells. Immunosuppression with sirolimus as well as IHPC with mitomycin-C do not alter healing of intestinal anastomosis in pigs.

Introduction

The number of patients under immunosuppressive therapy because of previous thoracic or abdominal transplantation has been steadily increasing in recent decades [1]. New potent immunosuppressive agents as well as different combinations of these drugs have led to a substantial increase in short-term and long-term survival of different grafts and of patients [2]. However, an increased risk of developing neoplasia in allograft recipients has been reported: After 20 years of immunosuppressive therapy, the risk for malignancies was calculated to be about 40% [3]. The overall cancer risk related to chronic immunosuppression is increased fourfold [4], and the risk of developing certain specific cancers may increase several 100-fold [5]. In consequence, cancer has become a major cause of death in patients in whom transplantation was otherwise successful [6]. Thus, long-term immunocompromised patients will increasingly be confronted with the risk of gastrointestinal diseases such as cancer [7] or diverticulitis [8], requiring bowel resection and bowel anastomosis.

Various immunosuppressant agents can severely disturb wound and especially anastomotic healing [9]. In a

previous study, we demonstrated that mycophenolate mofetil, a potent immunosuppressant, significantly impairs healing of left-sided colon anastomoses in an experimental model [10]. Sirolimus (Rapamune®, Rapamycin) another potent immunosuppressant, is widely used as a maintenance immunosuppressive agent in organ transplantation. It was first extracted as the active antifungal component from *Streptomyces* found in a soil sample on Easter Island [11], and initial observations demonstrated its antifungal activity, mainly against various *Candida* species, especially *Candida albicans* [12]. Sirolimus displays a mechanism of immunosuppressive action distinct from that of cyclosporine and tacrolimus. It acts in both the co-stimulatory activation and cytokine-driven pathways, inhibiting the mammalian target of rapamycin (mTOR). mTOR, a multifunctional serine-threonine kinase, is inhibited by the sirolimus-FK-binding protein complex, which results in an inhibition of proliferation and differentiation of B and T lymphocytes [13]. Several studies also revealed severe wound-healing complications associated with sirolimus administration [14–16].

Peritoneal carcinomatosis (PC) is a common consequence of advanced digestive carcinoma. Once diagnosis of PC is confirmed, the median survival time is approximately 6 months [17]. For this reason, PC is regarded as a terminal condition, and palliative therapeutic strategies are used. In the last decade, several treatment options targeted an improved prognosis of PC, including cytoreductive surgical procedures [18], intraperitoneal drug administration [19], or intraperitoneal hyperthermia. Hyperthermia has been shown to potentiate intraperitoneal chemotherapy [20], and encouraging results have been described, especially for a combination of these treatment strategies [21,22]. Intraoperative hyperthermic peritoneal chemoperfusion (IHPC) reduces progression of PC following oncological and cytoreductive intestinal surgery and leads to a considerably higher overall survival [23]. However, following IHPC, intestinal wound-healing problems, such as an increased anastomotic leakage rate, have been reported [24,25].

The initial aim of this work was to evaluate the possible adverse effects of oral sirolimus administration on the healing of bowel anastomosis in pigs. Another aim was to evaluate the possible adverse effects of IHPC with and without sirolimus administration (which may mimic the immunocompromised condition of a cancer patient) on the healing of bowel anastomosis in the pig.

Materials and methods

Ethical considerations

The Animal Care and Use Review Committee of the University of Bern approved the study in accordance with the standards set out in the Animal Welfare Act and other federal, state and local statutes and regulations related to animals.

Sirolimus and mitomycin-C

Sirolimus (Rapamune®) was manufactured and provided by Wyeth AG (Zug, Switzerland). Mitomycin-C was manufactured by Roche Pharma (Reinach, Switzerland).

Animals and sirolimus administration

Thirty healthy, female pigs (Swiss Edelschwein), obtained from Peter Reber, Uettlingen, Switzerland, weighing 25–30 kg, were used in the studies. Four pigs were used for a pilot study to evaluate the feasibility of the IHPC procedure (see below), and 26 animals were used in the main study. Animals were housed under stable conditions and fed a standard laboratory diet and water *ad libitum*. Pigs were fasted for 12 h prior to the surgical intervention. Sirolimus was administered orally once daily during 11 days perioperatively until killed on postoperative day 4. Treatment was initialized 7 days prior to surgery. Animals received a loading dose of 15 mg on day 1, 10 mg on days 2–7 and 5 mg on days 8–11. This protocol corresponds to a perioperative treatment of 7 days before and 4 days after surgery. The desired serum level was >15 ng/ml. Blood samples were taken intraoperatively from the gastroepiploic vein for sirolimus serum concentration measurements. Animals for the main study were divided into four groups ($n = 6$ each). Sirolimus (group SIR), IHPC (group IHPC), or the combination of both (group COMP) were administered, and results were compared with the sham-treated control group, CON. All animals not receiving IHPC ($n = 12$) underwent the correct control procedure with isothermic peritoneal physiologic saline perfusion.

Operative technique

General anesthesia consisted of ketamine, xylazine, atropine, and thiopental, followed by controlled ventilation with oxide/nitrous oxide 1:3 and isofluran. During anesthesia, animals were monitored according to a specific protocol. A fentanyl patch (50 μg) was applied for 3 days for postoperative analgesia. Animals were fasted overnight to clean the small bowel and colon and to minimize contamination of the peritoneal cavity and wound during the intervention. The abdominal area of the animals was shaved and prepared using standard aseptic techniques. For skin disinfection, a 10% povidone-iodine solution (Betadine®, Mundipharma Medical Company, Basel, Switzerland) was used. After sterile drapes were placed, a

10 cm abdominal midline laparotomy was performed. After the abdominal cavity was entered, the distal jejunum and sigmoid colon were identified and divided while vascular supply was preserved. Then, dissection margins were readapted by hand-sutured, continuous, single-layer, end-to-end anastomosis with 5/0 PDS II sutures (Ethicon, Neuchatel, Switzerland). Following peritoneal perfusion (see below), the laparotomy was closed using a PDS 1 (Ethicon) running suture and the skin using a 3-0 Prolene (Ethicon) continuous mattress suture. Transparent film dressing spray (OpSite, Smith & Nephew, Solothurn, Switzerland) was applied to the wound.

Technique of intraoperative peritoneal perfusion with heated mitomycin-C solution

Pilot studies with four animals were performed to evaluate the feasibility of IHPC in pigs. After small bowel and colonic anastomoses were performed, 12 animals underwent IHPC that was similar to the procedure described by Beaujard *et al.* [26]. To prepare the chemoperfusate, we dissolved 40 mg mitomycin-C in 4 l saline. Then, the chemoperfusate was pumped into the abdominal cavity through a 28-French flexible silicone drain (inflow drain) placed under the right diaphragmatic cupula. Another 30 French silicone drain (outflow drain) was inserted into the Douglas pouch. These two silicone drains exited through the midline incision and were connected to a sterile closed circuit. Using a thermal heat exchanger (Polystan Safe-mini, Maquet, Germany) connected to the heating circuit (Fumedica AG, Muri, Switzerland), chemoperfusate was heated up to a 43 °C inflow temperature. Active circulation of the perfusate into the peritoneal cavity was achieved by means of roller pumps of a heart-lung machine (HL20, Maquet, Germany). Perfusion was maintained at a flow rate of 500–600 ml/min for 60 min with close monitoring of respiratory and hemodynamic parameters. During the procedure, the open abdominal cavity was covered with transparent adhesive foil (30 × 28 cm, OpSite, Smith & Nephew, Solothurn, Switzerland). In control animals (n = 12), an isothermic saline perfusion solution (0.9% NaCl) was circulated.

Bursting pressure technique

Animals were killed with potassium chloride administered intravenously under general anesthesia. At killing, a 20 cm segment of the small bowel and the left colon respectively, including the anastomosis, were dissected and isolated. Adherent tissue to the anastomotic site was dissected out en bloc with the specimen to preserve anastomotic integrity. Bursting pressures (BPs) were evaluated by two investigators blinded to the treatment groups as described previously [27]. After dissection of the anastomotic site, one end of the extracted bowel was ligated using a Vicryl 2-0 (Ethicon) tie, and the other end was fixed over a Shiley needle using a Vicryl 2-0. The entire bowel was then submerged in a physiologic saline bath, and BP was measured using a sphygmomanometer with an in-line pressure transducer, increasing intraluminal pressure in increments of 10 mmHg over 10 s at intervals of 10 s. BP was determined by noting leakage of air or gross rupture at or near the anastomosis.

Histological assessment and morphometric analysis

The extracted bowel segment was opened longitudinally, fixed in 5% formalin, and embedded in paraffin. Transverse sections of the embedded tissue were stained with hematoxylin and eosin and histological assessment performed. Morphometric analyses were conducted with a Leica DMRB microscope equipped with a color video camera (Leica DC500; Leica Microsystems, Heerbrugg, Switzerland) and connected to a video-based computer-linked system. A computed measurement (Leica Qwin Standard V2.6, Leica Microsystems, Heerbrugg, Switzerland) of the ileum villous height (IVH) and colonic crypt depth (CCD) was performed. Ten random measurements were performed at least 5 mm from the anastomotic site where glands were perpendicular to the underlying muscularis.

Assessment of proliferating cells: immunostaining with bromodeoxyuridine (BrdU) and KI-67

All pigs received an intravenous injection of BrdU (Sigma-Aldridge Chemie Gmbh, Steinheim, Germany) at a dose rate of 50 mg/kg body weight, 120 min before killing. The small and large bowel were removed and attached to a piece of cork to allow exact longitudinal cuts of the mucosa and to inhibit shrinking. Specimens were transferred into a phosphate-buffered formalin solution (5%). After 24 h, three cross-sectional pieces were cut from each sample, transferred to an alcohol solution (70%), and embedded in a paraffin block. Paraffin sections were dewaxed in xylene and rehydrated in a series of graded alcohols according to histological standards. To recover antigenicity masked by formalin fixation, heat-induced antigen retrieval was applied. For BrdU staining, deparaffinized sections were heated in a microwave oven in 10 mM sodium citrate buffer at pH 6.0 for 10 min at 89 °C (350 W). For KI-67 staining, antigen retrieval was performed in a pressure cooker filled with preheated boiling citrate buffer (10 mM, pH 6.0) in which slides were heated for 6 min.

After antigen retrieval processing, the slides were allowed to cool slowly to room temperature (RT) for

20 min. Sections were then rinsed three times for 5 min in phosphate-buffered saline (PBS) before and after incubation and with 3% hydrogen peroxide-PBS (10 min) to block endogenous peroxidase activity. As indicated in the instruction manual of the BrdU In Situ Detection Kit (Becton Dickinson AG, Basel, Switzerland), slides were then incubated overnight with the diluted biotinylated anti-BrdU antibody (1:20 in diluent buffer) or for KI-67 staining, with diluted primary antibody (MIB-1, mouse anti-human Ig, 1:100 in PBS) in a humidified chamber at 4 °C. The following day, after PBS washes (3 × 5′), sections for KI-67 staining were promptly incubated with biotinylated secondary antibody (Rabbit Anti-Mouse IgG, diluted 1:300 in Tris–Hcl buffer) for 60 min at RT and afterwards washed again (PBS 3 × 5′). After the PBS washes, BrdU sections were incubated with streptavidin-horseradish-peroxidase and rinsed abundantly in PBS before staining for approximately 3 min in a diaminobenzidine (DAB) substrate working solution (Becton Dickinson AG) until the desired color intensity developed. KI-67 sections were concurrently incubated with avidin and the biotinylated horseradish peroxidase complex (ABC reagent, ABC Kit, Vectastain Elite, REACTOLAB, S. A., Servion, Switzerland) for 30 min at RT. After another set of PBS washes (3 × 5′), slides were stained for 3 min in a DAB substrate chromogen solution (Sigma-Aldrich). Following immersion in water to stop the reaction, all slides were counterstained in hematoxylin for 30–60 s and again rinsed thoroughly in water. Finally, slides were dehydrated in three changes of xylene and a graded alcohol series and mounted.

Two independent investigators blinded to treatment groups counted the total number of proliferating cells (KI-67/BrdU) and the total number of cells per crypt at a magnification of 1:100 in 10 longitudinal cuts of colonic crypts. Results were expressed as the ratio (%) between BrdU- or Ki-67-positive stained cells and total cells per crypt, respectively.

Statistical analysis

Results are expressed as mean ± SD. Significance of differences was assessed by ANOVA and Tukey's test. P values of <0.05 were considered statistically significant. SPSS Inc, version 12.0.1 (SPSS Inc., Chicago, IL, USA) was used for these calculations.

Results

Clinical survey and sirolimus serum concentration measurement

Two animals died after the initial surgical intervention, one because of ventricular fibrillation during recovery from anesthesia, and the second on the third postoperative day with ataxia and cyanosis of unclear etiology. Both animals underwent immediate necropsy, but no specific reason for death, especially no anastomotic leak, was found. Both animals were replaced by two others. All other animals tolerated the intervention well. After killing, the abdomen in all animals was reopened, and inspection revealed inconspicuous anastomoses and peritoneal cavity. Intraoperatively (time of killing) measured sirolimus serum concentrations were 18 ± 12 ng/ml (median ± SEM) for group SIR and 27 ± 4 ng/ml for group COMP ($P > 0.05$).

Macroscopic and microscopic pathological assessment

At necropsy, no anastomotic leaks were noted in any treated group. Development of adhesions next to the bowel anastomosis was distributed nonspecifically among groups, and there was no evidence for peritonitis or intra-abdominal abscess. Computed microscopic measurements of IVH (in μm) and CCD (in μm) revealed no significant difference in intestinal mucosa thickness (ileum: $P = 0.53$; colon: $P = 0.18$) between the groups. Ileum villous height (μm) was 518 ± 163 in group SIR, 515 ± 81 in group IHPC, 484 ± 102 in group COMP, and 580 ± 89 in group CON. Colonic crypt depth (μm) was 427 ± 81 in group SIR, 428 ± 48 in group IHPC, 501 ± 64 in group COMP, and 456 ± 59 in group CON (Figs 1 and 2).

Bursting pressure assessment

Bursting in the jejunum and the colon occurred in all animals at or near the anastomotic site. BPs were not significantly lower in groups treated with sirolimus and/or with IHPC when compared with the control group on postoperative day 4 (ileum: $P = 0.82$; colon: $P = 0.88$). BP (mmHg) was 125 ± 50 and 96 ± 15 in group SIR, 135 ± 29 and 101 ± 35 in group IHPC, 117 ± 48 and 97 ± 10 in group COMP, and 138 ± 40 and 91 ± 18 in group CON in the ileum and colon, respectively (Fig. 3).

Mucosal proliferation assessment (KI-67 and BrdU staining)

This study revealed in the ileum a significantly lower number of proliferating, KI-67-labeled cells in the sirolimus-treated group (group SIR) when compared with the control group (group CON) ($P = 0.045$). However, these findings could not be confirmed either in the IHPC-treated group or in the combination-treatment group when compared with the control group. Furthermore, colonic crypts in animals that underwent sirolimus and/or IHPC

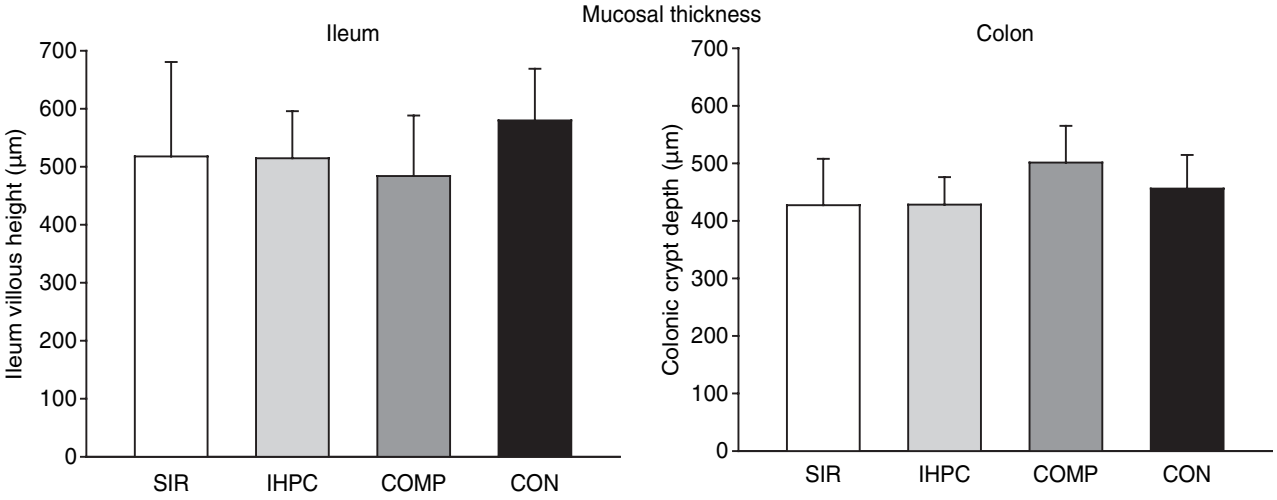

Figure 1 Ileum villous height (IVH, µm) and colonic crypt depth (CCD, µm) in sirolimus (SIR), IHPC and sirolimus/IHPC (COMP) groups compared with control (CON) animals ($P > 0.05$).

treatment did not contain significantly fewer proliferating cells than the control group (Figs 2 and 4). We also did not identify a marked difference of proliferation, expressed by a decrease in the number of BrdU labeled cells, in the sirolimus- and/or IHPC-treated tissue when compared with the control group. The crypt proliferation ratio (in %) for KI-67 was 40 ± 6 and 24 ± 8 in group SIR; 45 ± 5 and 29 ± 5 in group IHPC; 41 ± 5 and 22 ± 7 in group COMP; and 49 ± 5 and 23 ± 7 in group CON in the ileum and colon, respectively. The ratio (%) for BrdU was 33 ± 9 and 14 ± 5 in group SIR; 34 ± 6 and 17 ± 4 in group IHPC; 32 ± 2 and 14 ± 4 in group COMP; and 30 ± 8 and 12 ± 3 in group CON in the ileum and colon, respectively (Figs 2 and 5).

Discussion

Intestinal anastomotic leakage is one of the most important and feared complications following intestinal surgery. As a result of novel medications and new perioperative treatment strategies, the clinically apparent anastomotic leakage rate might increase in the future. The present experimental studies were performed to evaluate the influence of the immunosuppressant sirolimus and IHPC with mytomycin C on intestinal anastomotic healing and stability. We chose pigs as bioavailability and pharmacokintetic measures are similar to those in humans. Granger *et al.* [28] were able to show good immunosuppressive effectiveness (long time graft survival without obvious toxicitiy) of Sirolimus in pigs with mean trough blood levels of 9.3 ng/ml. This target level is very similar to the one nowadays being used in humans [29]. To secure an effect on anastomotic healing, we aimed at higher trough levels in our studies. Postoperative day 4 has been chosen for comparison based on previous experimental data [10,27,30] suggesting the anastomotic healing process to be most vulnerable at this timepoint. Results of a clinical study from Wind *et al.* [31] provide further evidence for a critical phase of anastomotic healing around postoperative day 4.

Sirolimus inhibits growth factor-induced proliferation of several cell types, including endothelial cells, fibroblasts, and smooth muscle cells, an antiproliferative and antimitotic impact on cell populations that are essential for granulation tissue formation and wound healing [13,32]. Therefore, after gastrointestinal surgery the immunosuppressive, antiangiogenic, and antiproliferative properties of sirolimus may be deleterious in healing wounds and especially in healing intestinal anastomoses. Indeed, numerous reports show increased wound-healing complications after administration of sirolimus in combination with other immunosuppressants [14,16,33], although sirolimus without concomitant corticosteroid or mycophenolate mofetil administration did not reveal increased wound healing complications in transplant patients [34]. Dunkelberg *et al.* did not observe increased wound-healing complications in 170 liver transplant patients after primary immunosuppression with sirolimus and with the 3-day corticosteroid taper [35]. The synergistic or even additive effect of steroids and sirolimus on delayed wound healing has also been nicely demonstrated in an experimental model with rats [36]. However, scant data addressing wound healing are available in patients with a sirolimus regimen alone or in combination with

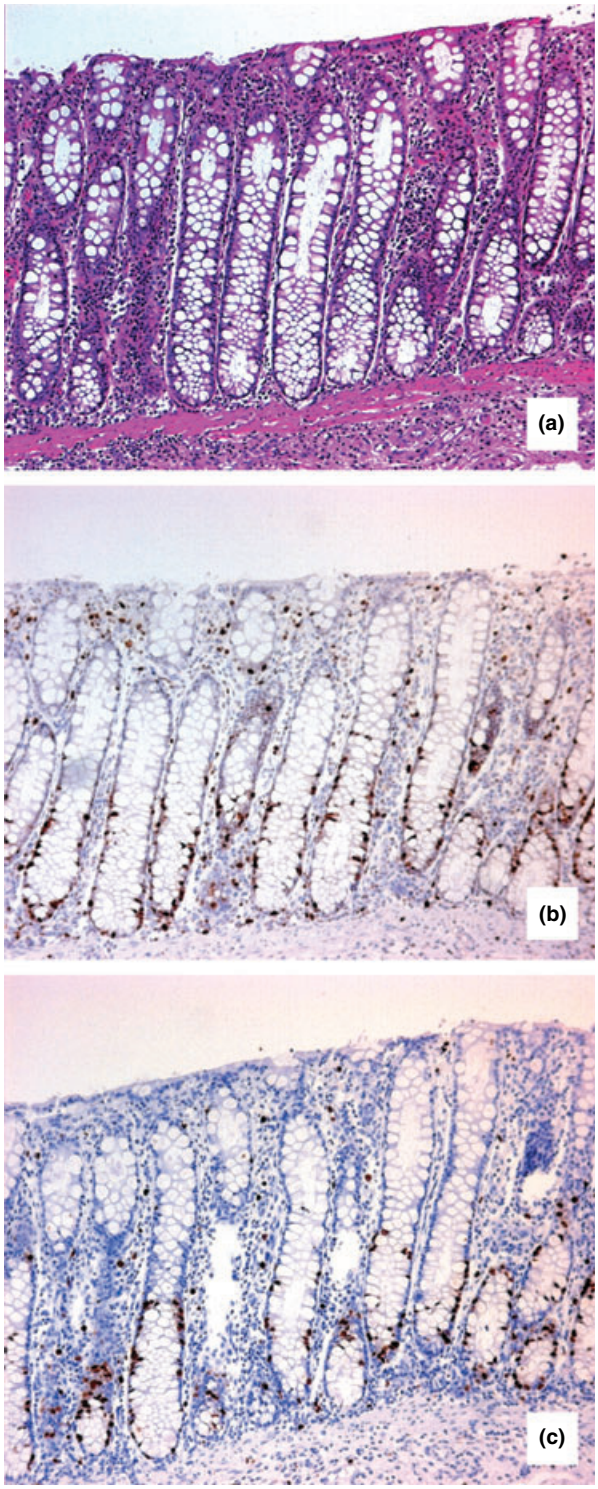

Figure 2 A through C: hematoxylin and eosin, KI-67 and bromodeoxyuridine staining in sirolimus treated colon (SIR), which did not demonstrate significant differences in mucosal thickness and proliferation rate compared with intraoperative hyperthermic peritoneal chemoperfusion (IHPC), sirolimus/IHPC (COMP) and control (CON) histologies (not shown) (Original magnification ×30).

other immunosuppressants or with sirolimus under corticosteroid avoidance. Grim *et al.* [37] were able to demonstrate that transplant patients with sirolimus and corticoid avoidance have had a rate of wound complications similar to a control group with corticosteroids. However, both groups received mycophenolate mofetil, a immunosuppressant, which is known to alter wound as well as anastomotic healing [10].

An unaltered mucosal healing process in the colon is the most important factor for normal anastomotic stability during the early postoperative period [10]. In this study, administration of sirolimus did not impair the mechanical stability of a bowel anastomosis, and we observed no wound healing problems. The measured BPs, around 120 mmHg for the ileum and 90 mmHg for the colon, suggested a completely stable intestinal anastomosis. Mucosal architecture expressed by CCD and IVH remained unaffected, and there was a completely unaltered mucosal cell proliferation in treated animals when compared with the control group. Based on these results, we may hypothesize that sirolimus administration does not seem to impair reparative mucosal cell proliferation and therefore also does not impair anastomotic stability. Puglisi *et al.* have even shown that sirolimus provides some protective effect in ischemic small bowel [38]. Interestingly, in comparison with our study findings Van der Vliet *et al.* [39] have recently published contradictory results showing that the sirolimus derivative everolimus compromised the restoration of strength in healing intestinal anstomosis. The fact that there are several differences concerning the methodology of these two studies makes results difficult to compare. First, they used a different method of performing BPs (air leak versus methylene blue leak). Secondly, there was a different animal model used (pig versus rat). Thirdly, pharmacokinetics of both sirolimus and everolimus display wide intra- and interindividual variability [40,41]. Clinical data suggest that adverse events and their associated severity are correlated with blood trough concentrations [42,43]. Thus, measurement of blood trough concentrations is of great importance to be capable of estimating the real therapeutic or toxic effect of mTOR inhibitors, such as sirolimus and everolimus. Unfortunately, there is lack of information about the trough blood levels of everolimus in the van der Vliet study. It could be speculated that the doses used in that study led to very high effective blood levels, which even may have been far in the toxic range concerning anastomotic healing. However, further evaluation of anastomotic healing under the influence of mTOR inhibitors with measurements of blood trough levels are mandatory.

Patients with advanced tumors and PC appear to have a condition similar to that of transplant patients under an immunosuppressive regimen. Tumor-derived factors

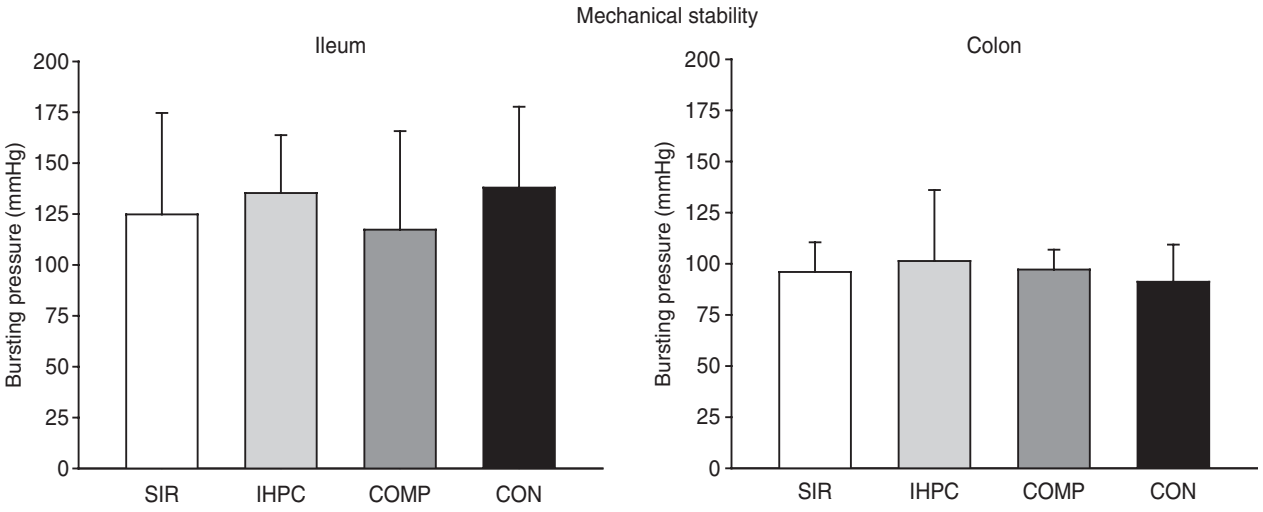

Figure 3 Bursting pressure (BP, mmHg) of ileum and colon anastomoses in sirolimus (SIR), intraoperative hyperthermic peritoneal chemoperfusion (IHPC) and sirolimus/IHPC (COMP) groups compared with control (CON) animals ($P > 0.05$).

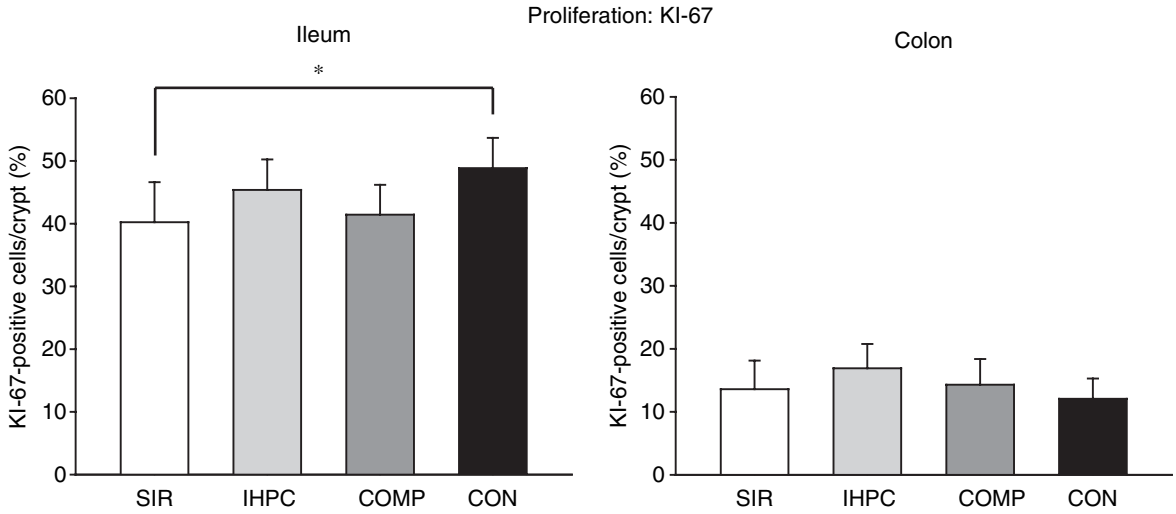

Figure 4 Proliferation rate (%) of KI-67-positive stained cells in comparison to total amount of cells per crypt in sirolimus (SIR), intraoperative hyperthermic peritoneal chemoperfusion (IHPC) and sirolimus/IHPC (COMP) groups compared with control (CON) animals ($P = 0.039$; Group CON > group SIR: $P = 0.045$).

drive the evolution of an immunosuppressive network which ultimately extends immune evasion from the primary tumor site to peripheral sites in patients with cancer [44]. In such patients, IHPC following cytoreductive intestinal surgery has been shown to lead to a considerably higher overall survival [23]. However, IHPC is regarded as another relevant disruptive factor in anastomotic healing. An increased leakage rate following IHPC has also been reported [24,25], with a published anastomotic leakage rate of 28%, an overall morbidity rate of 35%, and a mortality rate of 5% [45]. In our studies, however, we found no destructive effect of IHPC on intestinal anastomotic healing although hyperthermia and mitomycin-C concentrations were comparable with those used in other work [46]. Even the combination of sirolimus and IHPC, mimicking the immunosuppressive condition of patients with advanced cancer, did not reveal any differences in the evaluated healing parameters.

In summary, sirolimus as a monotherapeutic regimen does not disturb wound or anastomotic healing. These

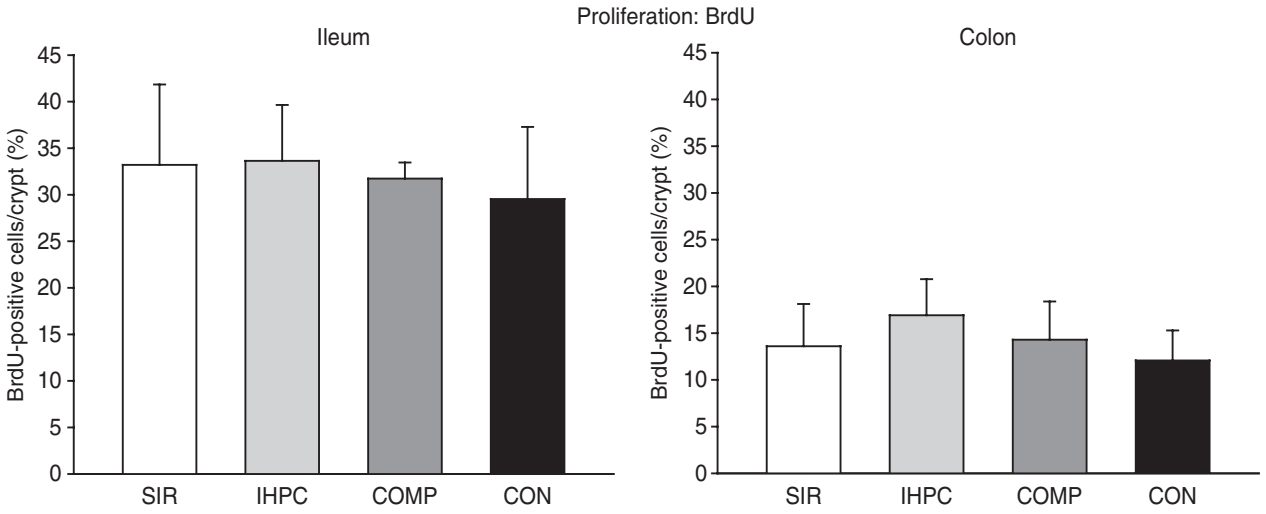

Figure 5 Proliferation rate (%) of bromodeoxyuridine-positive stained cells in comparison with total amount of cells per crypt in sirolimus (SIR), IHPC and sirolimus/IHPC (COMP) groups compared with control (CON) animals ($P > 0.05$).

findings may give confidence to surgeons performing bowel surgery in transplant and other patients on an immunosuppressive regimen with only sirolimus and with no corticosteroids. Furthermore, these results may also give confidence to surgeons wishing to perform more frequently cytoreductive surgery and use IHPC in patients with PC, while lowering the fear of the occurrence of anastomotic complications. At our institution, IHPC is now performed more often in patients with PC, even in patients undergoing multiple bowel resection and anastomosis. Thus far, only minor complications have been observed without any obvious anastomotic problems. However, further studies evaluating these findings in a larger patient population are warranted.

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Authorship

OJW, RAI, BE designed and performed research/study. OJW, RAI, SBK, DM, MB, DC, BE contributed important reagents. OJW, RAI, BE collected data. OJW, RAI, SBK, DC, BE analyzed data. OJW, RAI, BE wrote paper.

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