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The Effect of Mycophenolate Mofetil on Early Wound Healing in a Rodent Model

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Background. Immunosuppressant agents are inevitable for solid organ recipients, but may have a negative effect on wound healing that is difficult to measure because of clinical use of a polydrug regime. The evidence on mycophenolate mofetil (MMF) is scarce and contradictory. This study aims to investigate the effect of MMF administration on wound healing.

Methods. Ninety-six male Wistar rats divided into 4 groups underwent anastomotic construction in ileum and colon at day 0. Three groups received daily oral doses of 20 or 40 mg/kg MMF or saline (control group) from day 0 until the end of the experiment. Half of each group was analyzed after 3 days and half after 7 days. Another group started the medication 3 days after the laparotomy and was analyzed after 7 days, half of this group received 20 mg/kg and half 40 mg/kg MMF. Wound strength in anastomoses and in the abdominal wall was measured using bursting pressure, breaking strength, and histology. Trough levels were measured.

Results. Significant differences in wound strength were seen in ileum tissue after 3 days, which surprisingly showed a stronger anastomosis in the experimental groups. Bursting pressure as well as breaking strength was higher in the low-dose and high-dose MMF group compared with the control group. A negative effect was measured in abdominal wall tissue for the highest-dose group, which disappeared when the medication was delayed for 3 days. Histology showed poorer bridging of the submucosal layer and more polymorphonuclear cell infiltration in the ileum specimens of the control group compared with the treatment groups.

Conclusions. As a single agent in a preclinical wound healing model in the rat, MMF has no negative effect on healing of bowel anastomoses but might have a negative effect on the healing of abdominal wall.

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Due to a steady rise in numbers of solid organ transplantation, as well as a marked increase in efficacy and safety of the immunosuppressant treatment scheme, recipients of solid organs rise in number and age. In fact, the number of people living with a functioning kidney transplant in the United States doubled between 1995 and 2004 and almost doubled again by the end of the year 2012. This rise can not only be due to the rise in total numbers of kidney

transplants and therefore represents the cumulative effect of a better long-term outcome in past decades.^{1,2} Side effect of this development is the fact that surgeons are increasingly confronted with transplant patients in need of an operation, either related to their transplanted organ or to other diseases, such as aneurysms or malignancies,³⁻⁶ because it is well established that solid organ recipients are at a higher risk of developing malignancies compared to the general population.⁵⁻⁷ Immunosuppressant therapy is presently inevitable in solid organ recipients, and most drugs in the polydrug regimen of medication have been associated with wound healing complications. A well-known regimen of immunosuppressant drugs in solid organ transplantation consists of a calcineurine inhibitor such as tacrolimus or cyclosporine (CsA), an antiproliferative agent such as mycophenolate mofetil (MMF) or azathioprine and a steroid. With different immunosuppressant regimens, wound complications of the initial organ transplant operation have been reported up to 52%.⁸⁻¹²

Many clinical studies have been carried out to identify immunosuppressant drugs with a high complication rate.⁸⁻¹² However, it is difficult to entangle the effects on wound healing in a clinical study, where a polydrug study regimen is used. Furthermore, most clinical studies report adverse events, without consistent focus on wound healing complications. Despite all this, cumulating evidence has led to some modifications in clinical practice. In recent years, loading doses of mammalian target of rapamycin (mTOR) inhibitors,

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such as sirolimus and everolimus, are avoided if possible in the light of the wound healing complications. An increasing awareness of the risks of wound complications of mTOR inhibitors has limited its use in the immediate postoperative phase. In this light, it is important to know the effect on wound healing of the other agents that are frequently used in the direct postoperative phase.

MMF has been used as immunosuppressant for more than a decade. It is also frequently used to treat acute rejection after transplantation. MMF efficacy is attributed to its inhibitory activity on lymphocyte functions. After oral administration, MMF is rapidly absorbed and then converted to mycophenolic acid (MPA) which is the active immunosuppressant. MPA inhibits the activity of inosine monophosphate dehydrogenase (IMPDH), a rate-limiting enzyme for the *de novo* synthesis of guanosine nucleotides. Both adenosine and guanine are derived from inosine monophosphate (IMP) which is the first compound in the pathway to have a completely formed purine ring system. The function and proliferation of lymphocytes is more dependent on *de novo* purine nucleotide synthesis compared with other cell types; therefore, IMPDH inhibitors may provide a more lymphocyte-targeted immunosuppression. By depleting the intracellular concentration of guanosine nucleotides, MPA acts as a potent inhibitor of lymphocyte proliferation.

Roos et al¹³ have demonstrated that MMF inhibits collagen gene expression and fibroblast migration. Preclinical studies with this drug have been carried out *in vitro*^{13,14} as well as in pulpal tissue healing in dogs.¹⁵ A well-known and validated preclinical model for wound healing is a bowel anastomosis model in rats. Earlier studies have measured a negative effect of MMF on the healing of bowel anastomoses.^{16,17} However, an intractable difference in the regime of these studies with the clinical situation is the fact that in these studies MMF is started at days 3 and 7 before the operation, whereas in humans, immunosuppressant drugs start immediately after the transplantation operation. It is clinically relevant to find out if a negative effect on wound healing of MMF exists when starting MMF at the day of operation. In case of a negative effect of MMF on wound strength, it will be interesting to find out if any persistence of the effect is measured when delaying MMF for 3 days, as we have measured in a different experiment with Everolimus, in the same bowel anastomosis model.¹⁸

MATERIALS AND METHODS

Ninety-six male Wistar rats (body weight, 240-260 g; Harlan, Horst, the Netherlands) were randomly divided into 4 groups of 24 animals. The animals were housed 2 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 (Ssniff Spezialdiäten GmbH, Soest, Germany). Two groups (groups MMF20-0 and MMF 40-0) received MMF (Cellcept, Roche, Woerden, the Netherlands) orally in daily dosages of 20 and 40 mg/kg from the day of operation to the end of the experiment. Another group started the study medication on day 3 instead of day 0. Half of these animals received 20 mg/kg, and half received 40 mg/kg MMF daily until the end of the experiment (groups MMF20-3 and MMF 40-3.) A control group of 24 animals received saline orally from the day of operation until the end of the experiment. The study was

approved by the Animal Ethics Review Committee of the Radboud University Nijmegen.

Surgical Procedure

On day 0, a laparotomy was performed under general anesthesia using isoflurane 3%, in a mixture of oxygen and nitrous oxide. A midline laparotomy was followed by a resection of 1 cm ileum, 15 cm proximal to the ileocaecal junction, and 1 cm colon 3 cm proximal to the rectal peritoneal reflection. End-to-end anastomoses were constructed with 8 single-layer, inverting, interrupted 8-0 ethilon (Ethicon) sutures. The abdominal fascia 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. Fluid loss was compensated by administering 10 mL of 0.9% sodium chloride subcutaneously directly postoperatively. 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.W.).

Wound Strength

Of the control group and of the MMF-20-0 and MMF-40-0 groups, 12 rats were killed on day 3 and 12 rats on day 7 postoperatively. Of the groups with delayed medication, all rats were killed on day 7 postoperatively. The animals were killed by CO/CO₂ asphyxiation. 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. The anastomoses of ileum and colon were resected with adjacent bowel of approximately 4 cm in length and the suture line in the middle. The intestinal segments were carefully resected, including surrounding tissues and adhesions, and washed in saline. Bursting pressure and breaking strength were measured in the same segment as described previously.¹⁹ In the abdominal wall, breaking strength was measured in the same way; from each segment of the abdominal wall, 2 separate strips of 1 by 2 cm were collected, with the suture line in the middle, and breaking strength was measured in both. The measurement of the least strong segment was used. In each group, 2 rats were used for hematoxylin-eosin staining and histologic description. 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.

Histology

Intestinal samples of approximately 1-cm length containing the anastomosis in the middle were carefully resected en bloc, opened at the mesenteric side, and washed gently in 0.9% NaCl. They were spread out and immobilized, and the samples were immediately fixed in 4% (v/v) phosphate-buffered formaldehyde, pH 7.3. Each anastomosis was divided into 2 or 3 longitudinal strips. Specimens were dehydrated and embedded in paraffin. Sections of 4 mm in thickness were stained with hematoxylin and eosin. Sections were analyzed and assessed by histological parameters for anastomotic repair as described before.²⁰

Mycophenolate Mofetil

Of 4 rats in each of the following groups: control, MMF 20-0, and MMF 40-0 blood was sampled to determine trough levels of MPA. Because we did not need to sample blood for other reasons, we chose to sample blood by direct heart puncture when the animals were killed for humane reasons. MPA levels were determined by high performance liquid chromatography, the detection limit was 0.4 mg/mL.

Statistical Analysis

Trough levels are expressed as mean \pm standard deviation. To analyze differences in body weight, a 1-way analysis of variance was used. The software used for statistical analysis was SPSS 23.0. For testing normality, the Shapiro-Wilk test was used. Differences of bursting inside or outside the suture line of the anastomosis were analyzed with Fisher exact test. Data of breaking strength and bursting pressure were analyzed with the Wilcoxon-Mann-Whitney test. A *P* value less than 0.05 was considered a significant difference.

RESULTS

MPA Trough Levels

Of all 4 animals of the control group, the MPA trough level was under the limit of 0.4 mg/L. The mean MPA trough level in the MMF20-1 group was 0.5 ± 0.1 mg/L, mean MMF trough level in the MMF40-1 group was 0.8 ± 0.4 mg/L.

Weight

From the day of the operation, all the animals lost weight until the third or fourth postoperative day (Figure 1). The largest weight loss was seen in the group of high-dose MMF (MMF40): on day 4, the mean weight of the animals was 87.6% from starting point. On days 4, 5, and 6, the weight in the group MMF 40 was significantly lower compared with the control group. In each group of high-dose MMF (MMF 40-0 and MMF 40-3), 1 animal died for unknown reason. Autopsy in both animals revealed distended small bowel, which may be related to either the operation or the medication. These animals did not suffer from gastrointestinal signs, such as diarrhea. During relaparotomy at the end of the experiment, 2 other rats from the MMF 40-0 group, both operated on day 3, showed signs of ileus, with distended small bowel.

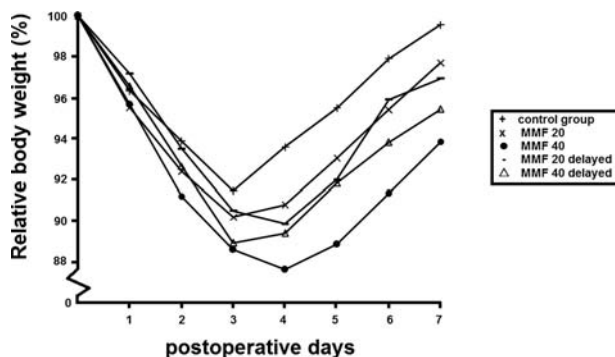


FIGURE 1. Postoperative course of body weight. Points represent average relative body weight, in relation to the weight before operation, for the control group (+) and the groups receiving MMF 20 (x), MMF 40 (●), MMF 20 with delay (-) and MMF 40 with delay (Δ).

Wound Strength

Individual values for bursting pressure of ileum and colon anastomoses and their bursting sites (within our outside the anastomoses) are given in Figure 2. Compared with the control group, the anastomoses of the ileum were stronger in the MMF-treated animals. This was significantly so in ileum tissue in both MMF-treated groups ($P = 0.034$ and 0.048 for MMF 20 and 40 groups, respectively) after 3 days. For colonic tissue, this was not the case ($P = 0.81$ and 0.17 , respectively). At 7 days, no difference could be noted for any of the groups ($P = 0.2$, 0.57 , 0.29 , and 0.41 for the control group versus MMF20-0, MMF40-0, MMF20-3-7, and MMF40-3-7 in ileum anastomoses and 0.71 , 0.05 , 0.41 , and 0.22 in colon anastomoses, respectively). The number of burst sites outside the anastomoses is again shown in Figure 3. We only show here the percentages after 7 days, when in many animals, the anastomoses have grown stronger than the surrounding tissue. The number of cases where the bursting takes place outside the anastomose is not different among the groups.

Breaking strength is shown in Figure 4. Here, the ileum anastomoses of rats of the MMF 20 and of the MMF 40 group are significantly stronger than those of the control group ($P = 0.014$ and 0.041 , respectively). After 7 days, no significant difference is measured ($P = 0.072$, 0.4 , 0.2 and 0.12 for control versus groups MMF20-0, 40-0, 20-3-7, and 40-3-7, respectively). In colon tissue, no difference can be measured between the control group and the experimental groups ($P = 0.57$ and 0.33 at 3 days and 0.42 , 0.94 , 0.65 and 0.87 at 7 days). In abdominal wall tissue, a decrease of strength is measured after 7 days in the high-dose experimental group that was treated from day 0 (MMF40-0), the *P* value was 0.022 . The low-dose group in this experiment just failed to show a significant difference but showed the same trend ($P = 0.05$). The negative effect disappeared when the medication was delayed for 3 days. *P* values at 7 days were, respectively, 0.41 , 0.36 , 0.37 , and 0.20 for groups MMF20-0, MMF 20-3-7, MMF 40-3-7, and MMF 40-1-3.

Histology

No obvious architectural differences were seen between the different groups of the colon tissue or fascia tissue. However, in the ileum, the accumulation of polymorphonuclear cells, macrophages, and lymphocytes was more profound in the control rats than in the MMF20 or 40 rats, indicating less progress in the healing of the anastomose. Even more striking was the fact that bridging of the mucosa and submucosal layer seemed to be less advanced in the control rats (Figure 5).

DISCUSSION

The introduction of new immunosuppressive agents in the last decades has significantly improved the outcome of solid organ transplantation but this improvement is accompanied with new adverse effects, such as nephrotoxicity, lymphoceles, or wound healing disturbances. Wound healing is a very complex process that is potentially influenced by all immunosuppressive agents, although this is difficult to assess in clinical studies, where patients receive a combination of immunosuppressant drugs. The problem with most clinical studies is that wound healing is not a primary endpoint but part of a standard adverse event listing that might be vulnerable to reporting bias. Compared with the vast amount of studies concerning

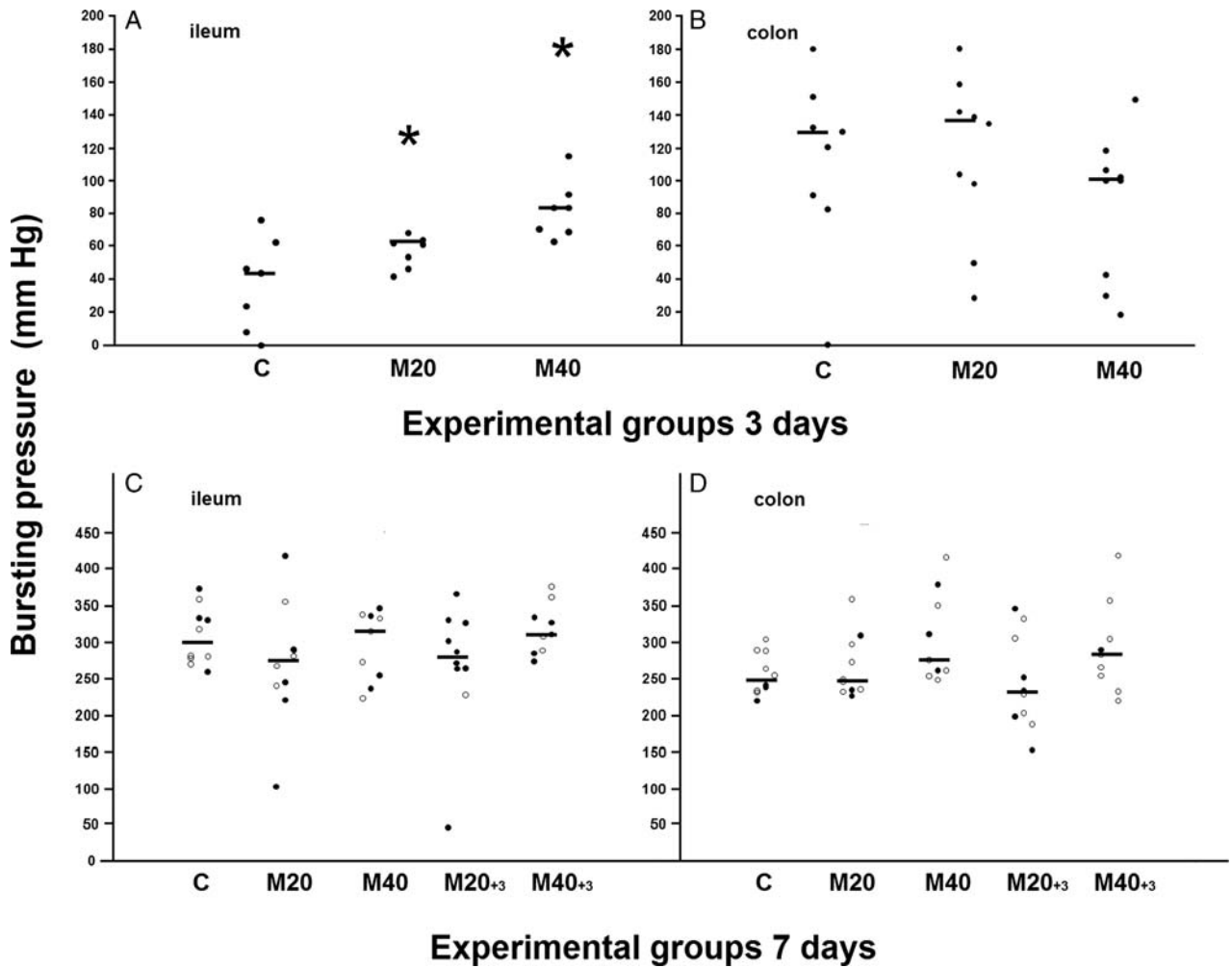


FIGURE 2. Anastomotic bursting pressure. Individual values and medians (horizontal lines) in ileum and colon. A, Ileum control group 3 days postoperative versus MMF 20 and 40; B, colon control group 3 days postoperative versus MMF 20 and 40; C, ileum control group 7 days postoperative versus MMF 20 and 40 7 days postoperative and at 7 days but after a delay of 3 days; D, colon control group 7 days postoperative versus MMF 20 and 40 7 days postoperative and at 7 days but after a delay of 3 days. X-axis: study groups. Open symbols denote rupture outside the suture line and closed symbols rupture inside the suture line. Significant *P* values are marked with an asterisk.

transplant survival, few studies or reviews have focused on wound healing. In 2012, Nanshan²¹ reviewed the literature of immunosuppressant agents and wound healing. Herein MMF does not seem to have a clear benefit over mTOR inhibitors, although the combination of MMF with mTOR inhibitors

may have an additive negative effect on wound healing, as concluded from the study of Pengel et al.²²

Very few preclinical studies have been carried out in the past to determine the effect of MMF on wound healing,^{13,16,17,21} These studies almost unanimously find a negative effect of

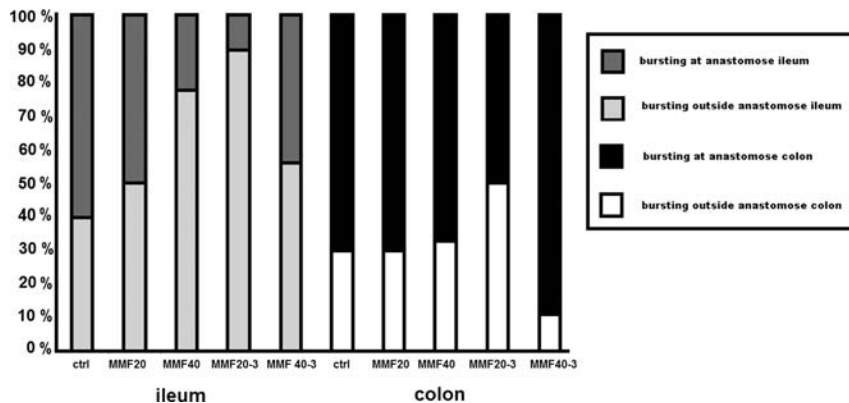


FIGURE 3. Data represent the frequency (percentages) of the bursting site outside and inside the actual suture line on day 7. Light gray represents bursting at the anastomose site in ileum, dark gray represents bursting outside the ileum anastomose. White represents bursting at the anastomose in colon and black represents bursting outside colon anastomose, all measured at day 7.

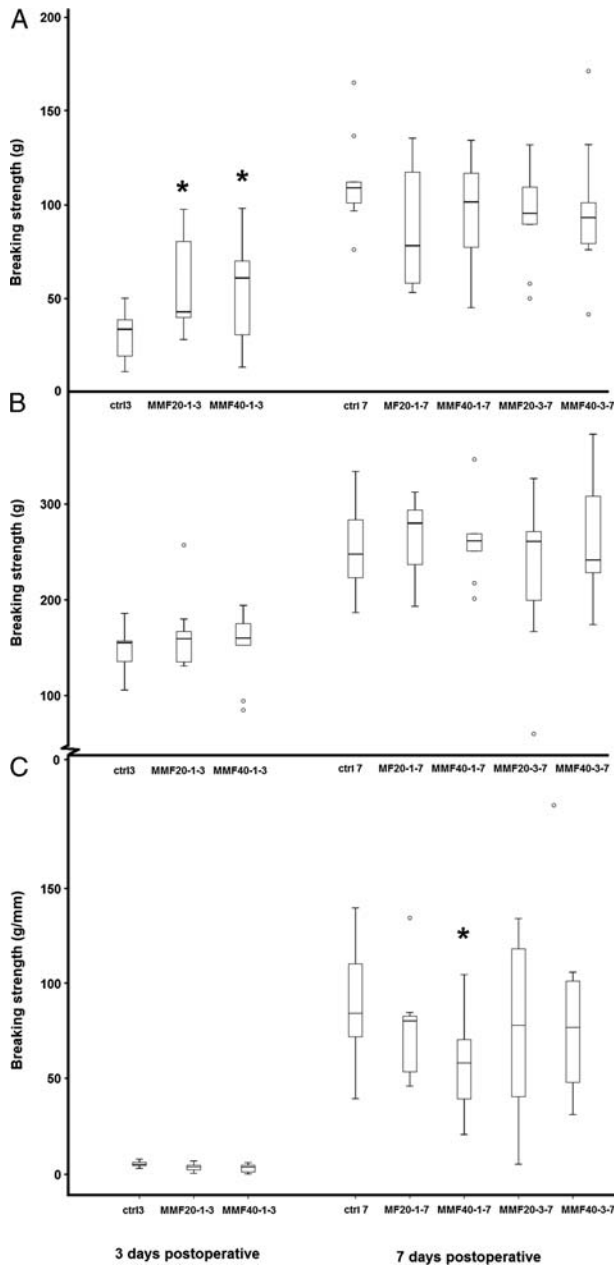


FIGURE 4. Wound breaking strength. Data represent median and range in all groups. A, ileum; B, colon; C, abdominal wall. *P* values that are significant are marked with an asterisk.

MMF. Zeeh et al¹⁶ were the first to show a negative effect on healing of bowel anastomoses in rats on systemic MMF, started 3 days before surgery. However, in this study, MMF was administered intraperitoneally, which may have had a direct effect on the colonic anastomoses and not mimic the orally administration of human organ recipients.

Mycophenolate mofetil is a “prodrug” which is metabolized in the active MPA, a specific inhibitor of IMPDH. This is a key enzyme for de novo synthesis of guanosine nucleotides, essential for DNA and RNA synthesis and necessary for maximum lymphocyte proliferation. The immune suppressant action is therefore based on a decrease of proliferation of T and B lymphocytes and monocytes. There is also evidence that MMF inhibits the action of fibroblasts and other cells that are not part of the immune system.¹³ A negative effect of MMF on wound healing is therefore plausible, even when started postoperatively, as is the case in new transplant recipients.

The experiment presented here is carried out to measure the effects of MMF on the healing of bowel anastomoses and abdominal wall after laparotomy. In the first postoperative week, wound strength is relatively low, and chances of complications are high. To guarantee that no effect of MMF is missed, 2 timepoints of measurement were incorporated: 3 days postoperative, at the end of the inflammatory phase, when wound strength is known to be at the weakest point and 7 days after operation, which is during the proliferation phase, when the wound strength is increasing rapidly. Mycophenolate mofetil is administered orally in 2 different dosages: 20 mg/kg and 40 mg/kg bodyweight. These dosages have proven to have a sufficient immunosuppressant action in rats and lead to clinically relevant MPA trough levels.^{23–25} Two rats in the high-dose group died prematurely of unknown reason but did have distended small bowel. This might be related to the study medication because the therapeutic window of MMF is narrow. In some preclinical studies, dosages of 40 to 60 mg/kg were not well tolerated by rats.^{23,24} The weight loss at 4, 5, and 6 days postoperative was significantly greater for the animals treated with the highest dose of MMF (MMF40). This observation is also made by Schuurman et al,²⁴ where they compared the MMF-CsA combination with CsA only. Mycophenolate mofetil is known for dose-dependent gastrointestinal side effects, such as diarrhea,²⁶ but although side effects were recorded, no more cases of diarrhea were seen in the experimental groups compared with the control group. At day 7, the difference between the MMF 40 group and the control group disappeared, thus showing an adequate recuperation of the rats in the MMF 40 group.

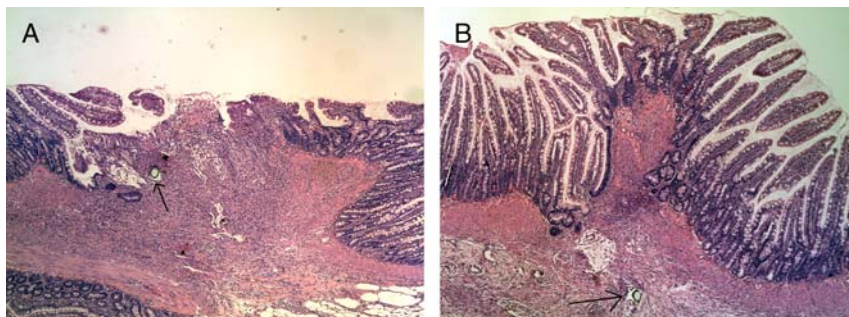


FIGURE 5. Histology example of hematoxylin-eosin–stained specimens. A, Ileum anastomose in control rat at 7 days; B, ileum anastomose in 40-0 rat at 7 days. Arrows indicate suture material.

The results presented here show a positive effect of MMF on the healing of ileum anastomoses, no effect on the healing of colon anastomoses and a negative effect when started at day 0 on the healing of the abdominal wall. Bursting pressure after 3 days in ileum anastomoses of the MMF treated animals is significantly higher than that in the control group. In the measurement of breaking strength in ileum after 3 days we see a similar pattern. In the colon, no significant difference in bursting pressure or breaking strength is noted between the control group and the experimental groups at 3 or 7 days. In the measurement of bursting pressure, the site of bursting indicates the strength of the anastomoses. After day 3, when the wound has gained a considerable amount of strength, the bursting site will often be outside the anastomosis. This means that the anastomosis has grown stronger than the adjacent tissue, and the measurement itself does not represent wound strength anymore. However, the rising proportion of bursting sites outside the anastomosis does indicate an increase in strength. This increase in strength is seen clearly in all groups comparing day 3 with day 7. Although a trend is seen toward a decrease in proportion of bursting sites outside the anastomoses, particularly in the high-dose groups (MMF40) and in the delayed low-dose group (MMF20-3), these differences were not statistically different. The only negative effect of MMF is seen in the abdominal wall tissue, where a significant decrease of breaking strength is seen in the highest-dose group, when the medication was started on day 0. The negative effect disappeared when the medication was delayed for 3 days. The abdominal wall model might therefore be a more sensitive model to wound healing than the bowel anastomose model.

The data presented here show a diverse effect of MMF on its own, dependent on the tissue that the effect is measured in. We have found no published data in the literature on a different effect of MMF according to tissue, because preclinical effects are usually measured on 1 tissue type only. Limitations of this study are the fact that we do not know if the dosages used are directly transferable to the human situation. Although pharmacokinetics in humans and rats are similar, the dosage of 40 mg/kg may have been too high. Furthermore, as in all animal studies, we do not know if these findings are directly transferable to humans.

CONCLUSIONS

As a single agent in a preclinical wound healing model in the rat, MMF in high dose has a negative effect on healing of the abdominal wall, which could possibly be prevented by delaying the medication for 3 days. No negative effect was found on the wound healing of bowel anastomoses.

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