European Surgical Research

Research Article

Eur Surg Res 2021;62:97–104
DOI: 10.1159/000515867

Received: January 28, 2021
Accepted: March 15, 2021
Published online: May 21, 2021

Distal Pancreatic Resection with Splenectomy in the Rat: A Pancreatic Fistula Model to Investigate Postsurgical Damage?

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Keywords
Pancreatic surgery · Pancreatic fistula · Leakage · Animal model · Surgical model

Abstract
Background: Postoperative pancreatic fistula (POPF) is a major complication in pancreatic surgery and can cause considerable postoperative morbidity. Advanced surgical-technical approaches to prevent POPF did not yield a substantial improvement. To investigate innovative treatments, experimental animal models of distal pancreatic resection and pancreaticoduodenectomy are of fundamental importance. After a failed attempt to replicate a previously described rat model for pancreatic fistula induction, we proceeded to distal pancreatic resection with splenectomy to provoke pancreatic leakage and generate a suitable animal model. ***Methods:*** Distal pancreatic resection with splenectomy was performed in 40 rats. The rats were sacrificed on postoperative day (POD) 1, 2, 4, 6, 8, or 10, and the abdominal cavity was explored. Ascites probes were collected pre- and postoperatively for the detection of pancreas amylase and lipase. Tissue samples from the naïve pancreas (POD 0) and the postoperatively harvested remnant were evaluated histologically. The extent of necrosis was determined, and samples were examined for neutrophil infiltration. TUNEL staining served for the verification of necrosis in distinct cases. Immunohistochemistry of Ki67, von Willebrand factor, and CD68 was performed to evaluate proliferation, blood-vessel sprouting, and macrophage invasion. ***Results:*** The rats showed no clinical symptoms or severe complications in the postoperative course up to 10 days. Abdominal exploration revealed adhesions in the upper abdomen, but no intra-abdominal fluid accumulations were found. Signs of inflammation and tissue damage were evident at the pancreatic resection margin on histological examination whereas the naïve pancreatic tissue was widely unaffected. Statistically significant differences were seen between the preoperative and postoperative

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extent of necrosis, the presence of neutrophil infiltrate, and levels of ascitic amylase and lipase. Immunohistochemical staining on Ki67, von Willebrand factor, and CD68 did not reveal any workable results on nonstatistical examination, and it was therefore not considered for further analyses. ***Conclusion:*** Creating a functional animal model of pancreatic fistula that reflects the clinical and pathophysiological impact of pancreatic leakage in humans has not been achieved. Our approach of left pancreatic resection recapitulated inflammation and tissue damage, early events in the development of fistulas, and it could be suitable for the experimental testing of novel targeting methods.

Introduction

Ever since surgical resections have been performed on the pancreas, postoperative pancreatic fistula (POPF) has been a major complication. Digestive juices persistently leak from a resection margin or from an inadequate pancreatic anastomosis. POPF causes damage to surrounding tissues and can trigger inflammatory complications, sepsis, and massive bleeding, and it thus results in high postoperative morbidity rates [1–3]. Numerous clinical trials have explored the influence of surgical techniques, devices, and preparations on POPF development but with no substantial reduction in fistula rates [4–13].

Tanaka et al. [14] were the first to describe the establishment of a rat pancreatic fistula model by comparing the effects of transecting each of the four pancreatic ducts. Indocyanine green injection into the portal vein and temporary common bile duct ligation led to dye congestion and pancreatic duct visualization (Takayuki Tanaka, pers. commun.) [14]. They concluded that splenic duct transection, without tissue resection, represented the optimal approach for the induction of pancreatic fistula in the rat, as it involved a moderate intra-abdominal change, a high degree of adhesions and ascites amylase levels >100 times greater than normal on postoperative day (POD) 1.

Here, we detail our experience with the generation of a rodent pancreatic fistula model and present our concerns regarding the preclinical suitability of the previously described model, where we did not observe fistula-related clinical and biochemical alterations. To generate a more suitable model, we resorted to distal pancreatectomy with splenectomy in rats, to facilitate future studies on pancreas damage and regeneration as well as fistula-preventive strategies.

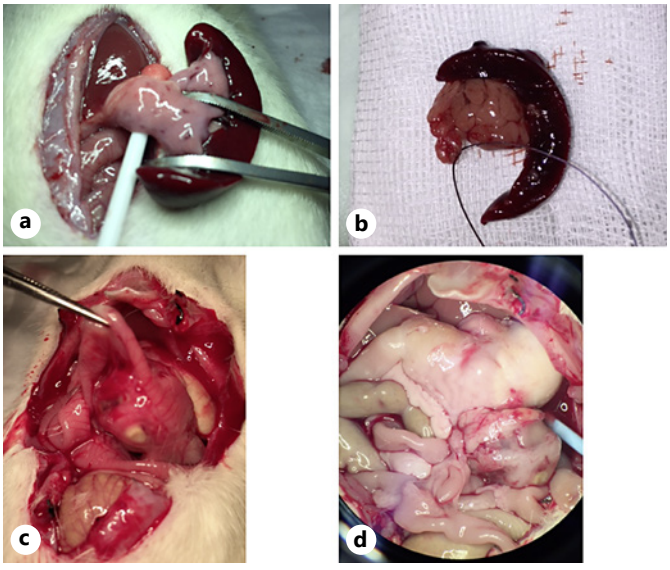

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Fig. 1. a A 2-pronged hook is holding the spleen to the left side to allow a preferably standardized transection line. **b** The distal part of the pancreas (approx. 8–10 mm) is resected en bloc with the spleen. **c**, **d** Postoperative examination of the abdominal cavity shows moderate to strong intra-abdominal changes and adhesions in the left upper abdomen around the pancreatic stump, predominantly involving the large bowel, greater curvature of the stomach, and liver.

Material and Methods

Animals

Forty male Sprague-Dawley rats weighing 250–300 g (Charles River, Sulzfeld, Germany, and Calco, Italy) were maintained under standard conditions in the Daniel Swarovski Laboratory animal facility with a 12-h light/dark cycle and unlimited access to water and rodent chow pre- and postoperatively.

Distal Pancreatic Resection Model

The rats received metamizol (100 mg/kg body weight) subcutaneously before isoflurane inhalation anesthesia (3–4% for induction, 0.5–1.5% for maintenance, O_2 flow 3–5 L/min). For perioperative analgesia, fentanyl (0.005 mg/kg body weight) was injected subcutaneously. After fixation, shaving, and skin disinfection, an abdominal midline incision was performed. Abdominal lavage with 5 mL of 0.9% NaCl solution was done to collect ascites for enzyme measurement. In the distal pancreatic resection model, neither the bile duct nor the duodenum was ligated. Abdominal attachments were dissected and the spleen and left part of the pancreas were exposed. A 2-pronged hook held the spleen to the left side to allow a preferably standardized resection line (Fig. 1a, b). Using microscissors, part of (8–10 mm) the distal pancreas was resected en bloc with the spleen prior to fixation. A bipolar forceps served for punctual hemorrhage control in the case of relevant bleeding. The abdomen was closed using a Vicryl® 3.0 running suture for the muscles and Seralon® 4.0 interrupted sutures for the skin.

Table 1. Number of rats sacrificed on each postoperative day (POD)

	POD 1	POD 2	POD 4	POD 6	POD 8	POD 10
Rats sacrificed, *n*	22	7	3	2	3	3

Animal Harvest and Exploration of the Postoperative Situs

Postoperative analgesia was administered as tramadol in drinking water (0.5 mg/mL) and subcutaneous metamizol (100 mg/kg body weight) every 8–12 h. General condition and wound healing were checked twice daily. Animals were sacrificed by anesthetic overdose and cardiac puncture on POD 1, 2, 4, 6, 8, or 10 (Table 1). The abdominal cavity was explored for signs of inflammation, fluid accumulation, adhesions, and alterations of the surrounding organs. Ascites was collected by abdominal lavage as described above. The pancreas remnant was removed quickly for subsequent fixation. If signs of tryptic necrosis were present in neighboring organs, a probe was resected for histological assessment.

Ascites Amylase and Lipase Detection

Ascites probes taken before pancreas resection served as controls for samples taken on the day the animals were sacrificed. Ascitic amylase and lipase levels were measured at the Central Laboratory of the University Hospital of Innsbruck according to standard procedures.

Histology

The resected specimens of the first operation served as controls for the pancreas remnant taken on the day the animals were sacrificed. All specimens were fixed in 4% buffered formaldehyde (SAV Liquid Production, Germany) and embedded in paraffin. Sections, 4-µm-thick, were cut, and stained with hematoxylin & eosin (HE). Histomorphological examination was performed by 2 pathologists, in blinded fashion, following a 4-eye-principle. Pancreatic necrosis was quantified as a percentage of the specimen. Neutrophil infiltration was evaluated (present or not present).

Immunohistochemistry

Paraffin-embedded tissue sections were prepared for immunohistochemical processing. We used an antibody against CD68 (FLEX monoclonal mouse anti-human CD68, DAKO, Denmark) to detect macrophages, an antibody against von Willebrand factor (vWF; FLEX polyclonal rabbit anti-human von Willebrand factor, DAKO) to detect blood-vessel sprouting, and an antibody against Ki67 (FLEX polyclonal mouse anti-human Ki67 antigen, clone MIB-1, DAKO) to evaluate proliferation. TUNEL staining (ApopTag® plus peroxidase in situ apoptosis detection kit, EMD Millipore Corp., Temecula, CA, USA) was performed to distinguish thermic tissue damage from true necrosis in uncertain cases.

Statistical Analyses

Generalized estimating equations (GEE) were used to analyze repeated measures comparing pre- and postoperative data [15]. Data are expressed as mean ± SEM in diagrams. $p < 0.05$ was considered statistically significant. Statistical analyses were performed using the software IBM SPSS Statistics 21 (IBM Corporation Armonk, NY, USA).

Results

Limitations of a Previously Published Rat Fistula Model

As previously described by Tanaka et al. [14], we opened the abdomen and slowly injected up to 5 mL indocyanine green antegradely into the portal vein. The common bile duct was ligated using a fine thread after carefully removing surrounding pancreatic tissue, which, in our hands, caused relevant collateral damage. The thread was difficult to remove afterwards so we tried duct-clamping using a microvessel clamp or duodenal clamping to both sides of the papilla of Vater to provoke dye distribution into all pancreatic ducts. However, duct visualization did not work reliably in our attempts and we were unable to identify distinct pancreatic ducts. Hence, we decided on transecting uncolored ducts that we detected. After up to 9 days, exploration of the abdominal cavity did not show adhesions or obvious alteration of the neighboring organs, and the abdominal lavage samples did not reveal an increase in pancreatic enzymes. Histological examination of the pancreas did not demonstrate significant areas of necrosis or inflammation (data not shown).

Distal Pancreatic Resection Model: Intra- and Postoperative Course and Macroscopic Situs

To make progress in the establishment of a functioning rat pancreas fistula model, we performed the operative procedures as described in Material and Methods. No relevant intraoperative complications occurred. Eventual bleedings were managed by gentle compression or punctual electrocauterization. Postoperatively, all animals presented clinically well, and ate, drank, and moved around in the cage without signs of discomfort. No intra-abdominal fluid accumulations were found when the animals were sacrificed. Moderate to strong adhesions and intra-abdominal changes were observed around the pancreatic stump, involving the large bowel, greater curvature of the stomach, and liver (Fig. 1c, d). The longer the postoperative follow-up, the more often adherent structures could not be separated easily using cotton swabs but had to be dissected with microscissors.

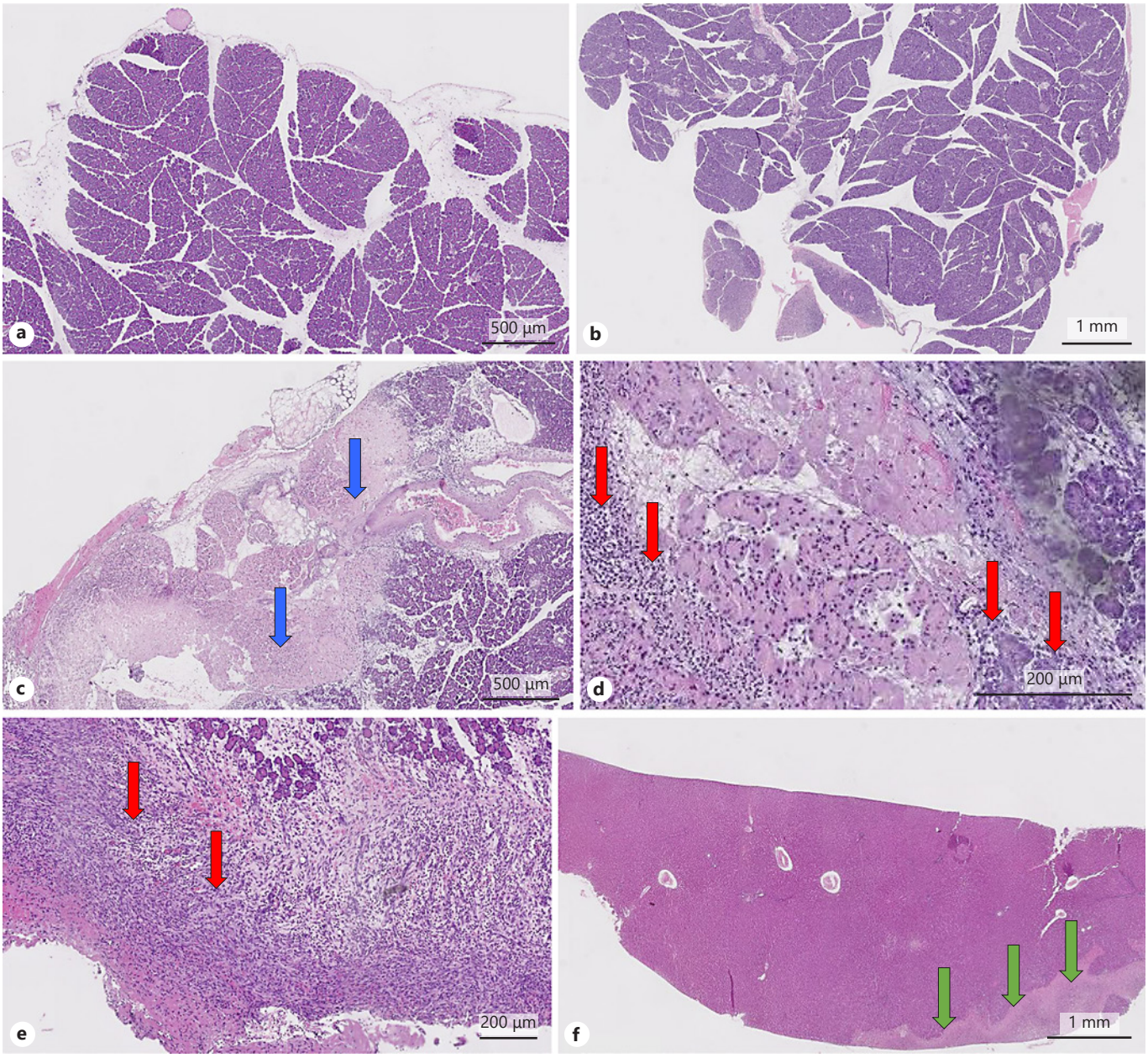

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Fig. 2. **a**, **b** Histological evaluation of sections of the unaffected naïve pancreas on POD 0. HE. Evaluation on POD 2 (**c**, **d**) and POD 4 (**e**) reveals signs of inflammation and tissue damage, i.e., neutrophil infiltration (red arrows) and areas of necrosis (blue arrows), at the site of the pancreatic resection plane. **f** Histological examination of a neighboring part of the liver confirmed the affection of the organ (green arrows) by pancreatic juices in the abdominal cavity on POD 1.

Histological and Molecular Characterization

Histopathological evaluation of the pancreas remnant revealed signs of inflammation and tissue damage at the resection site, characterized by necrotic areas, neutrophil infiltration, and cell deterioration with vanishing nuclei (Fig. 2c–e). The rest of the organ showed normal unaffected parenchyma. In some cases, histological examination of the neighboring liver was performed to confirm organ affection by leaking digestive juices (Fig. 2f). POD 0 pancreas samples barely showed signs of tissue damage or inflammation (Fig. 2a, b).

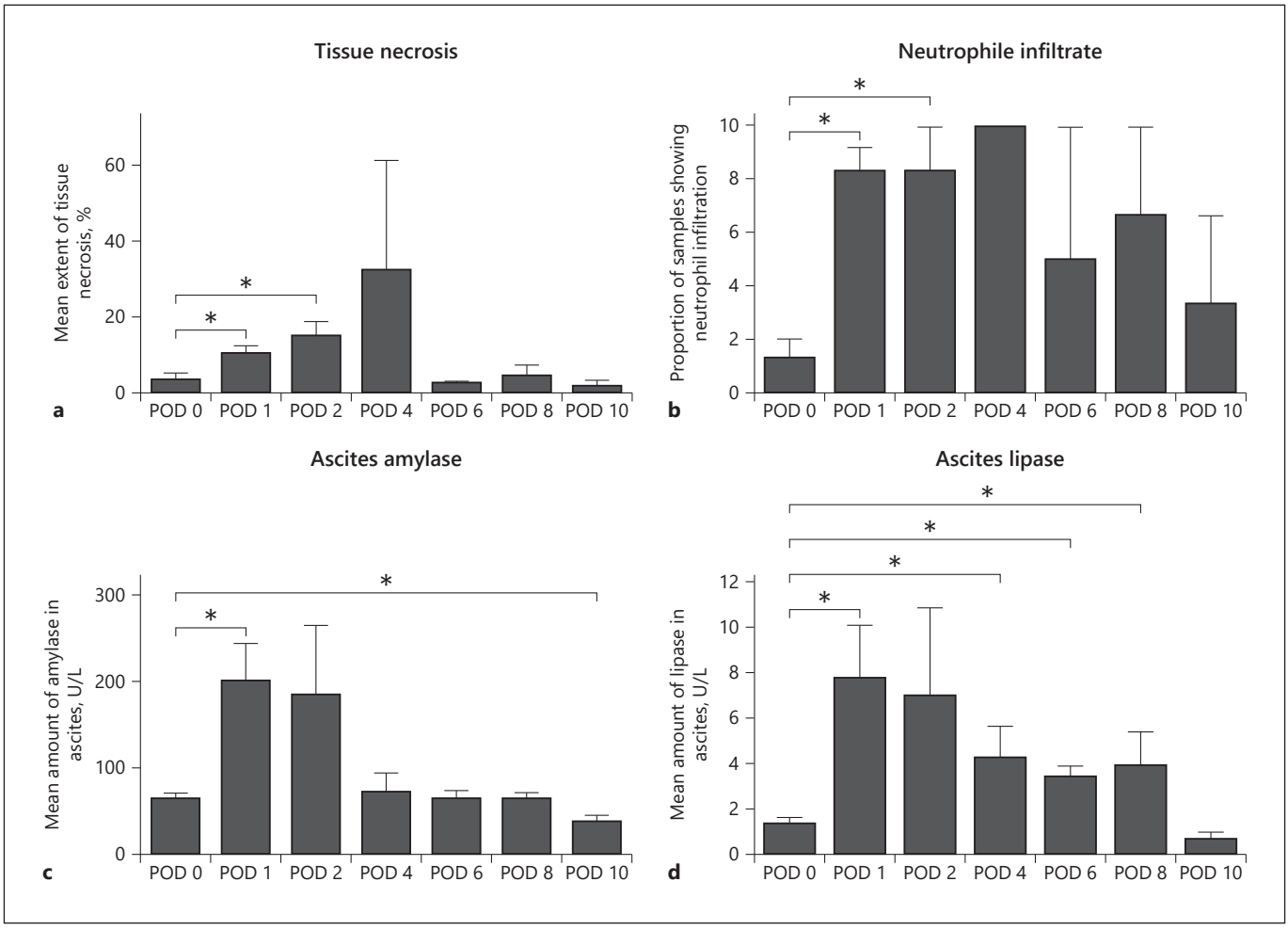

Fig. 3. a The extent of pancreatic tissue necrosis at distinct time points. The area of tissue necrosis was determined by histological examination. Mean size of the area was 3.35% of the pancreatic specimen on POD 0 ($n = 23$), 10.28% on POD 1 ($n = 18$), 15.0% on POD 2 ($n = 7$), 32.33% on POD 4 ($n = 3$), 2.5% on POD 6 ($n = 2$), 4.33% on POD 8 ($n = 3$), and 1.67% on POD 10 ($n = 3$). Significant difference was shown between preoperative and postoperative extent of necrosis, in general ($p = 0.015$), and between necrosis on POD 0 and POD 1 ($p = 0.024$) and between POD 0 and POD 2 ($p = 0.002$). **b** The presence of neutrophil infiltrates in the pancreas specimen at distinct time points. Infiltration of neutrophil granulocytes was observed in 3/23 samples (13%) on POD 0, 15/18 samples (83.3%) on POD 1, 5/6 samples (83.3%) on POD 2, 2/2 samples (100%) on POD 4, 1/2 samples (50%) on POD 6, 2/3 samples (66,7%) on POD 8, and 1/3 samples (33.3%) on POD 10. When considering the proportion of samples showing neutrophil infiltration on each day, a statistically significant difference was seen between POD 0 and all postoperative days ($p < 0.001$), between POD 0 and POD 1 ($p < 0.001$), and between POD 0 and POD 2 ($p = 0.004$). Levels of postoperative ascitic amylase (**c**) and lipase (**d**) measured in the abdominal lavage are shown. There was a statistically significant difference between preoperative and postoperative amylase, in general ($p = 0.001$), and between amylase levels on POD 0 and POD 1 ($p = 0.001$), and on POD 0 and POD 10 ($p = 0.004$). A statistically significant difference was seen between preoperative and all postoperative lipase samples ($p < 0.001$), and between lipase levels on POD 0 and POD 1 ($p = 0.005$), POD 0 and POD 4 ($p = 0.015$), POD 0 and POD 6 ($p < 0.001$), and POD 0 and POD 8 ($p = 0.027$). * $p < 0.5$.

The mean extent of pancreas necrosis and the proportion of samples showing neutrophil infiltration on each POD appear in Figure 3a, b.

Immunohistochemical staining of 31 pancreatic tissue samples did not reveal positivity for Ki67, CD68, or vWF (Fig. 4a–c). TUNEL staining confirmed the presence of DNA strand breaks indicating cellular necrosis on several samples (Fig. 4d).

Mean pancreatic amylase (Fig. 3c) and lipase (Fig. 3d) levels in ascites probes were, respectively, 62,74 and 1.37 U/L on POD 0 ($n = 19$), 195.81 and 7.95 U/L on POD 1 ($n = 21$), 180.29 and 7.14 U/L on POD 2 ($n = 7$), 70.0 and

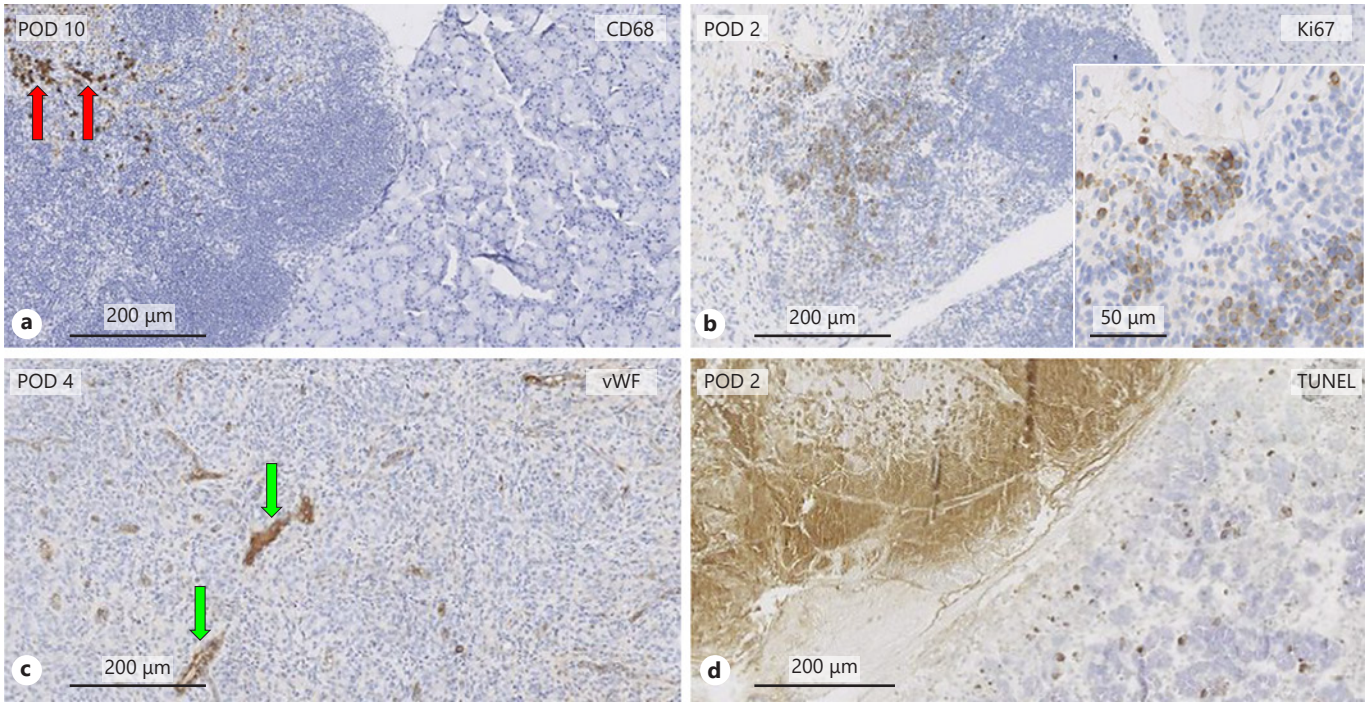

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Fig. 4. Immunohistochemical staining for CD68 (**a**), Ki67 (**b**), vWF (**c**), and TUNEL (**d**) on pancreatic tissue at distinct time points. Results of immunohistochemical staining for CD68, Ki67, and vWF did not reveal the expected signs of postinjury tissue proliferation and neoangiogenesis. On POD 10, CD68 is expressed in a lymph node (**a**, red arrows), demonstrating a positive control, but not in the pancreatic tissue. **c** Expression of vWF makes blood vessels visible in brown color (green arrows). **d** TUNEL staining served to confirm the presence of DNA strand breaks indicating cellular necrosis.

Table 2. Mean pancreatic amylase and lipase levels in ascites probes on each postoperative day (POD)

	POD 0	POD 1	POD 2	POD 4	POD 6	POD 8	POD 10
Samples analyzed, *n*	19	21	7	3	2	3	3
Mean amylase level, U/L	62.74	195.81	180.29	70.0	62.5	62.33	36.67
Mean lipase level, U/L	1.37	7.95	7.14	4.33	3.5	4.0	0.67

4.33 U/L on POD 4 (n = 3), 62.5 and 3.5 U/L on POD 6 (n = 2), 62.33 and 4.0 U/L on POD 8 (n = 3), and 36.67 and 0.67 U/L on POD 10 (n = 3) (Table 2). Amylase and lipase levels increased in the early postoperative days. Amylase returned towards a normal level around POD 4, but ascites lipase decreased only after POD 8.

Discussion

Only a few experimental studies have been published that describe animal models for investigations into POPF and respective prevention strategies. In 2013, a novel pancreatic fistula rat model, involving pancreatic duct visualization with indocyanine green and subsequent splenic duct transection, was reported [14]. When our group tried to reproduce this model, we were unable to induce duct visualization and did not achieve the same results regarding an increase in ascitic amylase and lipase. The Japanese group pointed out that ascitic and serum amylase levels would return towards normal by 7 days after the operation, making their approach an ideal short-term observation model. When we switched the surgical technique to distal pancreatic resection with splenectomy, irrespective of duct anatomy, we did not observe a dramatic clinical impact and no intra-abdom-

inal fluid collection. As also described by Tanaka et al. [14], we needed to perform abdominal lavage to collect sufficient ascites for enzyme measurement. We saw a significant postoperative increase in ascites enzyme levels and moderate to strong intra-abdominal changes and adhesions. The extent of necrotic areas and neutrophil infiltrates in the pancreatic remnant was significantly elevated in the early postoperative days. After 4 and 10 days, respectively, ascites amylase and lipase levels returned to normal. The extent of necrosis showed a marked decrease on POD 6, probably pointing to tissue degradation at the resection margin.

Nowadays, more and more experimental investigations into innovative POPF-preventive strategies are based on rat models [14, 16–19]. While our work was in progress, Kim et al. [18] compared 3 options of fistula manifestation. When they divided the rat pancreas at the intersection of the common pancreatic duct and bile duct (I), all the rats died early. When the splenic duct was transected at the pancreas tail level (III), rats survived the 10-day duration of the study but showed no abdominal fluid. Division of the gastric and splenic duct through pancreas transection along the portal vein (II) led to continuous secretion of pancreatic juice with an ascites amylase level >3 times the normal serum value, but 100% 10-day-survival. This approach was considered adequate to assess the efficacy of materials for POPF prevention. However, the procedure implies identification of distinct pancreatic ducts as well, which, in our opinion, is a challenging task. Kim et al. [18] acknowledged the difficulty of creating an animal model that fully represented clinically POPF but considered their model to be the most accurate at the time. When comparing with their technique, our approach of distal pancreatectomy most likely corresponds to a model between II and III regarding the anatomical transection plane. But the fact that we did not observe relevant ascites production or continuous leakage of pancreatic juices, respectively, might indicate that we should have determined the transection line closer to the portal vein.

As we could confirm in our rat model of distal pancreatic resection with splenectomy, inflammation and necrosis occur at the resection site postoperatively, demonstrating tissue damage at the resection site. Tanaka et al. [14] described similar changes in their study. Postoperative elevation of ascites enzymes points to leakage from the pancreatic resection margin, which is known to trigger a cascade of major further complications in humans [20–22]. We also found moderate adhesions 24 h after pancreatic resection, and even stronger adhesions the longer the follow-up period. Around POD 6, the upper abdominal organs could only be separated by microscissors. However, the clinical and biochemical impact of pancreas resection seems much milder in the rat than in humans when it comes to general condition, pain, fever, or peritoneal affection with ascites production. In our study, the rats presented clinically well after the operation and no fluid collections, abscesses, or severe complications such as bleeding were found when examining the abdominal cavity for up to 10 days postoperatively. Our findings suggest that the distal pancreatic resection rat model may facilitate investigations into the early cellular processes and molecular pathways that are activated at surgical transection and lead to impaired tissue healing in a milieu obtained by digestive juices. Establishing an internationally adopted rodent model of POPF would be of great value to allow investigations focusing on the inflammatory process that occurs at the pancreatic resection margin, and to lay the foundation for testing of innovative preventive means.

Acknowledgement

We acknowledge the essential support provided by our colleagues in the clinic and in the research laboratory during the work on this project. The help of Ruth Baldauf in the preparation of this manuscript is greatly appreciated.

Statement of Ethics

The animal experiments were approved by the Austrian Federal Ministry for Science, Research and Economy (application No. BMWFW-66.011/0120-WF/V/3b/2015) and conducted according to the Guidelines for the Care and Use of Laboratory Animals.

Conflict of Interest Statement

S.S. received grants/research support from Koehler Chemie, Novartis, Roche, Sandoz, Bridge to Life, Chiesi, Neovii and Organ Recovery, speaker's fees/honoraria from Astellas, Sanofi, Chiesi, BMS, OrganOx and Novartis, and consulting fees from Astellas, Novartis, Teva, Sandoz, Merck, Atara, NefroHealth, and ITB. I.E.K. received speaker's honoraria from Takeda and a fee for a book chapter from Springer. None of these declarations are related to this particular study. The other authors declare that they have no conflicts of interest.

Funding Sources

There was a donation of EUR 5,000 from SIGNA Holding GmbH, a real-estate company not affiliated with the Medical University of Innsbruck or any pharmaceutical institutions and that derived no benefit from the realization of the project. The donation was explicitly dedicated to conducting this study, for the purchase of equipment and animals, but not for personnel costs, and was deposited into an official university account.

Author Contributions

Study conception and design: S.K., J.T., I.E.K., and S.S. Animal experiments (surgical procedures and harvesting): S.K. Perioperative animal care and asservation of samples: S.K. and T.K. Analysis and interpretation of data: S.K., P.M., H.M., S.M., T.H., and J.T. Drafting of the manuscript: S.K. Critical revision: J.T., S.S., I.E.K., T.H., and D.Ö.

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