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Acetazolamide-eluting biodegradable tubular stent prevents pancreaticojejunal anastomotic leakage

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ABSTRACT

Postoperative pancreatic fistula at the early stage can lead to auto-digestion, which may delay the recovery of the pancreaticojejunal (PJ) anastomosis. The efficacy and safety of an acetazolamide-eluting biodegradable tubular stent (AZ-BTS) for the prevention of self-digestion and intra-abdominal inflammatory diseases caused by pancreatic juice leakage after PJ anastomosis in a porcine model were investigated. The AZ-BTS was successfully fabricated using a multiple dip-coating process. Then, the drug amount and release profile were analyzed. The therapeutic effects of AZ were examined in vitro using two kinds of pancreatic cancer cell lines, AsPC-1 and PANC-1. The efficacy of AZ-BTS was assessed in a porcine PJ leakage model, with animals were each assigned to a leakage group, a BTS group and an AZ-BTS group. The overall mortality rates in these three groups were 44.4%, 16.6%, and 0%, respectively. Mean α-amylase concentrations were significantly higher in the leakage and BTS groups than in the AZ-BTS group on day 2–5 ($p < 0.05$ each all). The luminal diameters and areas of the pancreatic duct were significantly larger in the leakage group than in the BTS and AZ-BTS groups ($p < 0.05$ each all). These findings indicate that AZ-BTS can significantly suppress intra-abdominal inflammatory diseases caused by pancreatic juice leakage and also prevent late stricture formation at the PJ anastomotic site in a porcine model.

1. Introduction

Pancreaticoduodenectomy (PD) is performed for the treatment of periampullary and pancreatic head neoplasms [1–4]. Owing to the improvements in surgical techniques, postoperative care, and the performance of this operation, the indication of PD has expanded to include benign or low-grade malignancies in addition to malignant neoplasms [1,2]. Therefore, the long-term survival after PD has improved, and the quality of life after surgery is being given increased importance [3]. PD with a pancreaticojejunal (PJ) anastomosis is currently the most common therapeutic strategy for malignant disease of periampullary and pancreatic head neoplasms [1–4]. However, leakage and subsequent fistula and strictures formation at the PJ anastomosis still occur after pancreatic reconstructive surgery despite improvements in operation

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methods [5–7], pharmacological approaches [8], and the use of newly developed biodegradable polymers [9–11]. Internal and external drainage using a trans-anastomotic stent for pancreatic-enteric anastomoses can decrease the incidence of anastomotic leakage and stricture formation after PD with PJ [12,13]. However, the choice of internal or external drainage and/or type of stent remains controversial and should depend on local expertise.

Postoperative pancreatic fistula (POPF) at the early stage can lead to auto-digestion, which may delay the recovery of the PJ anastomosis [14]. The pancreatic juice has an alkaline pH of approximately 8 because of bicarbonate (HCO3-) secreted from the pancreatic cells [15]. The highly acidic gastric juice that is transported from the stomach to the duodenum helps digestive enzymes from the pancreas to be active at a neutral pH [16]. After the surgical procedure, the gastric juice cannot neutralize the pancreatic juice because the stomach is reconstructed below the pancreas. Thereby, trypsin, an enzyme which digests protein (pH range, 7.9–9.7) [15] is activated, and it can cause auto-digestion of surrounding tissues, including the PJ anastomosis, as well as inducing intra-abdominal inflammation that can lead to abscesses and/or adhesion, and postoperative pancreatitis, resulting in dehiscence of the PJ anastomosis [11,17].

Considering the activation of the protease, a carbonic anhydrase (CA) inhibitor, called acetazolamide (AZ), was used in this study. Cells produce several CA enzymes to control intracellular pH [18]. AZ can prevent autolysis of the anastomosis because of acidification that reduces the activity of the pancreatic juice [19,20]. To confirm the efficacy of the AZ, a PJ anastomotic leakage porcine model was established by reducing the number of sutures at the PJ anastomosis. Herein, we hypothesized that an AZ-eluting biodegradable tubular stent (AZ-BTS) might decrease the self-digestion and development of inflammatory diseases adjacent to the PJ anastomosis caused by pancreatic juice leakage via acidification and reduced activity of the pancreatic juice. Therefore, the purpose of this study was to investigate the efficacy and safety of AZ-BTS for the prevention of self-digestion and intra-abdominal inflammatory diseases caused by pancreatic juice leakage after PJ anastomosis in a porcine model.

2. Materials and methods

2.1. Materials

AZ was purchased from Sigma-Aldrich (St. Louis, MO, USA). Poly (lactide-*co*-glycolic acid, LA: GA, 75:25, MW 25,000–35,000 Da) (PLGA) was purchased from Polyscitech (West Lafayette, IN, USA). Dimethylsulfoxide (DMSO), acetone, and acetonitrile (ACN) were purchased from Duksan (Ansan, Korea). The porcine pancreatic alpha-amylase enzyme-linked immunosorbent assay (ELISA) kit was purchased from the Bioassay Technology Laboratory (Shanghai, China). The pHrodo Red AM Intracellular pH indicator was obtained from Thermo-Fisher Scientific (Waltham, MA, USA).

2.2. Preparation of AZ-BTS

The BTSs used in this study were manufactured by using poly (dioxanone-*co*-trimethylene carbamate-co-glycolide) terpolymers consisting of poly *p*-dioxanone (PDO), trimethylene carbonate (TMC), and glycolide. The BTSs (diameter, 2 mm; length, 30 mm) were prepared as in our previous report [9]. Two radiopaque markers at each end of the stent were used to identify the stent location under fluoroscopy. A 5 Fr dilator was used as a stent introducer (Cook, Bloomington, IN, USA). The BTSs were coated by using a multiple dipping technique. The drug-containing layer solution consisted of PLGA (LA:GA, 75:25, MW 25,000–35,000 Da) and AZ in acetone and DMSO 9:1 (*v*/v). The ratio of the polymer to the drug was 12:5. The BTS was fixed on a mandrel, which was rotated during the coating process, and the BTS was dip-coated five times with a 1 cm/s speed at room temperature. After each coating, the BTS was dried under vacuum for 2 h. The residual solvent was eliminated in vacuum overnight after the final drug-containing layer coating process. The covering layer solution consisted of PLGA (LA:GA, 75:25, MW 25,000–35,000 Da) in acetone at an 8% (*w*/*v*) concentration. The covering layer was coated two times using the same technique. Finally, the AZ-coated BTS was dried overnight under vacuum (Fig. S1A, Supplementary Material).

2.3. Morphological analysis of AZ-BTS

The morphological structure of the AZ-BTS was analyzed by using a scanning electron microscope (SEM, SNE-3200 M, SEC, Suwon, Korea). The AZ-BTS was cut horizontally and vertically. The samples were fixed on an aluminum pin stub mount using carbon tape, and then the surface and thickness of the samples were analyzed to ensure proper distribution of the coating solution over the stent surface.

2.4. In vitro degradation assay of AZ-BTS

To evaluate the degradation test of both the non-coated BTS and AZ-BTS, the samples were placed in a 15 ml glass tube with 10 ml of phosphate buffer saline (pH 7.4, 1×). The stent was placed in a shaker set at 37 °C, 50 rpm. The surface morphologies of the samples were assessed using SEM at 1, 2, 3, 4, 6, and 8 weeks.

2.5. Drug amount and release of AZ-BTS

AZ-BTS was inserted into a 10 ml volumetric flask, and then we added 5 ml of ACN to dissolve the PLGA polymer. The AZ-BTS was sonicated at 37 °C for 30 min. And then, 5 ml of DMSO was added into the volumetric flask and sonicated for an additional 30 min. The solution was filtered with a 0.45 μm syringe filter and diluted 10 times with DMSO to quantify the drug content per stent by high-performance liquid chromatography (HPLC). To analyze the drug release profile of the AZ-BTS, the elution medium was prepared by using phosphate buffer saline (pH 7.4, 1×). The mobile phase was a mixture of 10:90 ACN per 20 mmol/L sodium acetate (pH, 4.1; *v*/v). To determine the AZ concentration, the AZ-BTSs were placed in 15 ml falcon tubes containing 10 ml of elution medium. The tubes were placed in a shaking bath (37 °C). The released drug solution in each tube was withdrawn, and the drug content was measured using HPLC up to 4 weeks. The solution was replaced with fresh buffer after each sampling. An HPLC apparatus equipped with an ultraviolet (266 nm) detector (Waters, Milford, Mass) was used for the AZ quantitation.

2.6. Pharmacokinetic study of AZ-BTS

AZ eluting film was fabricated by a casting method and surgically embedded in the peritoneal region of five. The AZ dose was adjusted to 100 mg/kg to ensure its detection by ultra performance liquid chromatography (UPLC, Waters Acquity UPLC System, Milford, MA). Blood was collected from the retro-orbital venous sinuses of these mice. Plasma (50 μl) was obtained by mild centrifugation and deproteinized by adding 400 μl of cold methanol, with sulfadiazine selected as the internal standard. The samples were centrifuged, and the supernatants were vacuum-dried and reconstituted with mobile phase solution for UPLC analysis.

2.7. In vitro cytotoxicity and therapeutic effects of AZ-BTS

AsPC-1 (A human pancreatic adenocarcinoma cell line) were maintained as a monolayer cultured in RPMI-1640 medium supplemented with 1% penicillin-streptomycin (Gibco, Thermo Fisher Scientific, USA) and 10% fetal bovine serum (Gibco). PANC-1 (a human pancreatic ductal epithelioid carcinoma cell line) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 1% penicillin-streptomycin (Gibco) and 10% fetal bovine serum (Gibco). AsPC-1 and PANC-1 cells were placed into 96-well plates at 1×10^5 cells per well. AZ was dissolved in the DMSO solution and diluted with RPMI-1640 and DMEM medium to the indicated concentration. After 24 h incubation in the 96-well plate, 5 nM to 100 μM of AZ was applied to the cells for one day. The cell viability was quantified using WST-1. The cell proliferation assay was conducted according to the manufacturer's instructions. Each of the cell lines was incubated in a 96-well plate with 0.5×10^5 cells per well for 24 h. Using an intracellular pH Calibration buffer kit, AsPC-1 and PANC-1 cell intracellular pH (pHi) was calibrated, and then 50 nM, 100 nM, or 500 nM of AZ were added to the cells. After 24 h, the intracellular pHs of the AZ treated cells were quantified by pHrodo, measuring the fluorescence intensity with an AZ fluorescence intensity standard curve. AsPC-1 human pancreatic cancer cells were subjected to western blot analysis using polyclonal antibody against human CA IX (Invitrogen, Carlsbad, CA), with MG63 human bone osteosarcoma cells used as a negative control.

2.8. Animal study

This study was approved by the Institutional Animal Care and Use Committee of Asan Institute for Life Sciences (2017–14-190), and it conformed to the US National Institutes of Health guidelines for laboratory animals. Eighteen pigs, each weighing 34.4–38.1 kg (median weight = 36.3 kg), were subjected to PJ anastomosis with two-point sutures to artificially create an anastomotic leakage. The pigs were randomized into three groups of six pigs each, a leakage group, a BTS group, and an AZ-BTS group, with the latter two groups undergoing BTS and AZ-BTS placement, respectively. All pigs were supplied with food and water ad libitum and maintained at 22 ± 2 °C. The body weights of the pigs were measured before surgery and then weekly until sacrifice. The animals were euthanized using an overdose of xylazine hydrochloride (Rompun; Bayer, Seoul, Korea) at 4 weeks after surgery.

2.8.1. PJ anastomosis leakage model and stent placement

The techniques of the surgical procedure and stent placement have been described in detail previously [9]. PJ anastomosis via the duct-to-mucosa method was performed at four points with sutures in our previous study [9]. In this study, to create pancreatic juice leakage at the PJ anastomosis, the number of sutures was decreased from four to two points, and sutures between the pancreatic capsule and jejunal serosa were not placed. In the BTS and AZ-BTS groups, the stents were sutured to the jejunal mucosa to prevent their migration into the cut pancreatic duct or distal small bowel (Fig. S1B and C, Supplementary Material). A 10-Fr Levin tube with multiple side holes was placed around the PJ anastomosis and fixed at the abdominal wall and skin for drainage of abdominal fluids after the surgical procedure. The drainage was maintained as a natural drainage, not a negative pressure drainage.

2.8.2. Pancreatic alpha-amylase assay

To confirm the presence of pancreatic juice in the abdominal fluid, the α-amylase concentration was measured. After the procedure, the abdominal fluid was extracted daily from the Levin tube until no fluid was released. The volume of the accumulated abdominal fluids and the periods of drainage were evaluated and compared between the groups. To confirm the presence of pancreatic juice in the abdominal fluids in these groups, the α-amylase concentration was measured with a porcine pancreatic α-amylase ELISA kit (Bioassay technology laboratory, Shanghai, China). The assay procedure was performed according to the manufacturer's instructions.

2.8.3. Pancreatic ductography

A pancreatic ductography was performed, and the luminal diameter was measured with a calibrated catheter at the anastomotic site, head, upper and lower body, and the tail regions of the pancreas as in our previous report [9]. Analyses of the pancreatic ductographic findings were performed on the basis of the consensus of three observers who were blinded to the study.

2.8.4. Computed tomography (CT)

Enhanced abdominal CT (Sensation 16; Siemens, Muenchen, Germany) was performed before the surgical procedure and immediately before sacrifice to evaluate the placement of the stent and changes in the pancreatic duct.

2.8.5. Gross examination

Surgical exploration of the pancreas and jejunal loops, including the PJ anastomosis, was followed by gross examination to determine the status of the intraperitoneal organs, PJ anastomosis, and the presence of pancreatitis, abdominal abscess formation, and/or inflammatory tissue changes.

2.8.6. Histological analysis

The tissue samples were fixed in 10% neutral buffered formalin for 24 h and then embedded in paraffin and sectioned. The slides were stained with hematoxylin and eosin and Masson's trichrome. The detailed histological analysis follows our previous report.[9] The luminal areas of the pancreatic duct at the anastomotic site, head, body, and tail portions were measured to compare the pancreatic duct size between the groups. The analyses of histological findings were based on the consensus of three observers blinded to the groups.

2.8.7. Definitions and data analysis

Technical success was defined as successful creation of PJ anastomosis leakage with or without stent placement. PJ anastomosis leakage was defined as the detection of amylase content in the drained intra-abdominal fluid because of the dehiscence of the PJ anastomosis. A PJ anastomotic stricture was defined as the presence of a fixed narrowing at the anastomotic site, along with distal ductal and side-branch enhancement on pancreatic ductography. The degree of inflammatory response caused by PJ anastomotic leakage was subjectively determined, i.e., graded as 1, mild when there was occasional inflammatory infiltration visible; 2, mild-to-moderate when there was intense inflammatory infiltration visible; 3, moderate when there was intense inflammatory infiltration visible with abdominal abscess formation with adhesions; and 4, severe when there was intense inflammatory infiltration visible with abdominal abscess formation and ascites with adhesion.

2.8.8. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Differences between groups were analyzed using the Kruskal-Wallis or the Mann–Whitney *U* test as appropriate using SPSS software (version 24.0; SPSS, Inc., Chicago, IL). A p value of <0.05 was considered statistically significant.

3. Results

3.1. Morphological characteristics of AZ-BTS

AZ-BTS was successfully fabricated using the multiple dip-coating process (Fig. 1A). The surface morphology of the BTS revealed it was smooth and flawless. The thickness of the BTS was approximately 70 μm on vertical section images. The surface of the AZ-BTS showed a smooth surface without AZ crystallization. The coating layers, both the drug-containing and covering layers, was approximately 7 μm (Fig. 1B).

3.2. Biodegradable behavior of AZ-BTS

SEM images of the BTS and AZ-BTS surface showed that there were no major changes and no significant differences between the BTS and AZ-BTS at 1 to 2 weeks. However, at 3 to 4 weeks, swelling and a bubble-

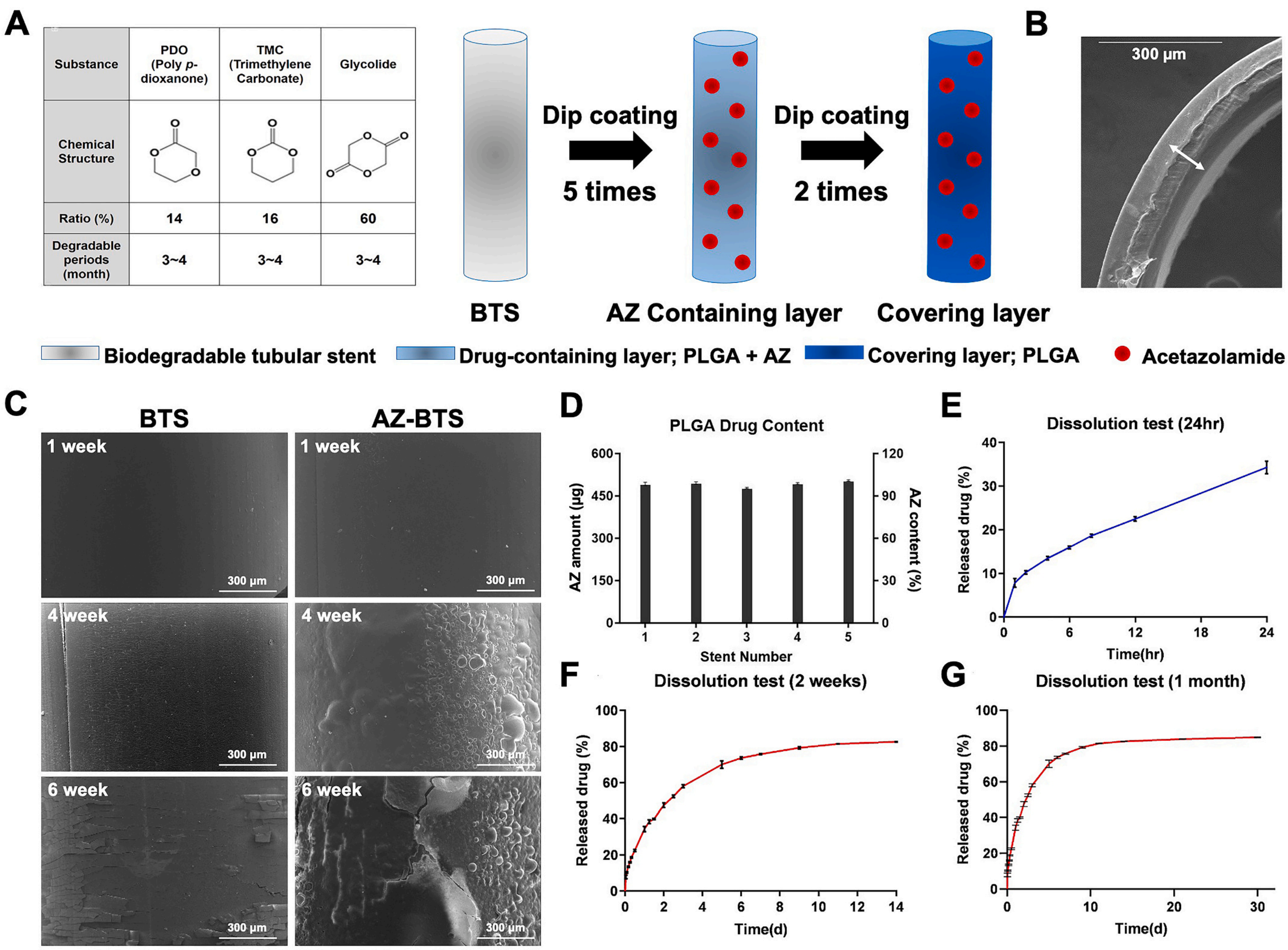

Fig. 1. Characteristics of AZ-BTS. A) AZ-BTS was prepared by using a multiple dipping technique. B) SEM image of the translational sectioned AZ-BTS shows the covering layers. C) Representative SEM images show the morphological characterization of the BTS and AZ-BTS samples during its degradation. D) The graph shows the drug content and uniformity. Curves show the in vitro release profiles of the drug from AZ-BTS at E) 24 h, F) 2 weeks, and G) 1 month.
Abbreviations: BTS, biodegradable tubular stent; AZ-BTS; acetazolamide-eluting BTS; SEM, scanning electron microscopy.

liked irregular layer on the AZ-BTS surface and minor cracks in the BTS surface became apparent. At 5 to 6 weeks, large cracks in the coating layers of the AZ-BTS surface were observed (Fig. 1C).

3.3. Drug content uniformity and release behavior of AZ-BTS

The drug content and its uniformity in the AZ-BTS was verified by HPLC. The mean (± standard deviation, SD) AZ amount of the stent was 593.09 ± 8.86 μg. The drug content uniformity in one batch of AZ-BTS was maintained in the range of −3.24% to 2.59%. Fig. 1D indicates that the same drug content among different batches of AZ-BTSs was consistently produced by the dip-coating method.

Fig. 1E, F, and G show the in vitro drug release profiles of the AZ-BTS. For 24 h, the initial drug release was 15.24% of the AZ from the stent, which indicated that there was an initial drug burst of AZ-BTS. Approximately 80% of the drug was released for a week and it showed first-order release kinetics.

Table S1 and Fig. S2 (Supplementary Material) show UPLC analysis of AZ in plasma samples, and Fig. S3 (Supplementary Mamterial) shows time-dependent plasma concentrations of AZ. The observed T_{max} was 6 h after stent embedding.

3.4. In vitro therapeutic efficacy of acetazolamide

The therapeutic effects of the AZ were examined in vitro using two kinds of pancreatic cancer cell lines, AsPC-1 and PANC-1. A pHrodo intracellular pH fluorescence dye was used to indirectly demonstrate that AZ inhibits bicarbonate ion production (Fig. 2A). The pH_i calibration curves were prepared for each cell line using the calibration buffer (Fig. 2B). In Fig. 2C, when both cell lines were treated with 50 nM of AZ, the pH_i went down from 7.4 to 7.0. In the PANC-1 cell line, the pH_i gradually decreased to 7.4, 6.8, 5.5, and 5.0 while the AsPC-1 cell was found to drop to 4.5 when treated with 500 nM of AZ. Small doses of AZ can dramatically inhibit bicarbonate ion release from pancreas cells. Fig. 2D and E show the in vitro viability of the cells after 24 h of treatment with AZ. The PANC-1 cell line maintained about 80% cell viability even after 100 μM of AZ treatment, while 50% of the AsPC-1 cells were killed by only 1 μM AZ treatment. The expression of CA IX on target pancreatic cells was confirmed by western blotting (Fig. S4, Supplementary Material).

3.5. Surgery outcomes from the animal study

The surgical procedures with or without AZ-BTS placement were technically successful in all pigs (Fig. 3A). In the leakage group, three (50%) of the six pigs died at 5, 4, and 2 days, respectively, after the

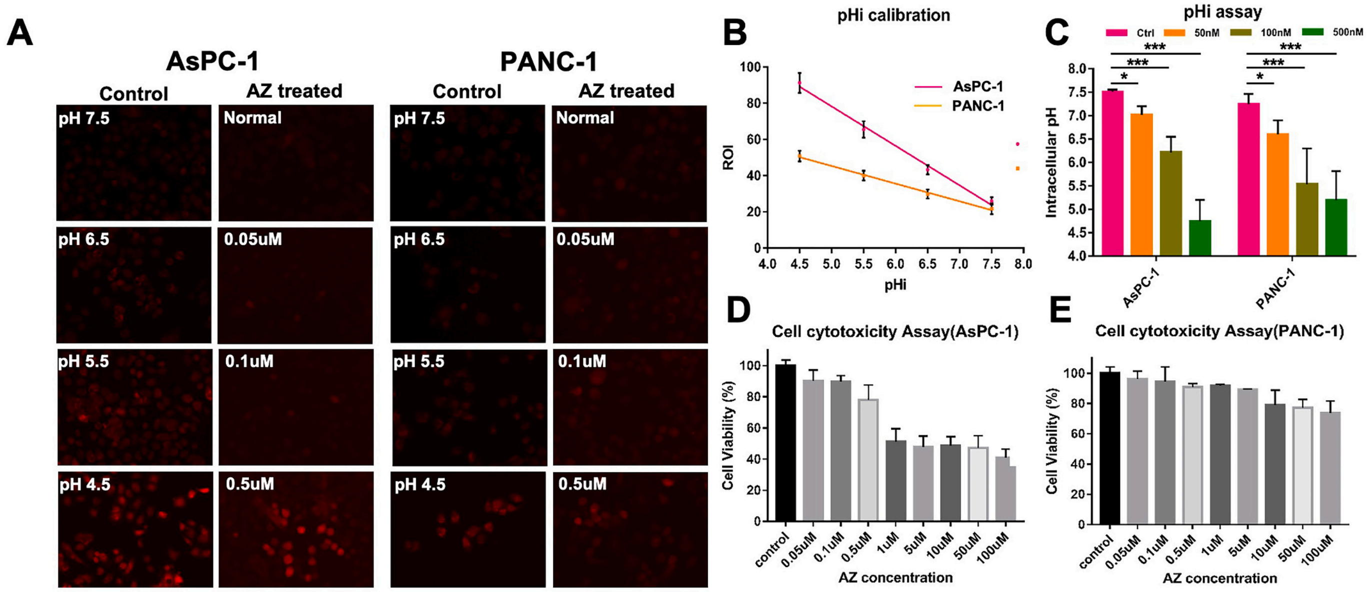

Fig. 2. In vitro cytocompatibility. A) Fluorescence microscopy images of the results of pH_i assays. B) pH_i fluorescence calibration curve. C) pH_i determination after acetazolamide (AZ) treatment showing the therapeutic effects of AZ. D, E) Cytotoxicity analysis of AZ after incubation with AsPC-1 and PANC-1 cells. Significance levels are indicated as *$p < 0.1$, **$p < 0.05$, *** $p < 0.001$.

surgery because of a severe POPF, which was confirmed by the disconnected sutures at PJ the anastomosis at the autopsy findings (Fig. S5, Supporting Information). In the second trial, an additional three pigs underwent PJ anastomosis for the leakage group. One (33%) of the three pigs died 7 days after the surgical procedure. One pig in the BTS group died 4 days after the surgery due to complete disconnection of the PJ anastomosis. The overall mortality rates were 44.4% in the leakage group, 16.6% in the BTS group, and 0% in the AZ-BTS group. The two pigs with surgery-related deaths were excluded from this study, and the remaining 16 pigs surviving until the end of the study.

All enrolled pigs experienced statistically insignificant weight loss at 0 ($p = 0.910$, Kruskal-Wallis test) and 1 ($p = 0.568$) week after the surgical procedure. Body weights in all three groups increased significantly at 2, 3, and 4 weeks after the surgery ($p < 0.001$ each). Mean weight at 2 weeks was significantly lower in the leakage than in the BTS and AZ-BTS groups, with these significant differences maintained at 3 and 4 weeks (Table S2, Supporting Information). Mean weight, however, did not differ significantly at these time points in the BTS and AZ-BTS groups. (Fig. S6, Supporting Information).

3.5.1. Efficacy of AZ-BTS for the prevention of pancreatic juice leakage

The mean volume of abdominal fluid was significantly greater in the leakage than in the BTS and AZ-BTS groups on days 1–5 ($p < 0.05$). The mean ($\pm$ SD) total volume of accumulated abdominal fluid was also significantly higher in the leakage group (49.0 $\pm$ 30.6 ml) than in the BTS (11.0 $\pm$ 2.8 ml, $p = 0.025$) and AZ-BTS (7.4 $\pm$ 3.2 ml, $p = 0.017$) groups (Fig. 3B). The mean ($\pm$ SD) period of drainage was also longer in the leakage group (5.4 $\pm$ 1.1 days) than in the BTS (4.4 $\pm$ 0.5 days, $p = 0.115$) and AZ-BTS (3.0 $\pm$ 0.7 days, $p = 0.006$) groups. The volume of abdominal fluid and the period of drainage did not differ, however, in the BTS and AZ-BTS groups. There was no drainage of abdominal fluid by 7 days after the surgical procedure in all pigs.

3.5.2. Alpha-amylase concentrations from the abdominal fluids

Mean α-amylase concentration was significantly higher in the leakage and BTS groups than in the AZ-BTS group on days 2–5 ($p < 0.05$ each) (Fig. 3C). Although the total volume of accumulated abdominal fluid differed significantly in the leakage and BTS groups, there was no difference in their mean α-amylase concentrations.

3.5.3. Pancreatic ductographic findings

The mean luminal diameters of the pancreatic duct at the head (5.28 $\pm$ 1.37 mm vs. 1.66 $\pm$ 0.32 mm vs. 1.74 $\pm$ 0.32 mm), upper (2.63 $\pm$ 0.83 mm vs. 1.35 $\pm$ 0.45 mm vs. 1.39 $\pm$ 0.34 mm) and lower (2.52 $\pm$ 0.63 mm vs. 1.04 $\pm$ 0.31 mm vs. 0.97 $\pm$ 0.21 mm) body, and tail (1.50 $\pm$ 0.56 mm vs. 0.71 $\pm$ 0.30 mm vs. 0.67 $\pm$ 0.18 mm) regions of the pancreas were significantly larger in the leakage group than in the BTS and AZ-BTS groups ($p < 0.05$ each) (Fig. 3D). However, the mean luminal diameters of the pancreatic duct at the anastomotic site was significantly smaller in the leakage (0.45 $\pm$ 0.30 mm) than in the BTS (1.19 $\pm$ 0.51 mm) and AZ-BTS (1.12 $\pm$ 0.43 mm) groups ($p < 0.05$ each) (Fig. 4A).

3.5.4. Gross findings

The excised specimens showed pancreatitis occurred in all (100%) pigs in the leakage group. However, none of the pigs in the BTS group, and only one (16.6%) in the AZ-BTS group, had pancreatitis (Fig. 4B). The PJ anastomosis was well repaired and intact without anastomotic fistula in all enrolled pigs. The grades of inflammatory response after the surgical procedure in the leakage group were determined as grade 4 in four pigs and grade 3 in the remaining one. Three pigs in the BTS group had grade 3 inflammation and two had grade 2 inflammation. In the AZ-BTS group, one pig had grade 3, four had grade 2, and one had grade 1 inflammation (Fig. S7, Supplementary Material.

3.5.5. Computed tomography (CT) findings

CT images from the leakage group revealed a dilatation of the pancreatic duct resulting from anastomotic narrowing and ascites in the splenorenal fossa caused by the PJ anastomotic leakage (Fig. 4C). The degree of dilation of the pancreatic duct was relatively smaller in the BTS and AZ-BTS groups than in the leakage group.

3.5.6. Histological findings

Histologic findings in the leakage, BTS and AZ-BTS groups are shown in Fig. 5. The mean luminal areas at the head (5.88 $\pm$ 2.43 mm^2 vs. 2.27 $\pm$ 0.59 mm^2 vs. 2.13 $\pm$ 0.96 mm^2) and upper (2.24 $\pm$ 0.53 mm^2 vs. 0.97 $\pm$ 0.30 mm^2 vs. 0.93 $\pm$ 0.36 mm^2) and lower (2.16 $\pm$ 0.88 mm^2 vs. 0.89 $\pm$ 0.45 mm^2 vs. 0.92 $\pm$ 0.46 mm^2) body regions of the pancreas were significantly larger in the leakage group than in the BTS and AZ-BTS groups ($p < 0.05$ each). Mean luminal areas in the tail region,

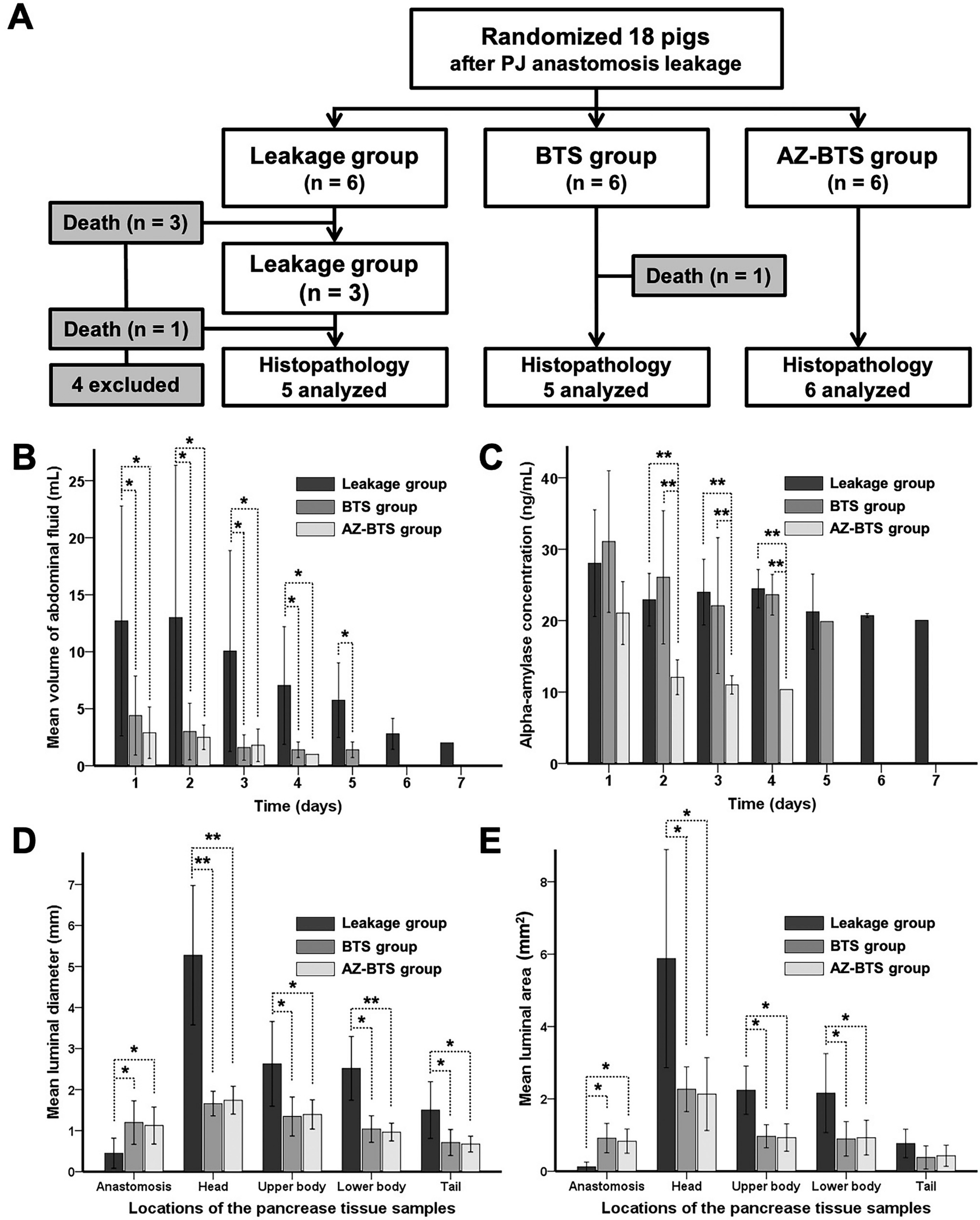

Fig. 3. In vivo study and outcomes. A) Flow diagram and in vivo study design show the randomization process and procedural outcomes. B) The mean volumes of abdominal fluid on days 1–5 were significantly higher in the leakage group than in the BTS and AZ-BTS groups. The mean period of drainage was also significantly longer in the leakage group than in the BTS and AZ-BTS groups. C). Mean α-amylase concentrations, D) mean luminal diameter on pancreatic ductography and E) luminal area on histology. Data are mean ± 95% confidence interval. Significance levels are indicated as * $p < 0.05$, ** $p < 0.001$. CI: confidence interval. Abbreviations: CI, confidence interval; PJ, pancreaticojejunal; BTS, biodegradable tubular stent; AZ-BTS, acetazolamide-eluting BTS.

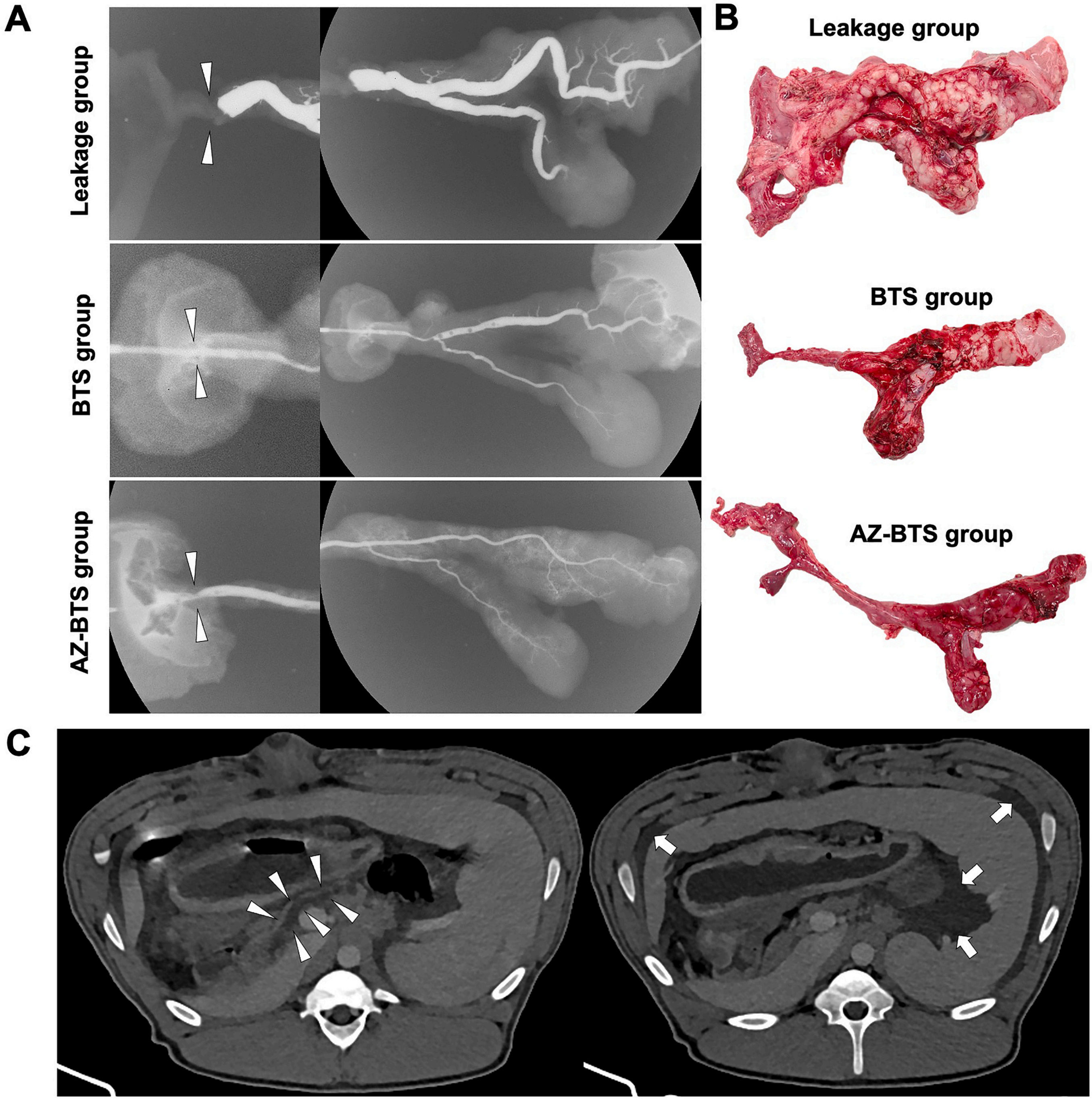

Fig. 4. In vivo efficacy and safety of AZ-BTS. A) Pancreatic ductographies show the significant differences in luminal diameters of the pancreatic duct, including PJ anastomosis (arrowheads) among the groups. Representative pancreatic ductography of one pig in the leakage group showed complete obstruction of the PJ anastomosis. B) Photographs obtained from gross examination, showing severe pancreatitis and a relatively enlarged pancreas in the leakge group compared with the BTS and AZ-BTS groups. C) Representative CT images obtained 4 weeks after the surgical procedure showing dilated pancreatic ducts and ascites in the leakage group.
Abbreviations: BTS, biodegradable tubular stent; AZ-BTS; acetazolamide-eluting BTS; PJ, pancreaticojejunostomy.

however did not differ significantly in these three groups (0.77 ± 0.32 mm^2 vs. 0.38 ± 0.30 mm^2 vs. 0.43 ± 0.28 mm^2) (Fig. 3E). The mean luminal area at the anastomotic site was significanlty smaller in the leakage group (0.12 ± 0.11 mm^2) than in the BTS (0.91 ± 0.39 mm^2) and AZ-BTS (0.83 ± 0.32 mm^2) groups ($p < 0.05$ each).

4. Discussion

Postoperative complications are common and typically related to the leakage of pancreatic exocrine secretions following anastomosis failure [21]. The development of POPF is the most serious postoperative complication. Amylase activity and the amount of the secretion have an effect on clinical or laboratory signs of intra-abdominal infection and may elicit abscess formation [11,21]. Pancreatic proteases and lipase leaking from the organ remnant attack the surrounding tissue, potentially leading to severe inflammation, tissue necrosis, and fistula formation [21]. Various trials of different operative anastomotic construction techniques, pharmacologic agents, and anastomotic

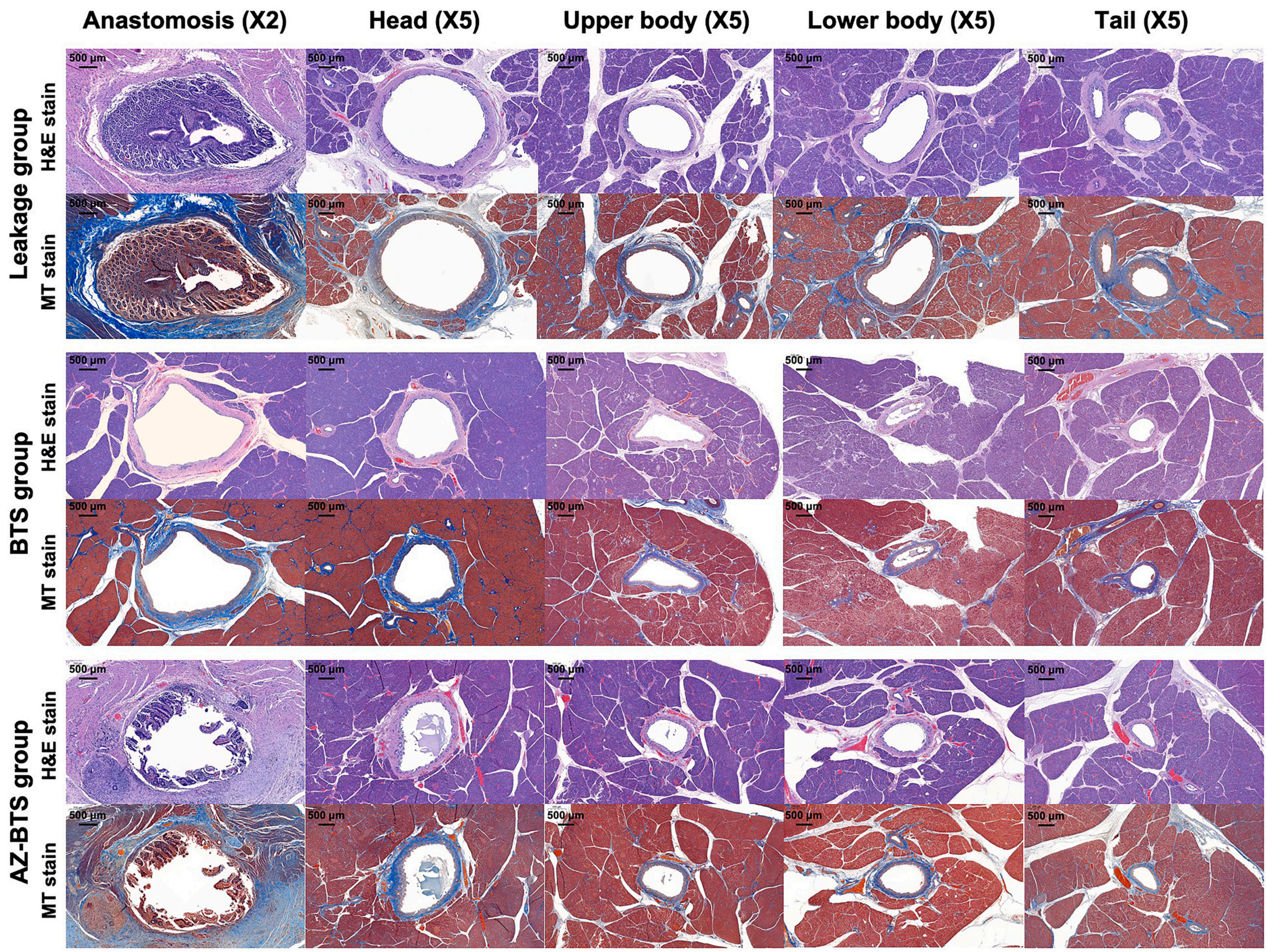

Fig. 5. Representative microscopic images of hematoxylin and eosin (H&E) and Masson's trichrome (MT) stained histological sections at the anastomosis site, and the head, upper and lower body, and tail portion of the pancreas obtained after 4 weeks in the leakage, BTS, and AZ-BTS groups.. (Anastomosis magnification ×2; magnification of the remaining sections ×5).
Abbreviations: BTS, biodegradable tubular stent; AZ-BTS, acetazolamide-eluting BTS.

stenting have been investigated for reducing or preventing POPF [22]. Pancreatic duct stenting at the time of anastomosis creation has been proposed to decrease or prevent pancreatic leak and/or fistula formation. Randomized trials have demonstrated that patients who underwent anastomotic stenting had a significantly lower incidence of pancreatic leak [8,13,23,24].

The benefits of stent-based drug delivery are to maximize the level of therapeutic agents in the local tissue, while using a lower drug dosage, and to minimize systemic toxicity [25,26]. It is well known that AZ can reduce the flow response of the pancreas to secretion and prevent autolysis caused by pancreatic juice leakage [19,20]. In the present study, we designed AZ-eluting biodegradable stents, which had a tubular structure, and investigated their efficacy in suppressing PJ anastomotic leakage and reducing pancreatic leak-related signs in an animal model. Our results suggest that AZ locally delivered by a stent successfully reduced intra-abdominal inflammatory symptoms secondary to pancreatic juice leakage via PJ anastomosis in a porcine model, as evidenced by a significantly lower volume and amylase concentration of the abdominal fluid and a shorter period of drainage in the AZ-BTS group than in the leakage group.

In a previous study, BTS placement successfully prevented PJ anastomotic strictures in a porcine model [9]. Consistent with our previous report, our results also revealed that luminal diameter and area were significantly lower in the BTS and AZ-BTS groups than in the leakage group. The findings of the stricture inhibitory effect in our study seem to be due to an effect of the BTS rather than the effect of the drug. Our results demonstrated that BTS and AZ-BTS successfully reduced the volume of accumulated abdominal fluid caused by leakage of pancreatic juice at the anastomotic site, indicating that the stent itself can reduce the rate of pancreatic leakage. Overall mortality rates in the leakage, BTS and AZ-BTS groups were 44.4%, 16.6%, and 0%, respectively, and the mean α-amylase concentrations on days 2–5 were significantly higher in the leakage and BTS groups than in the AZ-BTS group ($p < 0.05$ each). This finding indicates that AZ could effectively neutralize pancreatic juice and reduce the manifestations of pancreatic leakage. The BTS was made from commercially available materials, and biodegradation began 3 weeks after placement. It was completely degraded from 8 to 12 weeks in a rat study [9]. There were no significant differences in the overall degradation periods between the BTS and AZ coated BTS; however, the surface of the AZ-BTS showed swelling and a bubble-liked irregular layer at 3 weeks. AZ-BTS has therapeutic potential in the prevention of anastomotic stricture formation as well as the reduction of anastomotic leaks and its related symptoms.

The AZ-BTS was successfully fabricated by a multiple dip-coating technique, and PLGA, which is a biodegradable polymer approved by the FDA [27], was used for sustained AZ release. Utilizing multiple coatings can induce an increased burst release effect, but the overall drug release evened out at around 70–80% by day 11 [28]. In our study, approximately 80% of the drugs were released for a week. The problem with solvent-based coating techniques is that they result in bridging, pooling, and lack of uniformity [29–31], which causes difficulties in commercialization. Dip coating is usually used when performing introductory studies on a novel stent, but more advanced techniques may be required for commercial production of the stent.

In our study, a PJ anastomotic leakage porcine model was successfully established by reducing the number of sutures at the PJ anastomosis via the duct-to-mucosa method and sutures between the pancreatic capsule and jejunal serosa were not placed. However, the main drawback of this animal model was its high overall mortality rate (44%) in the first (50%) and the second (33%) trials because of severe POPF caused by the disconnected suture at the anastomosis. In the AZ-BTS group, all pig survived until the end of this study and body weights of the pigs at 3 and 4 weeks after the surgical procedure were significantly higher in the BTS and AZ-BTS groups than in the leakage group. The PJ leak was effectively prevented, and neutralizing the leaked pancreatic fluid seemed to help the pig recover. Furthermore, severe pancreatitis and intra-abdominal inflammation with a high amylase concentration and increased drainage volume were demonstrated in the leakage group. Although the mortality rate was rather high, the animal model induced a reproducible incidence of anastomotic leakage and leak-related symptoms and proved to be an efficient approach to stimulate PJ anastomotic leakage as a potential model for reproducing the mechanisms of the POPF.

In clinical practice, the first 1–2 weeks, especially the first week, of wound healing, consisting of stenting with or without drug release after PJ anastomosis, has critical significance in preventing pancreatic leakage or reduceing the detrimental effect of pancreatic juice after anastomosis. Moreover, a period of 6–9 weeks of stenting after PJ anastomosis was found sufficient to prevent stricture formation at the anastomotic site. The present study did not include in vivo drug release tests after PJ anastomosis because of the difficulties involved in assessing drug release in a porcine model. We found, however, that the concentrations of amylase after surgery were significantly higher in the leakage and BTS groups than in the AZ-BTS group during the early postoperative period, resulting in a plateau at a normal range of amylase 7 days after surgery. Thus, 1 week of drug release can be significant for preventing and reducing the detrimental effects of pancreatic leakage in clinical settings.

Our study has some limitations. First, the sample size was too small to conduct a robust statistical analysis, although many variables of interest reached statistical significance. However, the differences between the groups were indisputable. Second, we did not evaluate the mechanical properties of the AZ-BTS, such as radial force and tensile strength. Third, we used a single dose of the drug and could not conduct in vivo pharmacokinetic and dose range studies on the AZ-BTS.

5. Conclusion

Although additional studies are required to identify optimal drug doses, the results of this study support the basic concept of using the BTS with or without AZ to reduce or prevent PJ anastomotic leakage with the advantage of biocompatibility and the elimination of stent removal necessity. Our results demonstrated that direct and local treatment with AZ via BTS was effective and safe in suppressing intra-abdominal inflammatory symptoms secondary to PJ anastomotic leakage in a porcine model, and it possesses strong potential to inhibit the self-digestion and intra-abdominal inflammatory diseases caused by pancreatic juice leakage and prevent late stricture formation at the PJ anastomotic site.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2021.06.010.

References

[1] T. Sakaguchi, S. Nakamura, S. Suzuki, Y. Kojima, Y. Tsuchiya, H. Konno, J. Nakaoka, R. Nishiyama, Marginal ulceration after pylorus-preserving pancreaticoduodenectomy, J. Hepato-Biliary-Pancreat. Surg. 7 (2000) 193–197.
[2] Y. Park, D.W. Hwang, J.H. Lee, K.B. Song, E. Jun, W. Lee, J. Kwon, S.C. Kim, Analysis of symptomatic marginal ulcers in patients who underwent pancreaticoduodenectomy for periampullary tumors, Pancreas 49 (2020) 208–215.
[3] C.J. Wray, S.A. Ahmad, J.B. Matthews, A.M. Lowy, Surgery for pancreatic cancer: recent controversies and current practice, Gastroenterology 128 (2005) 1626–1641.
[4] K.B. Song, S.C. Kim, D.W. Hwang, J.H. Lee, D.J. Lee, J.W. Lee, K.-M. Park, Y.-J. Lee, Matched case-control analysis comparing laparoscopic and open pylorus-preserving pancreaticoduodenectomy in patients with periampullary tumors, Ann. Surg. 262 (2015) 146–155.
[5] J.L. Cioffi, L.A. McDuffie, A.M. Roch, N.J. Zyromski, E.P. Ceppa, C.M. Schmidt, A. Nakeeb, M.G. House, Pancreaticojejunostomy stricture after pancreatoduodenectomy: outcomes after operative revision, J. Gastrointest. Surg. 20 (2016) 293–299.
[6] Y. Azumi, S. Isaji, H. Kato, Y. Nobuoka, N. Kuriyama, M. Kishiwada, T. Hamada, S. Mizuno, M. Usui, H. Sakurai, M. Tabata, A standardized technique for safe pancreaticojejunostomy: pair-watch suturing technique, World J. Gastrointest Surg. 2 (2010) 260–264.
[7] M.D. Bai, L.Q. Rong, L.C. Wang, H. Xu, R.F. Fan, P. Wang, X.P. Chen, L.B. Shi, S. Y. Peng, Experimental study on operative methods of pancreaticojejunostomy with reference to anastomotic patency and postoperative pancreatic exocrine function, World J. Gastroenterol. 14 (2008) 441–447.
[8] C.J. Yeo, J.L. Cameron, K.D. Lillemoe, P.K. Sauter, J. Coleman, T.A. Sohn, K. A. Campbell, M.A. Choti, Does prophylactic octreotide decrease the rates of pancreatic fistula and other complications after pancreaticoduodenectomy? Results of a prospective randomized placebo-controlled trial, Ann. Surg. 232 (2000) 419–429.
[9] N. Bakheet, J.H. Park, S.H. Shin, S. Hong, Y. Park, I.K. Shim, C. Hwang, J.Y. Jeon, J.E. Lopera, H.Y. Song, S.C. Kim, A novel biodegradable tubular stent prevents pancreaticojejunal anastomotic stricture, Sci. Rep. 10 (2020) 1518.
[10] M. Aikawa, K. Okamoto, K. Okada, N. Akimoto, S. Yamaguchi, I. Koyama, Y. Ikada, Novel pancreatoenteric reconstruction using a bioabsorbable polymer sheet and biocompatible bond, J. Surg. Res. 183 (2013) 1–7.
[11] M. Parviainen, J. Sand, A. Harmoinen, H. Kainulainen, T. Välimaa, P. Törmälä, I. Nordback, A new biodegradable stent for the pancreaticojejunal anastomosis after pancreaticoduodenal resection: in vitro examination and pilot experiences in humans, Pancreas 21 (2000) 14–21.
[12] F. Motoi, S. Egawa, T. Rikiyama, Y. Katayose, M. Unno, Randomized clinical trial of external stent drainage of the pancreatic duct to reduce postoperative pancreatic fistula after pancreaticojejunostomy, Br. J. Surg. 99 (2012) 524–531.
[13] R.T.P. Poon, S.T. Fan, C.M. Lo, K.K. Ng, W.K. Yuen, C. Yeung, External drainage of pancreatic duct with a stent to reduce leakage rate of pancreaticojejunostomy after pancreaticoduodenectomy: a prospective randomized trial, Ann. Surg. 246 (2007) 425–433.
[14] N.O. Machado, Pancreatic fistula after pancreatectomy: definitions, risk factors, preventive measures, and management-review, Int. J. Surg. Oncol. 2012 (2012) 602478.
[15] M.C. Steward, H. Ishiguro, R.M. Case, Mechanisms of bicarbonate secretion in the pancreatic duct, Annu. Rev. Physiol. 67 (2005) 377–409.
[16] M. Roxas, The role of enzyme supplementation in digestive disorders, Altern. Med. Rev. 13 (2008) 307–314.
[17] M.C. Parviainen, J.A. Sand, I.H. Nordback, Coincidence of pancreatic and biliary leakages after pancreaticoduodenal resections, Hepatogastroenterology 43 (1996) 1246–1249.
[18] A. Grapin-Botton, Ductal cells of the pancreas, Int. J. Biochem. Cell Biol. 37 (2005) 504–510.
[19] P.A. Banks, P.T. Sum, Mode of action of acetazolamide on pancreatic exocrine secretion, Arch. Surg. 102 (1971) 505–508.
[20] W.P. Dyck, N.C. Hightower, H.D. Janowitz, Effect of acetazolamide on human pancreatic secretion, Gastroenterology 62 (1972) 547–552.

[21] C. Gouillat, J.F. Gigot, Pancreatic surgical complications–the case for prophylaxis, Gut 49 (2001) 32–39.

[22] H.F. Schoellhammer, Y. Fong, S. Gagandeep, Techniques for prevention of pancreatic leak after pancreatectomy, Hepatobiliary Surg. Nutr. 3 (2014) 276–287.

[23] J.M. Winter, K.A. Campbell, D.C. Chang, T.S. Riall, R.D. Schulick, M.A. Choti, J. Coleman, M.B. Hodgin, P.K. Sauter, C.J. Sonnenday, C.L. Wolfgang, M. R. Marohn, C.J. Yeo, Does pancreatic duct stenting decrease the rate of pancreatic fistula following pancreaticoduodenectomy? Results of a prospective randomized trial, J. Gastrointest. Surg. 10 (2006) 1280–1290.

[24] J.J. Xiong, K. Altaf, W. Huang, N.W. Ke, Systematic review and meta-analysis of outcomes after intraoperative pancreatic duct stent placement during pancreaticoduodenectomy, Br. J. Surg. 99 (2012) 1050–1061.

[25] M. Shaikh, G. Kichenadasse, N.R. Choudhury, R. Butler, S. Garg, Non-vascular drug eluting stents as localized controlled drug delivery platform: preclinical and clinical experience, J. Control. Release 172 (2013) 105–117.

[26] W. Khan, S. Farah, A.J. Domb, Drug eluting stents: developments and current status, J. Control. Release 161 (2012) 703–712.

[27] S.J. Siegel, Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier, Polymers (Basel) 3 (2011) 1377–1397.

[28] G. Acharya, C.H. Lee, Y. Lee, Optimization of cardiovascular stent against restenosis: factorial design-based statistical analysis of polymer coating conditions, PLoS One 7 (2012), e43100.

[29] R. Bakhshi, M.J. Edirisinghe, A. Darbyshire, Z. Ahmad, A.M. Seifalian, Electrohydrodynamic jetting behaviour of polyhedral oligomeric silsesquioxane nanocomposite, J. Biomater. Appl. 23 (2009) 293–309.

[30] Y. Levy, D. Mandler, J. Weinberger, A.J. Domb, Evaluation of drug-eluting stents' coating durability–clinical and regulatory implications, J Biomed Mater Res B Appl Biomater 91 (2009) 441–451.

[31] M. Livingston, A. Tan, Coating techniques and release kinetics of drug-eluting stents, J. Med. Device 10 (2019), https://doi.org/10.1115/1.4031718.