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ORIGINAL ARTICLE

In Vivo Healing Effects of Ankaferd Blood Stopper on the Residual Pancreatic Tissue in a Swine Model of Distal Pancreatectomy

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Abstract The aim of this study was to determine whether intraoperative Ankaferd blood stopper (ABS) application into the pancreatic channel and to the pancreatic remnant surface following distal pancreatectomy can or cannot prevent postoperative pancreatic fistula formation. Three pigs underwent distal pancreatectomy under general anesthesia. In two of the pigs, 0.5 ml of ABS was applied to the stump surface area after adding 0.5 ml of ABS into the pancreatic channel. The remaining one animal served as the control. The pigs were sacrificed on the seventh postoperative day for autopsy. The pancreatic remnants from the animals were then taken for histopathological analyses. It was observed that the oral intake had been broken and abdominal distention had developed in the control pig following on the third postoperative day. However, no significant clinical changes were observed in the ABS-applied pigs. In the autopsy, it was found that the control pig had generalized peritonitis with pancreatic necrosis. On the other hand, the ABS-applied pigs had either macroscopically and microscopically normal pancreatic tissue architecture with an occluded Wirsung duct at the pancreatic stump. It was concluded that application of ABS on the transected surface and into the pancreatic channel could prevent pancreatic fistula formation and improve wound healing in the residual pancreatic tissue following distal pancreatectomy.

Keywords Ankaferd blood stopper · Distal pancreatectomy · Pancreatic fistula · Wound healing · Histopathology · Amylase

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Introduction

The resections of the pancreas reaching the left side of the superior mesenteric vein are defined as distal pancreatectomy (DP). DP operations are performed particularly for the treatment of malignant neoplastic diseases of the pancreas, chronic pancreatitis, and damage to the pancreatic parenchyma following abdominal trauma.

In recent decades, advances in surgical techniques have reduced the operative mortality rate of pancreatic resections to below 5 %, but the morbidity rates have still remained unchanged, ranging from 30 to 50 % [1–3]. Pancreatic fistula has been reported as the most serious complication of pancreatic surgery often leading to morbidity and even mortality. A wide variety of further associated complications, including intra-abdominal abscess, wound infection, and hemorrhage, can also prolong postoperative hospital stay and increase the utilization of health care resources [4].

Ankaferd blood stopper (ABS) has been approved as a topical hemostatic agent of plant origin for the management of external hemorrhages and dental surgical bleedings in

Turkey [5–7]. In vitro and in vivo studies disclosed that ABS acts via a unique hemostatic mechanism. ABS promotes the formation of an encapsulated protein mesh, which acts as an anchor for vital erythroid aggregation [8, 9]. Recently, it has been shown that ABS has positive effects on early bone healing together with decreasing inflammation and necrosis and increasing new bone formation [10]. ABS has also in vitro antibacterial effects [11]. Furthermore, our previous study has demonstrated that adding ABS to the pancreatic fluid produces aggregates of protein network resulting in a solid layer over the pancreatic fluid like a frozen gel with important in vitro biochemical alterations [12].

The aim of this in vivo study was to determine whether intraoperative ABS application into the pancreatic channel and to the pancreatic remnant surface following DP can improve postoperative wound healing and prevent pancreatic fistula formation. Elucidation of the in vivo effects of ABS on pancreatic tissue can open new avenues for the operative management of pancreatic surgical diseases using this unique hemostatic agent.

Materials and Methods

Three 1-year-old female pigs, weighing 20 kg, were used in this experimental study. All pigs were housed in metal cages with a wire netting bottom which were maintained at a temperature of 23 °C (±5 °C). The animals were allowed free access to solid diet and tap water. The pigs were allowed to roam freely for an hour, twice daily, in a small garden.

The experimental study was conducted with the approval of the Fatih University Medical School Ethics Committee. All procedures were in full compliance with Turkish Law 6343/2, Veterinary Medicine Deontology Regulation 6.7.26, and with the Helsinki Declaration of World Medical Association recommendations on animal studies.

Anesthesia was induced by intramuscular injection of a combination of xylazine hydrochloride (1.1 mg/kg, Xylazine Bio 2 %, Bioveta, Czech Republic) and ketamine hydrochloride (15 mg/kg, Ketamidor, Richter Pharma AG, Wels, Austria), and maintained by inhalation of isoflurane (Abbott, Italy) and oxygen. Postoperative analgesia was provided on the first and second postoperative days by injecting 5 mg of morphine and paracetamol intramuscularly every 6 h. The pigs were placed in a dorsal recumbent position. The skin of the abdominal region was shaved and prepared for aseptic surgery. All surgical procedures were done under sterile conditions. The abdomen was entered via an upper midline incision. The tail of the pancreas was identified, and the distal 4 cm was surgically resected. In two of the three pigs, 0.5 ml of ABS solution was applied on the transected surface of the pancreatic remnant after injection of 0.5 ml of ABS solution into the pancreatic channel (Fig. 1a–d). The remaining one pig served as the control, and no action was performed to the pancreatic remnant and the pancreatic channel was left open. A closed suction drainage (Hemovac drain) catheter was inserted into each animal, adjacent to the pancreatic remnant and fixed with skin sutures. The abdominal fascia was closed with running 1 poly-*p*-dioxanone loop sutures, and the skin was closed with running 3/0 silk sutures. A large transparent waterproof dressing was then applied to the incision to prevent the pigs chewing on the drains and to protect the incision. All the animals were given water ad libitum for the first 24 h only and subsequently fed twice daily with pig chow. The amount of daily drainage was recorded, and fluid samples were taken from each animal from suction drainage catheters on the third and seventh postoperative days to measure fluid amylase levels. The animals were sacrificed using 500 mg intravenous Pentothal on the seventh postoperative day, and the pancreatic remnants were surgically resected for histopathological analyses.

Results

Intraoperative application of ABS into the pancreatic channel and to the transected pancreatic remnant surface formed, in only a few seconds, a thin gel matrix covering the surface area in the two pigs. Under the intraoperative observation for more than 15 min, no pancreatic fluid drainage occurred in either of the pigs administered ABS. However, a little drainage was seen in the control pig.

All three of the animals survived during the postoperative course. The mean drainage collected from the suction drain was 65.7±9.3 ml/day (median, 60; range, 55–80 ml/day) in the control pig, whereas 23.6±12.1 ml/day (median, 20; range, 15–45 ml/day) and 22.9±11.1 ml/day (median, 20; range, 10–40 ml/day) pigs administered ABS (Fig. 2). The drain amylase, which was taken on the third postoperative day, was 9,430 U/l in the control pig and 2,829 and 3,461 U/l in the ABS applied pigs, respectively. The oral intake had been broken after developing abdominal distention in the control pig on the third postoperative day, but no significant changes were observed in the pigs administered ABS. The drain amylase levels on the seventh postoperative day were 7,142 U/l in the control pig; 859 and 1,235 U/l in the ABS applied pigs, respectively. Blood biochemical analyses revealed normal liver enzyme and amylase levels in the ABS-applied pigs, whereas elevated aspartate aminotransferase, alanine aminotransferase, and serum pancreatic amylase levels were present in the control pig (Table 1). It should be considered that systemic baseline levels of serum amylase are much higher in pigs than in humans and were around 2,000 U/l, and levels up to 3,500 U/l are accepted as being within normal ranges [13].

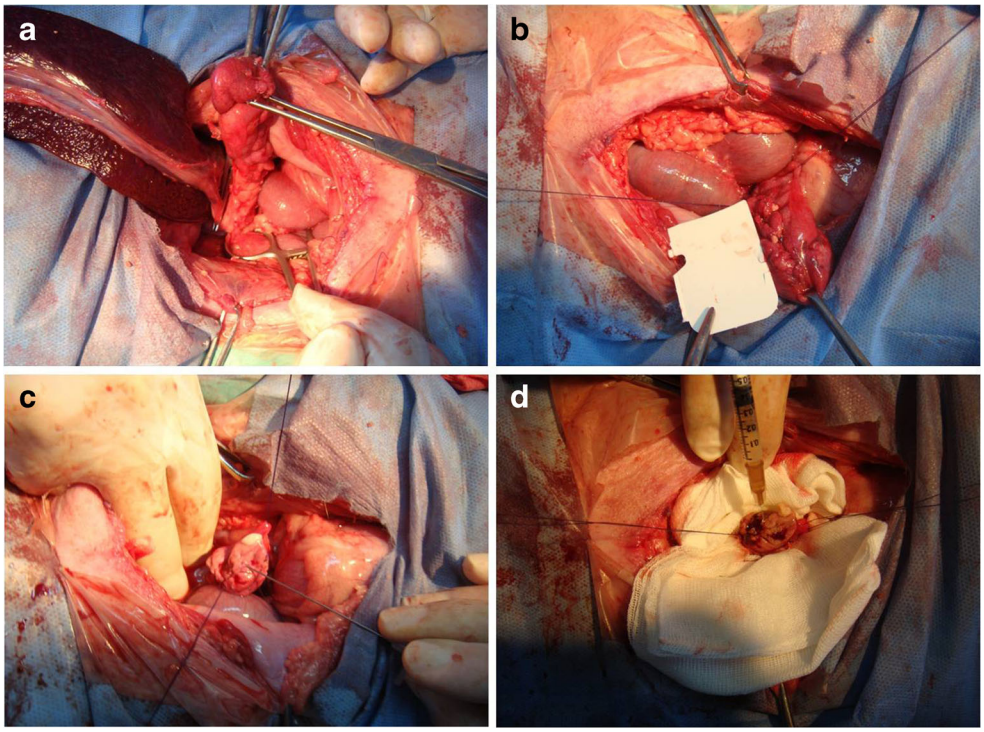

Fig. 1 The illustration of the operative procedure. The tail of the pancreas was identified and liberalized from the surrounding tissue (**a**). Resection of 4 cm of the pancreatic tail (**b**). The pinpoint shows the pancreatic channel (**c**). Application of ABS on the pancreatic channel and the stump surface area (**d**)

When the pigs were sacrificed, generalized peritonitis with free fluid collection and abscess formation around the pancreatic area and pancreatic necrosis were observed during the autopsy of the control pig (Fig. 3a). On the other hand, the two ABS-administered pigs had mild inflammation with limited fluid collection in the pancreatic bed. The architecture of the pancreatic remnant was protected (Fig. 3c, e). During the histopathological examination of the pancreatic specimens, tissue samples were taken from the operation area of the pancreas, and formalin-fixed and paraffin-imbedded tissue blocks were obtained. The slides were stained with hematoxylin and eosin. Microscopically, in the control pig, the pancreatic tissue disappeared with diffuse fat necrosis and marked distortion of the architecture (Fig. 3b). In the pigs administered ABS, the pancreatic parenchyma was well preserved, with only limited fat necrosis (Fig. 3d, f).

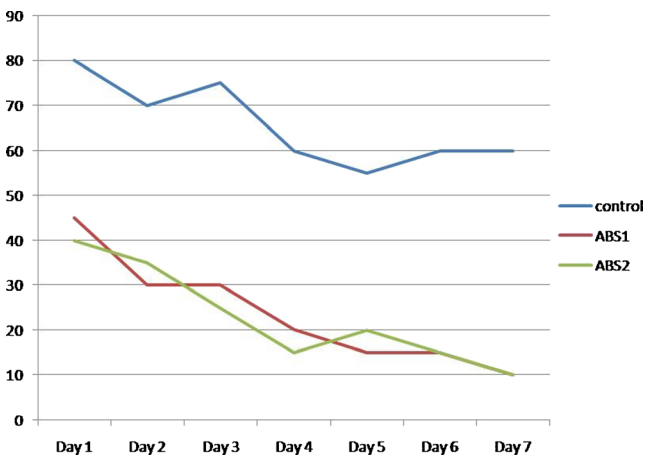

Fig. 2 Alterations of daily fluid drainage rates of the pigs

Discussion

Numerous prophylactic strategies to prevent postoperative pancreatic fistulas have been implemented for pancreatic transection and stump closure. Those include hand-sewn suture techniques, stapled closure techniques, ultrasonic dissection devices, pancreaticoenteric anastomosis, application of meshes, seromuscular and gastric serosa patches, and fibrin glue sealing of the pancreatic stump, or perioperative octreotide administration [14–19]. However, none of those procedures eliminates the risk of postoperative pancreatic fistula formation. Furthermore, ductal occlusion with neoprene or prolamine, both nonresorbable glues, has been used most often without anastomosis after DP operation but has been abandoned since permanent occlusion induces pancreatic atrophy and complete loss of exocrine function [20].

The ABS-induced formation of the protein network with vital erythroid aggregation covers the entire physiological hemostatic process [7, 21]. Mainly, there are distinct important components of the ABS-induced hemostatic network. Vital erythroid aggregation takes place with the spectrin and ankrin receptors on the surface of red blood cells. Those proteins and the required ATP bioenergy are included in the ABS protein library. Ankaferd also upregulates the GATA/-FOG transcription system affecting erythroid functions.

Table 1 Changes in laboratory values of three pigs

	Control Pig	ABS-1 pig	ABS-2 pig
Total bilirubin (mg/dl)	0.33	0.24	0.21
Direct bilirubin (mg/dl)	0.05	0.01	0.01
Aspartate aminotransferase (U/L)	110	28	105
Alanine aminotransferase (U/L)	70	29	31
Alkaline phosphatase (U/L)	140	101	79
Serum amylase (U/L)	3,872	1,345	1,643
Drain amylase (U/L) (postoperative 3rd day)	9,430	2,829	3,461
Drain amylase (U/L) (postoperative seventh day)	7,142	859	1,235

Urotensin II is also an essential component of Ankaferd and represents the link between injured vascular endothelium, adhesive proteins, and active erythroid cells [7, 22]. Those concepts have been developed via MALDI-TOF proteomic molecular analyses, cytometric arrays, transciption analysis, and SEM ultrastructural examinations as well as numerous investigations interacting with in vitro and in vivo research settings [7, 22]. Three ABS phase III studies performed in vascular port insertion bleedings, anterior epistaxis, and post-tonsillectomy hemorrhages have led to its approval as

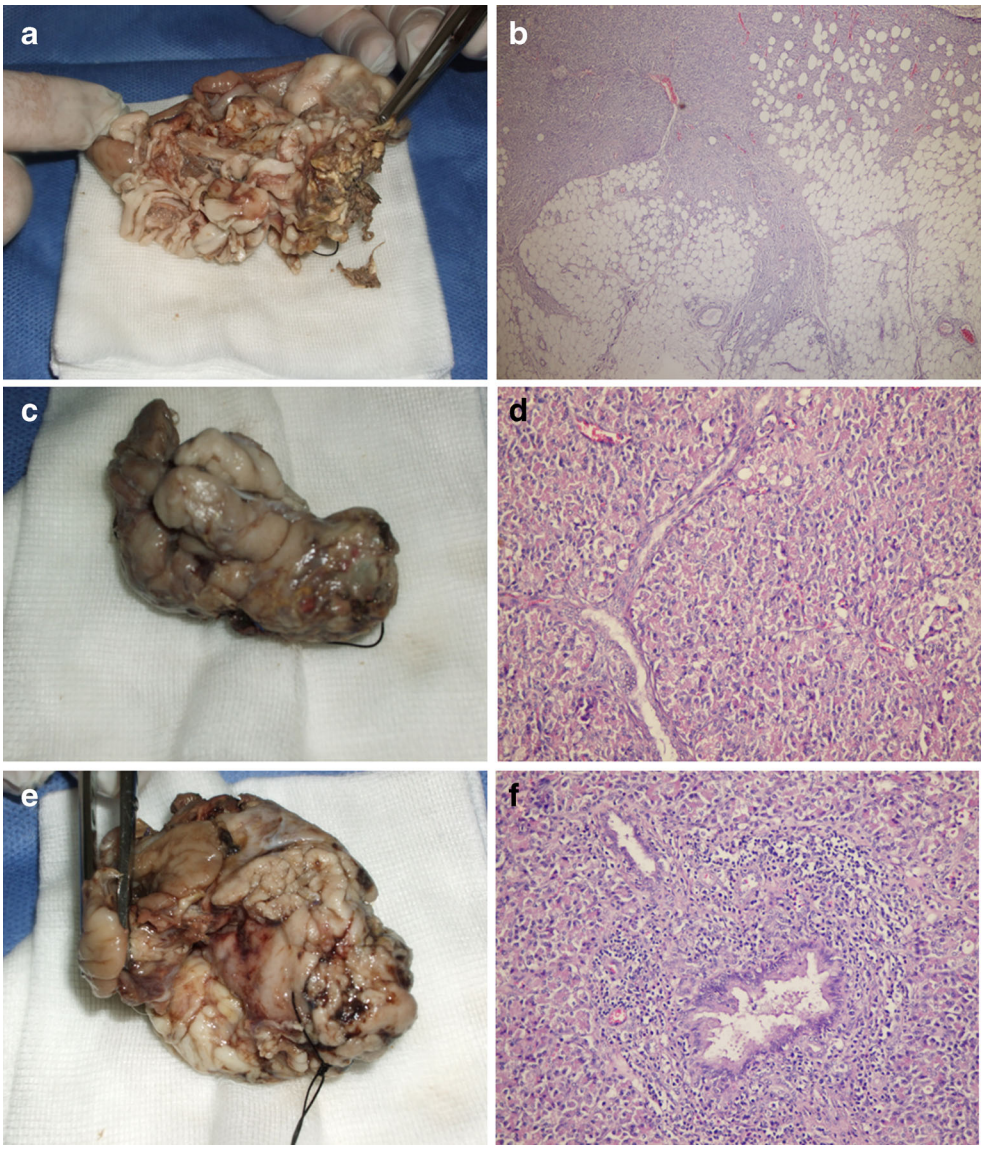

Fig. 3 Macroscopic and microscopic assessment of the pancreatic tissues. The pancreatic tissue from the control pig was scattered as though crumbling during display (**a**). Microscopically, in the control pig, the pancreatic tissue disappeared with diffuse fat necrosis and marked distortion of the architecture (**b**). Remarkably, as macroscopically, the structure of the pancreatic specimens of the ABS applied pigs was intact, and the Wirsung duct seemed to be occluded and covered with fibrotic tissue at the pancreatic stump surface (**c**, **e**). There was a preservation of the pancreatic parenchyma within the lobules, and inter-intralobular ducts were noticed adjacent to the fat necrosis (**d**, **f**)

a hemostatic agent in Turkey and Bosnia-Herzeginova [23–25]. Hence, ABS could be effectively used both in individuals with normal hemostatic parameters and in patients with deficient primary hemostasis and/or secondary hemostasis. In vitro data on the antibacterial [11] and wound healing profile of Ankaferd and bleeding control in the settings of gastrointestinal disorders [5, 6, 26–34] shed further light on that critical issue. Further investigations are underway to elucidate the place of ABS hemostatic effects on distinct tissues in various in vivo trauma models.

Approximately 2.5 l of clear, colorless, bicarbonate-rich pancreatic juice, containing 6–20 g of protein, is secreted by the human pancreas each day. With the possible exception of the lactating mammary gland, the exocrine pancreas synthesizes protein at a greater rate, per gram of tissue, than any other tissue. More than 90 % of proteins consist of digestive enzymes [35]. The protein-rich pancreatic fluid created the basis of our hypothesis that ABS can also promote the aggregation of the proteins in pancreatic fluid. Results of the previous in vitro study of adding ABS to pancreatic juice and other experimental studies associated with blood hemostasis support the hypothesis that ABS acts via protein aggregation [9, 12]. However, the in vivo effect of ABS on pancreatic tissue had not been previously examined until our present study.

The present study showed that applying ABS to the transected surface of the pancreatic remnant formed in seconds a visible gel matrix which covered the pancreatic stump. Furthermore, adding ABS in the pancreatic channel did not produce pancreatitis or toxic adverse effects. It could be considered that occlusion of the pancreatic channel by ABS can cause pancreatitis or pancreatic atrophy, which can lead to loss of exocrine functions as previously shown by using neoprene or prolamine [20]. Remarkably, we observed both macroscopically and microscopically that the architecture of the pancreatic tissue was not disturbed in the two ABS-applied pigs. Furthermore, our previous in vitro study showed a fast reaction in only a few seconds after adding ABS to pancreatic fluid, which resulted in a solid layer over the pancreatic fluid like a frozen gel. The architecture of the solid layer was observed in tubes ex vivo over 4 days without disruption. After 4 days, it began to resolve [12]. These observations suggest that adding ABS in the pancreatic channel did not cause a permanent occlusion but may seal the pancreatic stump for a few days, which allows healing and closure of the stump. This situation may also be extrapolated to surgery in humans where the stump would be surgically closed and the addition of ABS as an adjunct on the closed pancreatic remnant stump surface may prevent fistula formation.

In the present study, both in the experimental group pigs and the control pig, no suture ligation or stapler was used to close the remnant stump and the pancreatic channel. In addition to examining the effect of ABS in preventing a pancreatic fistula, we also aimed to investigate and to observe the effect of ABS on the pancreatic channel and pancreatic tissue. The sample size of the study is too small for a definite conclusion. However, we found our observations important for further studies in this area.

In conclusion, adding ABS to the transected surface of the pancreatic remnant as well as into the pancreatic channel seems to be a safe procedure that prevents pancreatic fistula formation without causing adverse side effects such as pancreatitis. These promising results should be supported with other in vivo studies in order to reach a definitive conclusion. The aim of this preliminary study was primarily to observe the effect of ABS on the main structure of the pancreatic tissue. Further studies should focus on larger controlled experimental animal studies specifically designed to elucidate morphological and/or functional alterations of the pancreas on exposure to ABS. Likewise, pancreatic stem cells represent another starting point to dissect molecular mechanisms of the ABS effect on the pancreas. Those investigations are not just academic. All of those observations should give promising results for the application of ABS around the pancreaticojejunal anastomosis to prevent anastomotic leakage in surgical practice.

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