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The experimental study for efficacy and safety of pancreatic cryosurgery ☆

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ABSTRACT

Objective: This study was designed to basic information concerning the efficacy and safety of cryosurgery for pancreatic cancer. Fifteen healthy pigs were used to perform biochemical analysis and histological assessment. *Methods:* Following anesthesia and laparotomy, an argon–helium cryoprobe was inserted into the pancreas. The introduction of argon gas induced a rapid decrease in temperature to $-160\,°C$ (Group I, 5 pigs) or $-110\,°C$ (Group II, 5 pigs), respectively, resulting in ice-ball formation of 15–20 mm diameter after 5 min. Following freezing, helium gas was circulated in the probe tip to increase the temperature to 10–20 °C over 3 min to thaw. The freeze/thaw cycle was then repeated. Group III (3 pigs) had a cryoprobe inserted, but without freezing, and Group IV (2 pigs) included untreated or normal control animals. Levels of serum amylase (AMY), IL-6 and C-RP were measured prior to freezing and for 7 days following the procedure. All pigs were euthanized 7 days post-treatment and pancreases were examined histologically. *Results:* Neither hyperaemia, edema or hemorrhage were observed in the un-frozen parts of the pancreas. Histological assessment revealed a significant level of necrosis in the central and lateral regions of the tissue frozen within the ice-ball. All cellular ultrastructure was destroyed and only observable as a few of remaining nuclei with broken crests and degranulated mitochondria and rough endoplasmic reticulum. There was a significant increase of serum AMY levels for a brief period in both "deep frozen" and the "shallow frozen" groups. However, the AMY also increased in two pigs in the "normal control" group and one pig from the "inserted cryoprobe without freeze" control group. All experimental pigs appeared healthy until the sacrifice time. *Conclusion:* Cryosurgery is a safe and effective ablative procedure for pancreatic tissue resulting in minimal complications.

Introduction

Pancreatic cancer is a malignancy with very poor prognosis and with few treatment options [5]. Cryoablation can be a useful therapeutic choice of pancreatic cancer [10,23]. However, little attention has been paid to experimental observation of the pancreas after cryosurgery. In an effort to provide the basic information for efficacy and safety of cryosurgery for the treatment of pancreatic cancer, we performed serial blood biochemical determinations, and light microscopic and electron microscopic observations of the pancreases of healthy experimental pigs before and after cryosurgery.

Materials and methods

Experimental animal

Fifteen certified healthy Tibet miniature pigs were obtained from the Animal Experimental Center of South Medical University, weighing 27–32 kg, with an average weight of 30 kg. For 2 weeks prior to the experimentation, the pigs were observed to ensure normal behavior.

Argon–helium cryosurgery system

This study utilized the EndoCare™ CryoSurgical System (CA, USA). This system relies on the Joule–Thomson effect, in which pressurized gas is permitted to depressurize through a narrow nozzle located at the tip of the probe. The depressurization of argon gas results in cooling of the cryoprobe tip to $-160\,°C$. Helium gas is used for probe tip heating (up to 67 °C).

Groups and experimental methods

Group I (deep freezing)

Five pigs were anesthetized with Isoflurane. Using sterile technique, the pancreas was exposed through abdominal access. A 2-mm diameter cryoprobe was inserted directly into the pancreatic body, argon flow was set to 100% yielding a cryoprobe tip temperature of -130 to -140 °C and an ice-ball of 15–20 mm in diameter. After 5 min, argon flow was stopped and helium gas introduced to raise the temperature from 10 to 20 °C for 3 min. This freeze/thaw cycle was repeated. The freezing process was completed under ultrasound monitoring. With ice-ball formation, the ultrasound echoes yielded images of ice expanding from the center of the frozen area adjacent to the cryoprobe and gradually expanded. The cryosurgery was considered a success when the ultrasound showed large areas of non-echo from the frozen area (Fig. 1). A silk suture was placed to mark the center of the frozen area. After removing the probe, the hole caused by the cryoprobe was filled with a thrombin gelatin sponge, and the abdomen was closed without drainage. Post-surgically, anesthesia was stopped, pigs were retuned to their cages, and offered their regular diet. Neither intravenous infusion, antibiotics, nor other medications was administered.

Group II (shallow freezing)

Five pigs underwent the same procedure as described above but the argon flow set to 10%. Following activation of cryoprobe, the indicated tip temperature was -110 to -120 °C resulting in a same size ice-ball.

Group III (inserted cryoprobe without freeze)

Three pigs underwent a sham procedure with only the cryoprobe being placed into the pancreatic body without any freezing/thawing procedures. The cryoprobe was held in place for 10 min, and then removed.

Group IV (normal control)

Two control pigs not experiencing either the sham or freeze treatments were sacrificed and pancreatic specimens were taken for light and electron microscope examination.

Observations

Clinically

The 15 experimental pigs were maintained on normal diets and their daily activities were observed.

Serial blood biochemical tests

Preoperative and post-operative determination following tests were performed for all of the experimental pigs:

(1) *Serum amylase (AMY):* serum amylase level was pre-operatively and post-operatively determined on days 1, 2, 3, 4, 5, 6 and 7.
(2) *Serum interleukin-6 (IL-6) and serum C-reaction protein (C-RP):* were determined post-operatively on days 1, 2, 3, 4, 5, 6 and 7 by solid-phase ELISA by the kits of Quantikine – R&D Systems (Minneapolis, Minnesota, USA) and Behring Nephelometer – Analyzer (Marburg, Germany).

Visual and histological observation

Group I and II: The 10 pigs were sacrificed by bloodletting at post-operative day 7. A laparotomy was immediately performed and the pancreas and its adjacent organs were observed. Two pancreatic specimens were taken at each three places within the primary freeze/insertion zone: (A) the central area of the ice-ball; (B) the lateral central area of the ice-ball; and (C) ice-ball edge area close to normal un-frozen tissue. The specimens were fixed in 10% formalin and 2.5% glutaraldehyde for light microscopic and electron microscopic examination, respectively.

Group III: The pancreatic specimens were taken from the 3 pigs, as above, for light microscopic and electron microscopic examination, respectively.

Group IV: The two pigs as normal control also had pancreatic specimens taken at operation and post-operative day 7, respectively.

Results

Clinically

All of the 15 experimental pigs exhibited normal eating, drinking and behavioral activities until their sacrifice. No bleeding tendencies appeared.

Biochemical tests

Serum AMY (Fig. 2): 13 (86.7%) of the 15 pigs had significant increased serum AMY. In all pigs of the Group I and II, serum AMY showed a prompt increased levels after cryosurgery and reached peak levels within 2–3 days before rapidly declining. However, by post-freeze day 6, serum AMY returned to normal. In Group III, two of three experimental pigs demonstrated no change in serum AMY levels, only pig (No. 13) had mild increase of serum AMY at post-operative day 5, and did not return to normal at post-operative day 7. Two pigs in Group IV, as normal controls, had significant increases in AMY level at post-operative day 1. In one of the two pigs, AMY returned to normal at post-operative day 4, while the other remained high up to post-operative day 7.

Serum IL-6: Of 15 pigs, 5 (33.3%, 2 in the Group I, 1 in the Group III and 2 in the Group IV) had mild increases in IL-6 level at
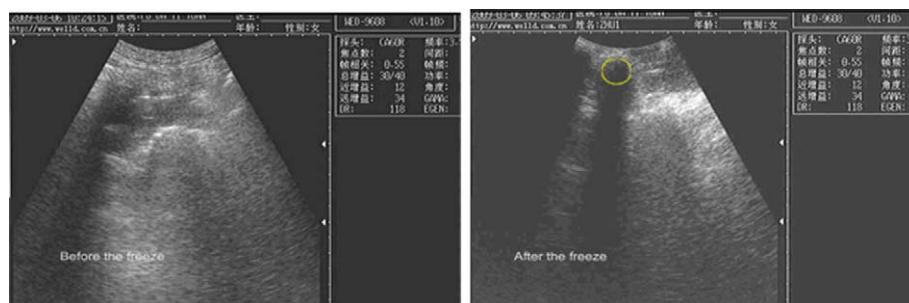

Fig. 1. The pancreatic ultrasound imaging before (left) and after (right) the freeze.
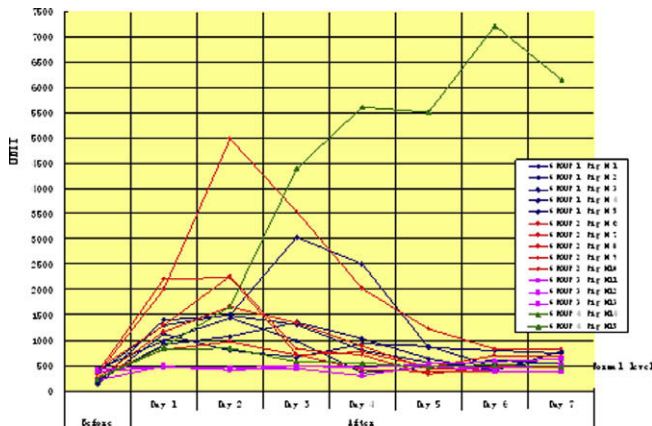
Fig. 2. Serum amylase levels before and after the experiment in the 15 pigs.

Fig. 4. The visualizing at post-freeze day 7.

post-operative day 7, day 1, day 1 and day 6, respectively. For one in the Group III, IL-6 level returned to normal the next day. The other four pigs had slight increases of IL-6 up to post-operative day 7. The average serum IL-6 levels in "deep freezing" and "normal control" groups were lower than in the "shallow freezing" and "insert cryoprobe without freeze" groups (Fig. 3).

Serum C-RP: All experimental pigs had no marked change before and after the experiment.

Visualizing

After the 15 pigs were sacrificed by bloodletting at post-operative day 7, a laparotomy was immediately performed. All un-frozen pancreatic tissue appeared normal with no hyperaemia, edema or hemorrhage observed (i.e., acute pancreatitis or pancreatic fistula). In Group I and II, there were obvious dark-brown necrotic areas around the silk suture on the pancreases with a definite boundary close to un-frozen pancreases (Fig. 4). In pig No. 1 of the Group I, there was a $2 \times 2 \times 3$ cm necrotic area on the colon wall close to the pancreatic frozen zone that had an extensive adhesion with the surrounding tissues, and a small volume of ascetic fluid within the abdominal cavity. In the Group III, the only obvious abnormity on the pancreas was some faintly visible damage along the needle insertion tracts.

Histology

By light microscopy (Fig. 5): H&E staining showed a significant zone of coagulative necrosis in all pancreatic specimens in the central and lateral central zones of the ice-ball in Groups I and II. More than half specimens had inflammatory infiltration in these zones. The severity of the necrosis and inflammatory lesions gradually

Fig. 3. Serum average IL-6 levels before and after the experiment (normal value is 5.9 pg/ml).

diminished from the center areas outwards to freeze zone edges. Conversely, granulation tissue hyperplasia appeared on 8 specimens at the edges, 4 specimens at the lateral central areas, and 2 specimens in central areas. In addition, pancreatic duct hyperplasia appeared in 3 specimens of the Group I, and 1 of the Group II, respectively. There was vasculitis on a specimen on the edge freeze zone in the Group I. No significant difference in the severity of necrosis between Group I and II was noted. Likewise, no abnormal changes were noted in Group III and IV.

By electron microscopy (Fig. 6): In the Group I and II, all cells became necrotic, all cellular ultrastructure was destroyed and disappeared in the central areas. In the lateral central zone of the freeze zone, most cells were necrotic with few relict cellular nuclei along with ruptured nuclear membranes. Organelles were persistent, mitochondria broken with degranulation. Also rough endoplasmic reticulum was degranulated. At the edge of the frozen areas, most of the cellular membranes had ruptured but most organelles remained including nuclei, rough endoplasmic reticulum and secretory granules. No significant differences between the severity of tissue necrosis and organelle damage between the Group I and II was observed. No abnormal changes were noted in Group III and IV.

Discussion

Pancreatic cancer is one of the most fatal cancers with a one-year survival rate of only 10% [7]. The preferred option of pancreatic cancer treatment is surgical resection. However, because of the difficulty of early diagnosis, less than 10–20% patients are able to accept radical surgery. Even if the cancer was resected, there is the operating trauma and complications in about 50% of the patients and a 3% mortality [20,13,21]. Cryosurgery is a minimally invasive surgery. Kovach et al. [12] reported on cryosurgery for 10 patients with un-resectable pancreatic cancer from 1995 to 1999, with no post-operative complications (i.e., pancreatitis, pancreatic fistulas, etc.) and no operative death. After cryosurgery the cancer pain was lessened in the all patients. Korpan et al. [9,11] reported that all patients with pancreatic cancer have responded well to cryosurgery without surgical complication (i.e., intra- or post-operative bleeding, fistulas or sepsis) or mortality directly associated with the cryosurgery. The authors postulate that since the cryosurgical method is far less invasive than conventional pancreatic resection with lower rates of complications and post-operative mortality, cryosurgery should be choice modality for most patients with pancreatic cancer. Xu et al. [23] have used percutaneous cryosurgery for patients with locally advanced pancreatic cancer under ultrasound guidance and/or computed tomography (CT) since 2001. Of the reported [24] 49 cases, the 12- and 36-month survival rates were 63.1% and 9.5%, respectively; 53.1% of cases surviving for 12-month or more with median survival of
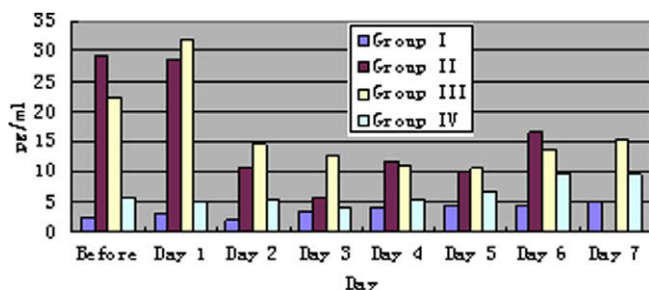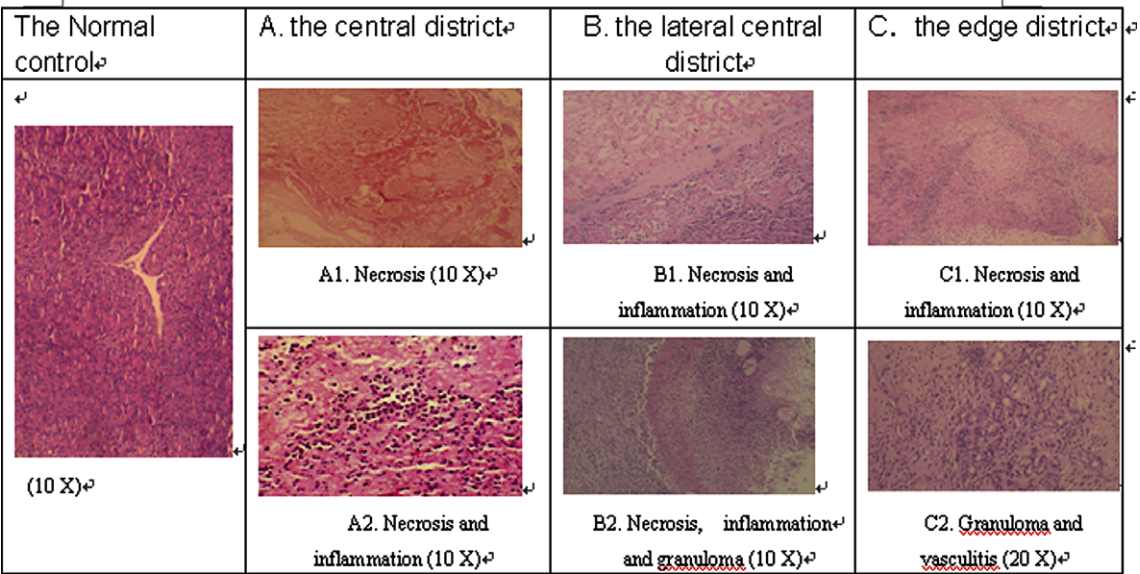
Fig. 5. The pancreatic tissues by light microscopy at post-freeze day 7. (H & E).
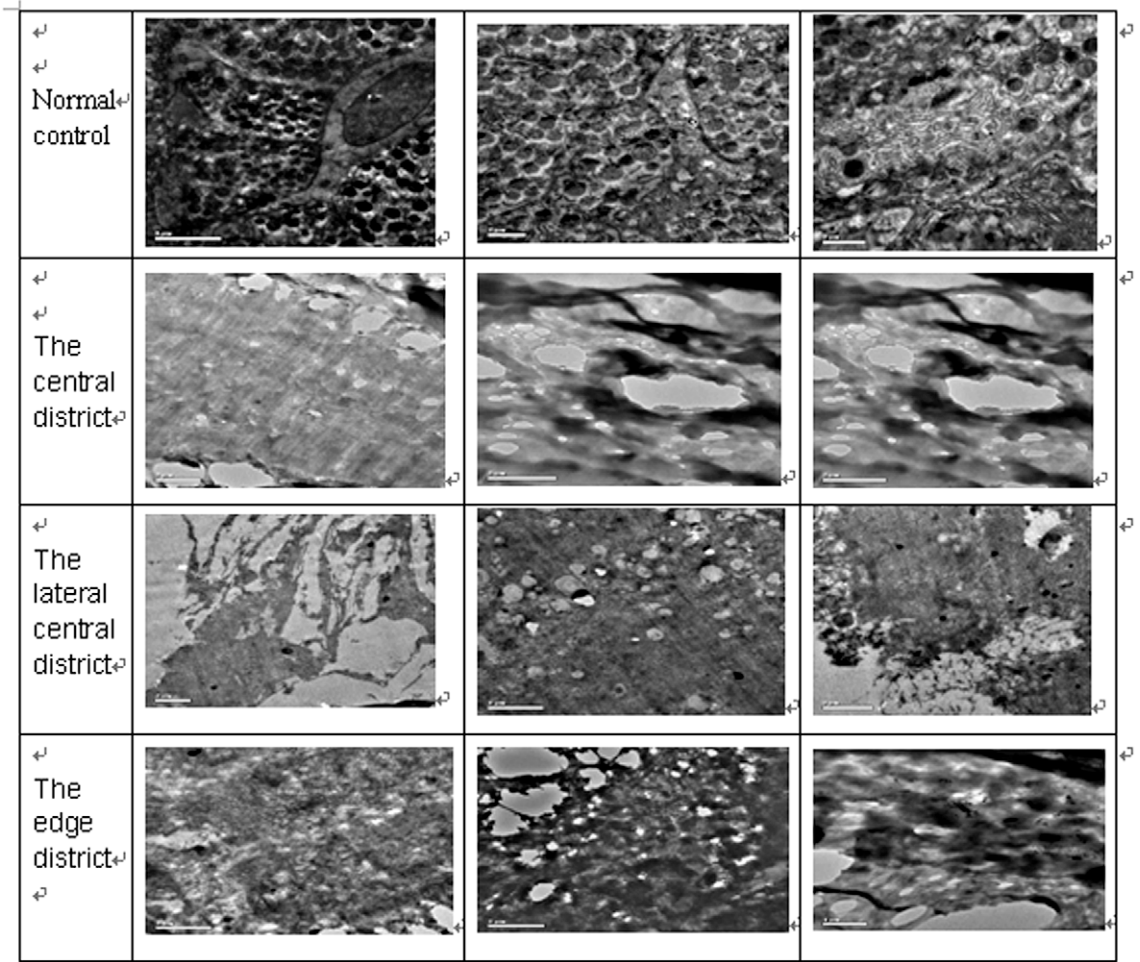

Fig. 6. The pancreatic tissues by electron microscopy at post-freeze day 7.

16.2 months. The longest surviving patient is 52 months. There was no therapy-related mortality; acute pancreatitis was seen in 6 cases, one of whom developed severe pancreatitis, 3 cases

(6.1%) had intra-abdominal bleeding. All adverse effects were controlled by medical management. However, the application of cryosurgery for pancreatic cancer is still in its primary stage. Its efficacy

and safety require more observation by experimental and clinical practice. Korpan [9] has used the disc cryoprobe with a diameter of 20 mm placed on a dog pancreas at a temperature of −180 °C for 9 min in a single freeze–thaw cycle. His results showed the immediate formation of an ice-ball with 12 mm in diameter around the cryoprobe and a line of demarcation of the cryozone. In the following hours, the area exposed to freezing showed aseptic necrosis with thrombus formation developed. After 4 weeks, loose connective tissue with numerous blood vessels had developed. After 9–12 weeks tight connective tissue developed. In the study, we used the argon–helium cryosurgical system which relies on the principle of the Joule–Thomson effect to freeze pig pancreas. The temperature of frozen pancreatic tissue reached −110 to −140 °C very quickly resulting in an ice-ball for 5 min, then a thaw for 3 min. There was a total of two cycles of freeze/thaw. After 7 days, there was an obvious necrotic area with definite boundaries in the frozen area. Histological examination by both light and electron microscopy show there was significant necrosis in the frozen areas and all of the cellular ultrastructure had been destroyed.

Generally, temperatures lower than −40 °C are necessary to cause the irreversible necrosis of the target cells. However, the temperatures of freeze edge close to normal un-frozen tissue are always higher −40 °C. In order to achieve complete ablation, freezing must be accomplished beyond the tumor edge by at least 1 cm, namely a "1 cm safe border" [17,14,19]. In this experiment, as with freezing liver and other solid organs, tissue necrosis and cellular structural damage in the frozen pancreas was related to proximity to the cryoprobe. The severity of cell necrosis was gradually reduced from the central frozen areas outwards to the edge of the freeze zone. This indicates that the frozen zone also should go beyond the tumor edge. Due to pancreas' small volume, a "1 cm safe border" may be difficult to achieve. This study shows that although most cellular membranes had ruptured, they were rich in organelles with rough endoplasmic reticulum and secretory granules at the edge of the frozen area. Also, there was significant granulation tissue hyperplasia and vasculitis at the edges indicating that the pancreatic damage was also severe even at the edge of the freeze zone. Therefore, the frozen area of pancreatic cancer may not necessarily require the "1 cm safe border". However, this conclusion requires further study. Our study includes "deep freezing" group and "shallow freezing" group, where the argon gas output was 100% and 10% with the final temperatures −140 and −110 °C, respectively. The freeze time was 5 min and the non-routine 10 min. However, the results of light and electron microscopy showed that there was no significant difference in the severity of tissue damage between "deep freezing" group and the "shallow freezing" group. This finding is an important observation for clinical treatment, and it indicates that the procedure of lower argon gas output power, shorter time and the relative non-extreme cold temperature can be used for pancreatic cancer cryotherapy. Accordingly, milder subfreezing temperatures may assure effective cryoablation for cancer while reducing excessive trauma to the pancreas. Because pancreas contains digestive enzymes, damage to pancreatic tissue may causes acute pancreatitis, pancreatic necrosis, pancreatic fistula and other serious co-morbidities. This is the most concerning clinical problem. To help solve this problem, we examined the levels of serum AMY, IL-6 and C-RP preoperatively and at post-operative day 1, 2, 3, 4, 5, 6, 7, respectively. Thirteen (86.7%) of the 15 pigs had a brief period increase of serum AMY. It reached peak levels within 2–3 days, then fell rapidly and returned to normal at the post-freeze day 6. Two pigs in Group IV, as normal control, had significant increases of AMY level at post-operative day 1. In one of the two pigs, the high AMY level remained up to post-operative day 7. Myers, etc. [15] also found the similar results in a pancreas frozen by liquid nitrogen in 15 healthy rhesus monkeys. The serum AMY had a prompt increase

4–5 times that of normal level on the first post-freeze day but returned to normal 3 days later without evidence of acute pancreatitis. Since many reasons can cause an increase of serum AMY [22], it is valueless to judge pancreatitis by serum AMY only. More articles [3,8,18,1,6] showed that unexplained only elevated serum AMY is not evidence of pancreas damage. Serum IL-6 and C-RP are considered valuable indicators of acute pancreatic damage [16,4,2]. The average serum IL-6 levels in "deep freezing" and "normal control" groups were lower than in the "shallow freezing" and "insert cryoprobe without freezing" groups. The undulation of serum IL-6 levels within each group were not regular. Serum C-RP of the all pigs showed no marked change. Both serum IL-6 and C-RP did not show complicating acute pancreatitis after the freeze. It is important to note that all experimental pigs exhibited normal characteristics post-operatively. After the aparotomy at post-operative day 7, all un-frozen pancreatic tissue appeared normal with no hyperaemia, edema or hemorrhage in the "deep freezing" and "shallow freezing" groups. These observation show that pancreatic cryosurgery is safe. As with any surgery, the safety of pancreatic cryosurgery is closely related to operative performance. Especially in percutaneous procedures, accurate positioning and appropriate freezing are very important. In one pig from the "deep freezing" group, there was a local necrotic area on the colon wall close to the pancreatic frozen zone, it was apparently too deeply frozen.

Acknowledgments

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