Pancreatic Head Cryosurgery

Safety and Efficiency In Vivo–A Pilot Study

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Objectives: Pancreatic cancer is the fourth leading cause of cancer-related death. Cryosurgery has emerged as a promising new technique for treatment. Although 80% of pancreatic cancers are located in the pancreatic head, no research has been conducted on the safety and efficacy of cryosurgery for these tumors.

Methods: Two groups of Tibetan miniature pigs (n = 4 per group) underwent cryosurgery to the pancreatic head with either the deep freezing protocol (100% argon output) or shallow freezing protocol (10% argon output), and compared to sham-operated pigs.

Results: Serum inflammatory factors and amylase increased during the 5 days after cryoablation in both groups but acute pancreatitis did not occur. Adhesions were observed between the pancreatic head and adjacent organs, and only minor trauma was caused to the stomach, duodenum, small intestine, and liver. Ice balls with a radius of 0.5 cm beyond the tumor edge were sufficient to cause complete necrosis of the pancreatic tissue, and decreased the degree of cold injury to surrounding tissues.

Conclusions: Shallow freezing protocol seemed to be safer than, and just as effective as, the deep freezing protocol. This preliminary study suggests that cryosurgery could potentially be an effective treatment of cancer of the pancreatic head.

Key Words: cryosurgery, pancreatic head, safety, efficacy, pilot study

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Cryosurgery is an efficacious treatment that is based on the specific cytotoxic effects of cold in tissue; it has been used as an alternative method to conventional surgery in solid tumors, including prostate,[1] liver,[2] lung,[3] kidney,[4] and breast[5] cancers. As a new technique in cancer treatment, numerous attempts have been made to expand its clinical application, particularly in pancreatic cancer. The preliminary clinical observations of Kovach et al,[6] Korpan,[7] and Korpan et al[8] all showed that cryoablation is far less invasive than the conventional pancreas resection; furthermore, the rate of complications and postoperative mortality was very low. Cryosurgery might therefore become the treatment of choice for most patients with pancreatic cancer.

Overall, approximately 75% of pancreatic carcinomas are located in the head and neck of the pancreas, 15% to 20% in the pancreatic body, and 5% to 10% occurs in the tail. As the pancreas is small, soft, and thin, and pancreatic head is close to several vital organs, including the duodenum, portal vein, and gastric fundus, cryosurgery is very difficult. Therefore, the use of cryosurgery for the treatment of pancreatic head cancer remains in a preliminary stage, and its safety and efficiency require investigation by both experimental and clinical practices.

In this paper, the safety and efficiency of pancreatic head cancer cryosurgery was assessed in a Tibet pig model. Damages to the pancreatic head and adjacent organs were assessed after cryosurgery, and multiple indexes of pancreatic damage and inflammatory response were analyzed, including inflammatory cytokines (tumor necrosis factor [TNF]-α, interleukin [IL]-6, IL-8, and C-reactive protein [CRP]), amylase (AMY), and routine blood tests. As the formation of an oversized ice ball will increase the complications and endanger the life of the animal, but an inadequately sized ice ball will lead to the recurrence of tumor at the margins of freezing, 2 programs of cryoablation were selected to analyze their different effects on the pancreatic head. We aimed to assess the conditions required to induce the formation of an optimally sized ice ball with minimum complications to the pig.

MATERIALS AND METHODS

Experimental Animals

Fourteen certified healthy Tibetan miniature pigs were provided by the Animal Experimental Centre of South Medical University, China, weighing between 27 and 32 kg. Study approval was obtained from the Research Animal Care and Use Committee of South Medical University, and all experimental studies were compliant with the Guide for the Care and Use of Laboratory Animals.

Argon-Helium Cryosurgery System

The cryosurgery equipment used was the Cryocare Cryosurgical System (Endocare, Irvine, Calif), which included the main tool and the cryoprobes. The system is based on the principle of the Joule-Thomson effect. Pressurized gas is depressurized through a narrow nozzle located at the tip of the probe. In accordance with the gas coefficient and the dimension of the nozzle, different gaseous elements generate different thermal exchange events at the area close to the nozzle.

Groups and Cryosurgery

Two programs were selected (the deep and shallow freezing programs), and 8 pigs were randomly divided into 2 groups

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according to the program applied. For the deep freezing program, the argon output power was set up to 100%, and to only 10% for the shallow freezing program. All pigs were anesthetized with isofluorane. With the use of sterile techniques, the abdomen was opened and the pancreas was exposed. Because the 2 pancreatic ducts join and connect with duodenum by common bile duct, the pancreatic ducts can be traced by the major duodenal papilla. A 2-mm-diameter cryoprobe was inserted 1 cm into the pancreatic head, keeping away 1 cm from the duodenum and avoid touching the 2 pancreatic ducts. The cryoablation protocol was a double freeze-thaw cycle, which consisted of a 5-minute freeze with a 3-minute thaw; this cycle was then repeated. The freezing temperature in the central point of the cryoprobe was recorded in detail with a temperature detector, and the size of the ice ball formation was calculated using vernier calipers and a ruler. After the cryosurgery was finished, the probe was retracted, and the pinhole was filled in with thrombin gelatin sponge and sutured with black silk thread. After the operation was completed, the pigs were returned to their cages, and regular food was offered. No intravenous infusion, antibiotics, or other medications were administered.

The third experimental group of 3 pigs was the nonfreezing group, in which the cryoprobe was inserted into the head of pancreas for 10 minutes without freezing. The final group was the control group of 3 pigs that underwent a sham laparotomy. Seven days after the cryosurgery, all pigs were killed and their pancreatic heads were resected. Any adhesions of the pancreatic head to adjacent organs were observed and the cryozones were inspected.

Routine Blood Analysis

Venous blood was extracted from the subclavian vein plexus at day 7 postoperatively. Routine blood samples from 14 animals were analyzed by CELL-DYN3700 (Abbott, Chicago, Ill).

Detection of Inflammatory Factors and AMY in Serum

Venous blood was extracted from the subclavian vein plexus on the day before surgery, as well as on days 1, 3, and 5 postoperatively. Sera were extracted from the venous blood and enzyme-linked immunosorbent assay (ELISA) detection was performed for all of the experimental pigs. Porcine ELISA kits for TNF-α, IL-6, IL-8, CRP, and AMY were all purchased from Langdun Biotechnology Co, Ltd (Shanghai, China); the purified and horseradish peroxidase-labeled detection antibodies were all from Bethyl (Montgomery, Tex), with the exception of the AMY kit (Thermo, Rockford, Ill). The detection was performed by the sandwich method according to the manufacturer's instructions.

Pathology

The pancreatic heads were inspected grossly. All tissues, including the surrounding adhesion tissues, were embedded in paraffin. Five-micrometer-thick slices were made and stained with hematoxylin and eosin. Histopathological analyses were performed in the cryozones and surrounding adhesion tissues. Pathological analyses were performed by an attending surgical pathologist (X.L.F.).

Statistical Analysis

All analyses were performed using the GraphPad Prism (GraphPad, Inc, San Diego, Calif) software. The ice ball lengths were measured and analyzed by the Student *t* test; multiple serum factors and routine blood analyses were analyzed by a 2-way analysis of variance and were expressed as the mean values (standard deviation). As multiple comparisons were made during the analysis, a Bonferroni correction was calculated and applied. A value of $P < 0.05$ was considered to indicate a statistically significant difference, with $P < 0.01$ and $P < 0.001$ indicating highly significant differences.

RESULTS

Measurement of Ice Ball Length and Cryolesion

The argon output powers of 10% and 100% were used in the freezing of the pancreatic head in both the shallow and deep freezing groups, respectively, and ice ball formation was seen during the operation (Fig. 1A).

Probe temperatures were recorded intraoperatively, and the temperature dropped to −70°C within 1 minute in the shallow freezing group; this continued to fall and reached −100°C

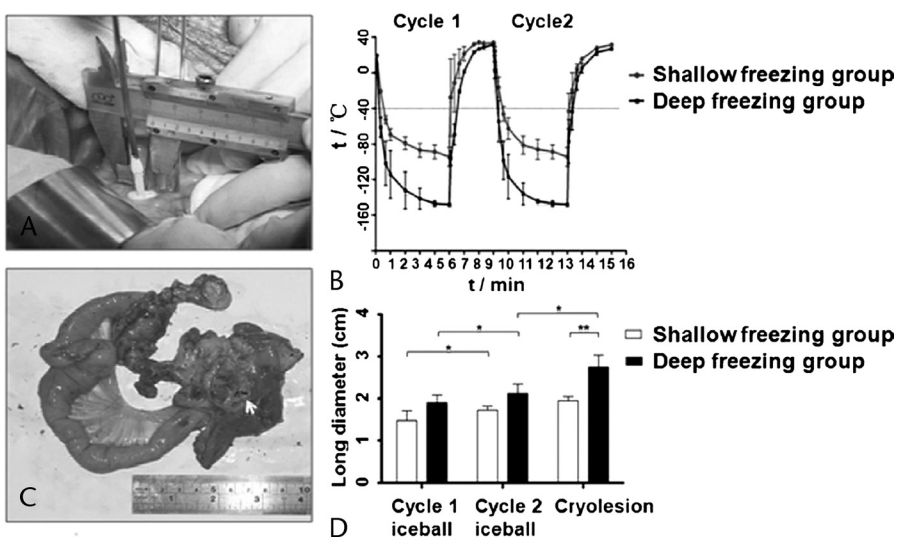


FIGURE 1. Long diameters of the ice ball and cryolesions. A, Ice ball formation can be observed during surgery; (B) probe temperatures of the 2 freezing programs. The transverse line located at −40°C represents the temperature for definite cell death; (C) a cryolesion can be observed 7 days postoperatively; a white arrow indicates the black line fixed at frozen center; (D) a comparison of the ice ball and cryolesion diameters after the 2 different ablation programs, *$P < 0.05$ and **$P < 0.01$.

within 5 minutes; after warming by helium gas for 1 minute, the probe temperature rose to 0°C, and then reached 30°C within 2 minutes. Similar changes of temperature were observed in the next cycle. In the deep freezing group, the cryogenic temperature of argon gas reached −120°C and −160°C within 1 and 5 minutes, respectively (Fig. 1B). After 2 freeze/thaw cycles, we observed that the ice ball diameters of the second cycle were longer than the first cycle in both groups (1.5 [0.24] vs 1.9 [0.3] cm in the shallow freezing group, $P < 0.05$; 1.73 [0.1] vs 2.13 [0.3] cm in the deep freezing group, $P < 0.05$; Fig. 1D).

The animals were killed and their pancreatic heads were resected 7 days later and cryozones in the pancreatic head were observed (Fig. 1C). Because the pancreas is soft and thin, the full thickness of the pancreas was almost perforated by cryoprobe, cryolesions can be observed in the front and back side of pancreas, and the cryolesions diameters of both sides were almost the same. The cryozone diameters in the deep freezing group were longer than those of the shallow freezing group (2 [0.1] vs 2.7 [0.3] cm, $P < 0.01$; Fig. 1D).

Serum Factors Expression Levels Changed Over Time

Blood was taken at day −1 preoperatively and days 1, 3, and 5 after cryosurgery, and sera were extracted from each sample. The expression levels of TNF-α, IL-6, IL-8, CRP, and AMY were detected (Fig. 2). All indexes in both freezing groups rose obviously at day 1, and then descended gradually (Fig. 2, merge). Every index of both freezing groups was higher than that of the nonfreezing and control groups on day 1. By day 3, CRP expression had decreased to normal but the other parameters remained higher. By day 5, IL-8 expression had decreased to normal and TNF-α, IL-6, and AMY levels in the freezing groups were higher than those of the nonfreezing and control groups. Although AMY expression in both freezing groups was still higher than the control, the expression did not reach 3 times the normal value, and therefore acute pancreatitis could not be diagnosed in these 2 groups.[9,10] No difference was observed for all indexes between the 2 freezing groups on each day after cryoablation.

Changes in Routine Blood Parameters After Cryosurgery

Blood was taken at day 7 after cryosurgery and routine blood analyses were compared between the 4 groups (Fig. 3). Only the total lymphocyte number and the percentage of lymphocytes in the deep freezing group were higher than the control group. There were no differences in the other indexes that reflected no obvious damage to pancreatic function, including white blood cell,[11] red blood cell,[12,13] platelet,[14] and polymorphonuclear.[15] Overall, the symptoms of pancreatitis were not observed at day 7 after cryoablation.

Adhesion of Pancreatic Head to Adjacent Organs

All the animals were killed 7 days after cryoablation, and pancreatic head tissue was resected for pathological observation. Different degrees of adhesions were observed between the pancreatic head and adjacent organs in both freezing groups (Fig. 4A), but the adhesions could be separated bluntly with ease. The pathological examination of adhesive sites were performed, and different degrees of fibroelastosis (small intestine and liver in the shallow freezing group, and all adjacent organs in the deep freezing group), focal bleeding necrosis (duodenum, small intestine, liver, and lymph nodes in the shallow freezing group, and all adjacent organs in the deep freezing group), and a large amount of neutrophil infiltration were observed (Fig. 4B).

Pathological Changes of Cryozones

Regardless of the deep (Fig. 5A) or shallow freezing group (Fig. 5B), the pathological phenomena of each area were as follows: (1) central necrosis, a large range of pancreatic tissue necrosis, in which there are large volume of necrotic cell fragments; (2) inflammation, large numbers of infiltrating neutrophils; (3) granulation, obvious fibroblast hyperplasia and neutrophil infiltration; (4) severe apoptosis, numerous visible apoptotic cells, with little residual pancreatic acini and ductal

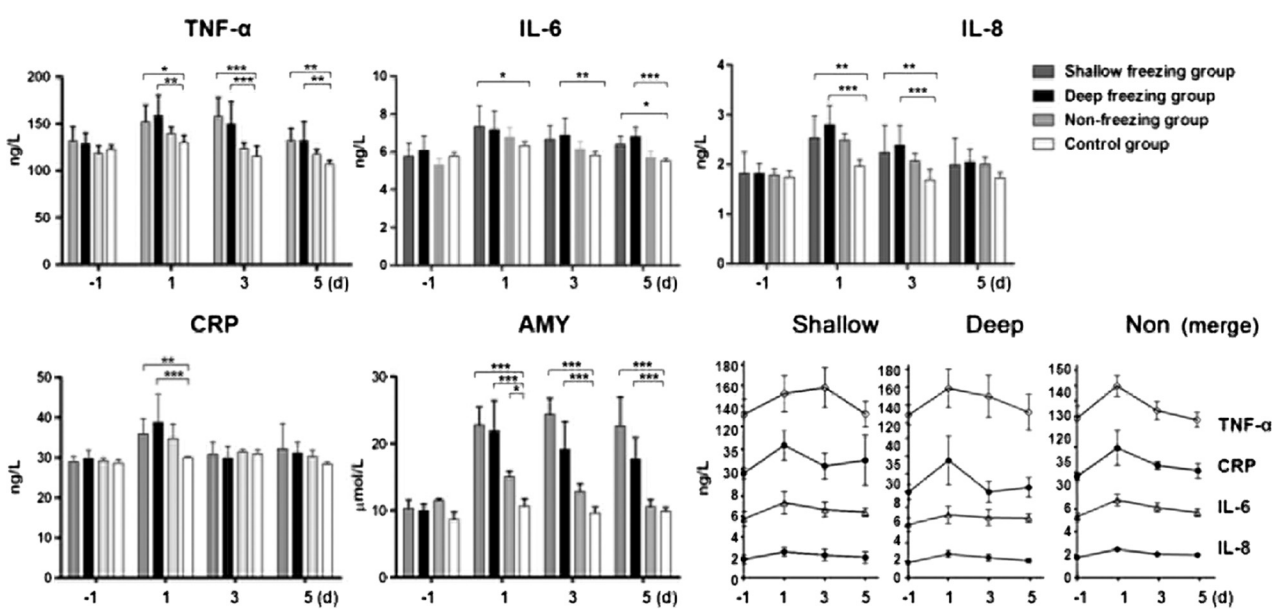


FIGURE 2. Expression of multiple serum factors changed over time. The ELISA analyses were performed to detect the expression levels of TNF-α, IL-6, IL-8, CRP, and AMY at days −1, 1, 3, and 5 after cryosurgery. ''Merge'' represents the overall trend in the change of all factors over time. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

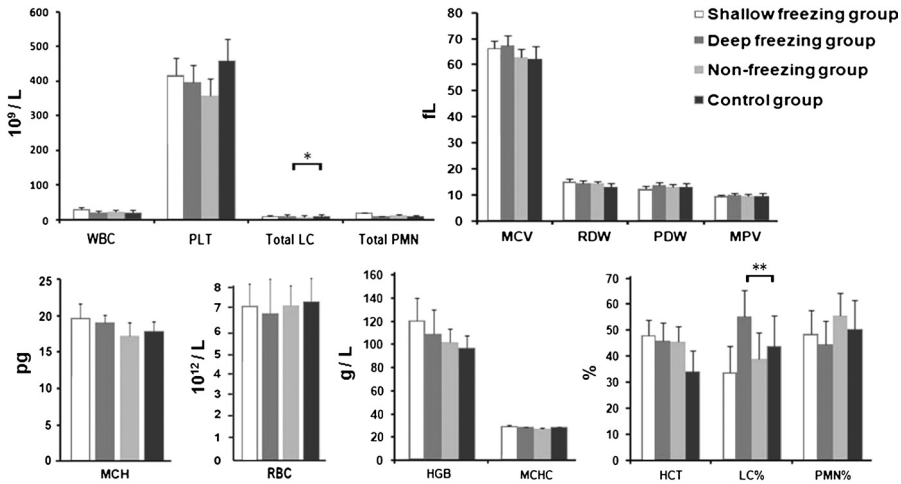


FIGURE 3. Blood routine analysis of experimental and control groups. HCT indicates hematocrit; HGB, hemoglobin; LC%, percentage lymphocyte; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; PDW, platelet distribution width; PLT, platelet; PMN%, percentage polymorphonuclear cell; RBC, red blood cell; RDW, red blood cell volume distribution; total LC, total lymphocyte; total PMN, total polymorphonuclear cell; WBC, white blood cell. $*P < 0.05$ and $**P < 0.01$.

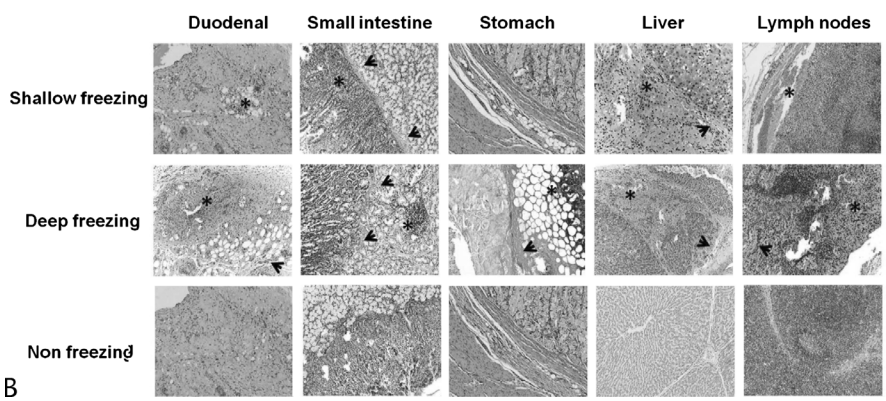
epithelial hyperplasia; and (5) slight apoptosis, visible normal pancreatic tissue structures, with recognizable acini between numerous infiltrating neutrophils, and more apparent apoptotic cells. Among these, central necrosis, inflammation and granulation areas were zones of complete necrosis, and severe and slight apoptotic areas were terms of apoptosis zones. The radii of the complete necrosis zones in the deep (6 [0.7] mm) and shallow freezing group (5.2 [0.6] mm) were similar ($P = 0.502$), and the radii of the apoptosis zones in the deep (6.5 [0.7] mm) and shallow freezing group (4.9 [1.2] mm) were different ($P = 0.043$). The typical pathological changes and ice ball diameters were then summarized (Fig. 5C), which showed that the ice ball radius was almost 0.5 cm longer than the radii of the complete necrosis zone in both the shallow and deep freezing groups.

DISCUSSION

Pancreatic cancer is the fourth leading cause of cancer-related death in Western societies, and the incidence of this tumor almost equals its mortality.[16] Pancreatic head cancer has one of the worst prognoses of all human malignancies, and approximately 75% are located in the head or neck of the pancreas. It is very difficult to treat this cancer with surgery alone, especially for patients with late-stage disease.[17] Currently, the

Side effects / Positive ratio / Groups	Injury of duodenal	Adhesion of small intestine	Adhesion of stomach	Adhesion of liver	Bleeding & necrosis of lymph nodes
Shallow freezing	2/4	2/4	0/4	1/4	2/4
Deep freezing	2/4	2/4	2/4	2/4	4/4
Non freezing	0/4	0/4	0/4	0/4	0/4

A



B

FIGURE 4. Adhesion of the pancreatic head to adjacent organs after cryosurgery. A, The proportions of adhesions in the 3 experimental groups; (B) typical pathological changes observed in the 3 experimental groups. Black arrows represent fibroelastosis, and asterisks represent areas of focal necrosis (all magnifications ×100). The images represent typical photographs from each group.

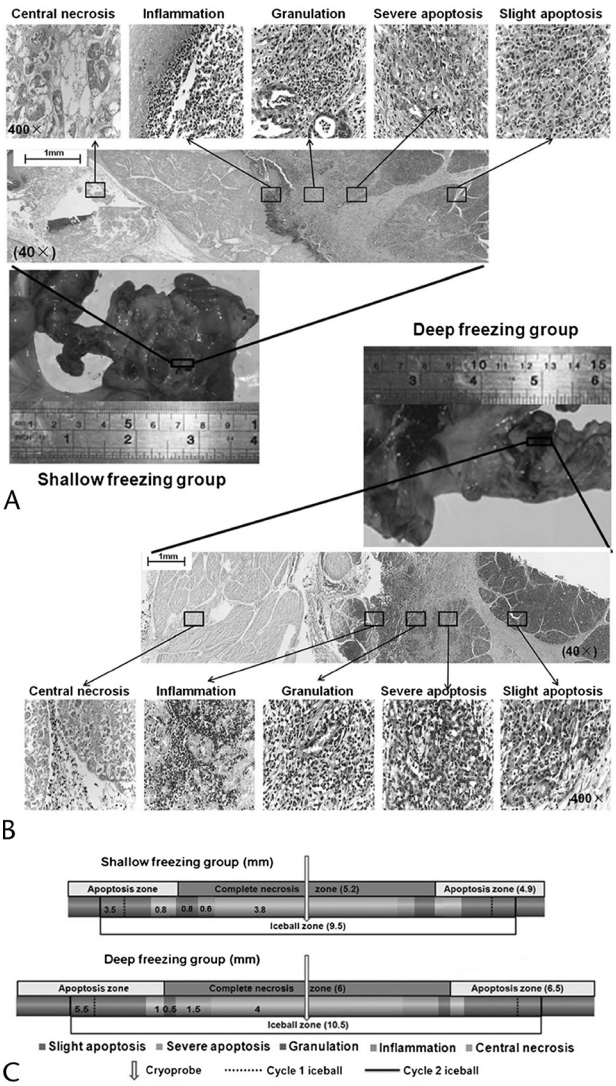


FIGURE 5. Five histological areas were evident at day 7 after cryosurgery. A, B, Histological manifestations of the shallow and deep freezing group, respectively; (C) the correlation of typical pathological changes and ice ball sizes, which represent the general pathological findings (per group, n = 4). Average radii of typical pathological areas, zones of complete necrosis and apoptosis, and ice balls are all indicated on the transverse bars.

treatment of stage IV pancreatic cancer includes palliative surgery,[18] chemotherapy,[19,20] and cryosurgery.[21,22] The first 2 methods are the standard treatment of late-stage pancreatic cancer.

Cryosurgery, which has been generally accepted as a technique that can cure many solid tumors, has emerged to be a novel technique in the treatment of pancreatic cancer. In 2002, Kovach et al[6] reported that 10 patients with unresectable pancreatic cancer who were treated with cryosurgery between March 1995 and March 1999 responded well; there were no surgical complications or mortalities directly associated with the cryosurgery, including no intraoperative or postoperative hemorrhages, fistula formation, or episodes of sepsis. In our hospital, percutaneous cryosurgery has been applied since 2001 to locally advanced pancreatic cancer under the guidance of ultrasound or computer tomographic imaging by Xu et al.[23] The 12- and 36-month survival rates were 63.1% and 9.5%, respectively, and 53.1% cases survived for 12 months or more. Acute pancreatitis was observed in 6 cases, and only 1 of these patients developed severe pancreatitis. In this study, we focused on the innovation of freezing protocol in this study according to the pathological damage of pancreatic head and localized and systemic complications.

As the pancreas is a thin and soft organ, it does not tolerate freezing well. In this experimental study, the freezing time was reduced to 5 minutes, rather than the common 10 minutes practiced in clinical situations; we established another freezing group which received only 10% of the argon output power, which also differed from the routine 100% output power used clinically.[24] After 2 freezing cycles, the ice ball diameters of both the shallow and deep freezing groups were 1.5 (0.24) and 1.9 (0.3) cm, respectively. The freezing point was 1 cm away from main pancreatic duct and duodenum, which are the most easily injured and deadly sites.[23,25] The ice ball edges contacted the adjacent organs, and physiologically, no severe damage were found in the animals of the experimental groups. Indeed, different degrees of adhesions between the pancreatic head and adjacent organs were observed and fibroelastosis and bleeding with areas of focal necrosis were detected pathologically. If the freezing trauma is too severe, intestinal necrosis and potential perforation could be serious complications of cryoablation. When cryosurgery is performed on pancreatic head, the patients should be fasted for at least 7 days postoperatively or liver function should be assessed daily when the tumor is very close to duodenum or liver. Because the pancreatic head volumes of pigs are smaller than human beings, the adverse effects of cryosurgery on adjacent organs may be less in human beings. With regard to adhesions of the pancreatic head to adjacent organs, the shallow freezing protocol seems to be better than the deep freezing protocol, as there was a higher probability of trauma to the adjacent organs in the deep freezing group in this study.

In pancreatic cryosurgery, AMY is a classic testing index for acute pancreatitis, and increased expression levels that are more than 3 times the normal value are usually considered to be the diagnostic standard. During 5 days postoperatively, AMY expressions in both freezing groups remained high, but did not reach 3 times the normal value, and so could not be diagnosed as acute pancreatitis. This phenomenon may be caused by the second necrosis of apoptosis cells within cryozone,[26] which is likely to be induced by inflammatory cells. As an inflammatory factor, TNF-α predominantly derives from activated macrophages and functions as a proinflammatory factor for neutrophil activation in acute pancreatitis.[27,28] In response to stimulation by TNF-α, IL-6 can be produced by a wide range of cells, including monocytes-macrophages, endothelial cells, and fibroblasts.[29–31] Interleukin-8, which is raised in acute pancreatitis, is a strong chemokine secreted from neutrophils and a predictor of disease severity.[32,33] Neutrophils and IL-6 can induce CRP synthesis in hepatocytes.[34] C-reactive protein increases dramatically in acute trauma and infection,[35,36] and is a reliable inflammatory evaluation index after 48 hours in the hospital.[37] These inflammatory factors increased sharply after cryosurgery in 2 freezing groups, and descended to normal level within 5 days, in accordance with the lymphocytes count at day 7 postoperatively. The inflammation or immune reactions may also induce lymph nodes bleeding and necrosis surrounding the pancreatic head because the lymph node is a little far away from ice ball and the freezing could not influence the lymph nodes. From the data previously mentioned, it seems that AMY is the more rigorous index than the other inflammatory factors for monitoring the complication of pancreatitis in cryoablation of pancreatic head.

In cryozone, necrosis and apoptosis are always simultaneously induced in the cryolesion. As not all cells can be killed within the zone of apoptosis, the remaining tumor cells may survive and induce cancer recurrence, which leads to the failure of therapy.[38] Therefore, only the size of the complete necrosis zone can determine the efficiency of the cryoablation procedure. In this study, the results of pathological investigations showed that there were similarly sized zones of complete necrosis and differently sized zones of apoptosis between the deep and shallow freezing groups, which suggested that the shallow was more efficient than the deep freezing protocol. Alternatively, ice ball formation is an important observation index for cryosurgery. As the temperatures of the freezing edge are almost 0°C[39] and temperatures lower than −40°C are necessary to cause the irreversible necrosis of target cells,[40] the area of ice ball does not equal the area of necrosis. To achieve complete necrosis of the cancer tissue, a 1-cm safe border has become a basic principle for the use of cryosurgery for hepatocellular carcinoma.[41–43] In this study, the radii of ice balls in the second cycle of shallow and deep freezing group were 0.95 (0.15) and 1.07 (0.15) cm, respectively, and the radii of the zones of complete necrosis in both the shallow and deep freezing groups were 0.52 (0.06) and 0.6 (0.07) cm, respectively. This indicated that the radii of the ice balls were almost 0.5 cm longer than the radii of the complete necrosis zones in both the shallow and deep freezing groups. For tumor cryosurgery, oversized ice ball formation will increase the complication rate and endanger the life of the patients, whereas an undersized ice ball can lead to recurrence of the tumor at the margins. Therefore, the optimum ice ball formation will give the best treatment outcomes with minimal complications. If the efficiency of cryosurgery and its safety is taken into account, a "0.5-cm safe border" may be sufficient for cryoablation of the pancreatic head, regardless of whether the shallow or deep freezing protocol is used.

In conclusion, the shallow freezing protocol not only ensured the effective cryoablation of the pancreatic head but also reduced excessive trauma to the pancreatic head and adjacent organs. It seems that if the ice ball extends 0.5 cm beyond the tumor edge, this is sufficient for the complete necrosis of the tumor tissue, and protects the surrounding tissues from severe ice damage. Acute pancreatitis should be monitored mainly by the detection of AMY. Research into the use of cryoablation for the treatment of pancreatic head cancer is only in its infancy and long-term observation of pancreatic head cryoablation in animal model still need be investigated. There is a great potential for the clinical application of cryosurgery for pancreatic head cancer, and our results provide basic evidence in this field.

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