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Comparison of dual- and triple-freeze protocols for hepatic cryoablation in a Tibet pig model

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ABSTRACT

The purpose of this study was to compare a dual-freeze protocol with a triple-freeze protocol for hepatic cryoablation in a porcine model. Eighteen cryoablations were performed over an exposed operation field in nine normal porcine livers, using dual- (10–5–10–5) and triple-freeze (5–5–5–5–10–5) protocols. Changes in the temperature of the cryoprobes and the diameter of the iceballs were recorded during the ablation, and pathological changes in the cryozones (zones of tissue destruction) were assessed seven days after the procedure. Use of two and three freeze–thaw cycles produced iceballs of different diameters. Seven days after cryosurgery, the triple-freeze protocol was associated with a larger zone of complete necrosis than the dual-freeze protocol, although the two protocols produced cryozones and cryolesions of similar length, and in both cases the cryozones contained five areas of destruction. With the same freezing time (20 min), the triple-freeze protocol may be a more powerful liver ablation method than the dual-freeze protocol.

Introduction

Transarterial chemoembolization, radiofrequency and cryoablation are now the primary modalities used in the clinical setting for primary and metastatic liver cancer [1]. Cryoablation has many advantages, including the ability to visualize the iceball [13,14] and to activate cryo-immunology in cancer [18], the absence of severe damage to the great blood vessels [12], lack of severe pain to the patient [1], better healing and minimal invasion. Experimental and clinical applications have shown that cryosurgery of the liver is safe and efficacious [8]. In unresectable liver cancer, cryosurgery can be applied with chemotherapy, with 3- and 5-year survival rates of 40% and 27%, respectively [21]. Iceball formation in liver cryoablation under ultrasound induces tumor tissue necrosis, which determines the effectiveness of the treatment effect [4]. Freezing protocols include the temperature, duration, and speed of both the freeze and thaw cycles, all of which factors influence the effect of freezing [8]. Hinshaw et al. demonstrated that, in lung cancer, three freeze cycles have a greater cytotoxic effect than two freeze cycles [10], but whether the protocol is suitable for hepatic cryosurgery needs to be investigated. In this study, dual- and triple-freeze protocols for hepatic cryoablation were compared in a Tibet pig model and pathologic examination conducted to assess the necrosis zones seven days after cryosurgery.

Materials and methods

Experimental animals

Nine certified healthy Tibet miniature pigs were provided by the Animal Experimental Center of South Medical University (Guangzhou, China), weighing 27–32 kg. Study approval was obtained from the Research Animal Care and Use Committee of our hospital, and all husbandry and experimental studies were compliant with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Argon–helium cryosurgery system

The cryosurgery equipment used was the Cryocare™ Cryosurgical System (Endocare, Irvine, CA), including the main body and cryoprobes. This system is based on 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 dimensions of the nozzle, argon and helium generate differing thermal exchange events in the area close to the nozzle.

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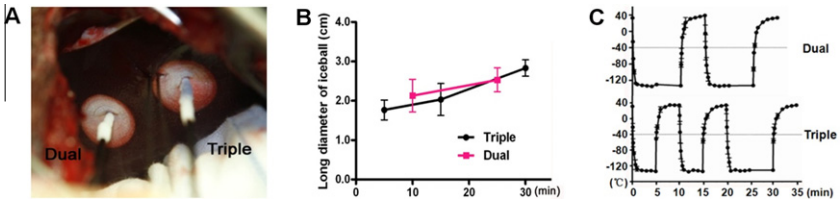

Fig. 1. Iceball formation under dual- and triple-freeze ablation protocols. (A) Iceball formation during the procedure. (B) Long diameter of the iceball at the end of the final freeze–thaw cycle. (C) Cryoprobe tip temperature changes with time.

Protocols and experimental methods

All pigs were anesthetized with isoflurane. Using sterile technique, the abdominal cavity was opened and the liver was exposed. Two cryoprobes of 2 mm in diameter were inserted 2 cm into the left liver lobe respectively, with their freezing points about 5 cm apart. The output power of the two gases was set to 100%. Dual- and triple-freeze protocols (10–5–10–5 and 5–5–5–5–10–5) were applied to the inside and the outside freezing points, respectively. The probes were then removed and the pinholes were filled in with thrombin gelatin sponge. The abdominal activity was closed without drainage. The pigs were returned to their cages and offered regular food. Neither intravenous antibiotic infusion nor any other medication was administrated. The diameter of the iceballs during cryoablation was measured using a vernier caliper. The temperature around each cryoprobe was recorded from its own temperature detector.

Pathology

The pigs were sacrificed seven days after cryoablation and the livers were removed for further examination. Cryolesions were inspected for their gross anatomic appearance and their diameter measured with a ruler. The cryolesions and surrounding tissues were then removed and embedded in paraffin. Five micrometer thick slices were made and stained with hematoxylin and eosin. Pathologic analysis was performed by an attending surgical pathologist.

Statistical analysis

All analyses were performed using Graphpad Prism (Graph-Pad, Inc., CA) software. All data were expressed as means ± standard deviation, and cryolesion surface length and depth, and cryozone areas were analyzed using the paired *t*-test. Values of $P < 0.05$ were considered to indicate a statistically significant difference, with $P < 0.01$ and $P < 0.001$ indicating highly significant differences.

Results

Iceball diameter under the different ablation protocols

When dual- and triple-freeze protocols were performed simultaneously in the central right liver, iceballs were seen to form rapidly (Fig. 1A). Their measured diameters under the dual-freeze protocol were 2.1 ± 0.4 cm and 2.5 ± 0.3 cm and those under the triple-freeze protocol were 1.8 ± 0.3 cm, 2.0 ± 0.2 cm and 2.8 ± 0.2 cm (Fig. 1B). The long diameter of the final iceball under the triple-freeze protocol was longer than that under the dual-freeze protocol ($P = 0.035$). Under both protocols, the probe temperature dropped sharply to below −120 °C and then reached more than 40 °C during the freeze–thaw cycle, with no significant differences among cycles (Fig. 1C).

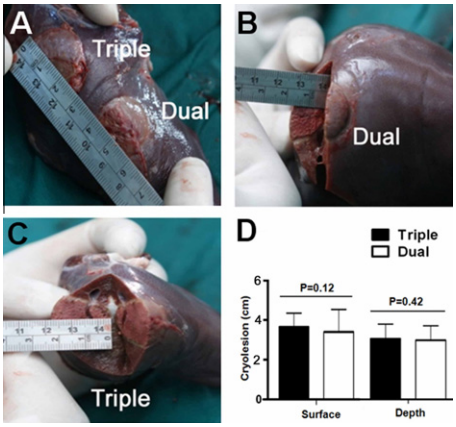

Fig. 2. Surface length and depth of cryolesions in the liver. Seven days after cryoablation, the cryolesions produced under the two protocols were contrasted. (A) Surface cryolesions, black suture threads represent probe insertion sites. (B) Depth of cryolesion produced under the dual-freeze protocol, the thin line near the ruler is the probe insertion track. (C) Depth of cryolesion produced under the triple-freeze protocol, the thin line near the ruler is the probe insertion track. (D) Comparison of the surface length and depth of the cryolesions.

Surface length and depth of cryolesions

All nine animals were sacrificed seven days after the cryoablation, at which time boundaries were visible between cryolesions and the surrounding tissue. The cryolesion diameters were similar under the dual- and triple-freeze protocols (3.4 ± 0.6 cm and 3.5 ± 0.4 cm, respectively). Because the freezing points were 5 cm apart during the procedure, the two cryolesions were about 1.5 cm apart after cryoablation (Fig. 2A). The two cryozones were sectioned vertically, revealing oval cryolesions. Measured with a ruler in the direction of placement of the needles, the depth of the cryolesion was 3.0 ± 0.3 cm for both the dual- and the triple-freeze protocol (Fig. 2B and C); no statistically significant differences in the surface length or depth of the cryolesions were observed (Fig. 2D; $P = 0.12$ and $P = 0.42$, respectively).

Pathologic changes in cryozones

The cryozones were removed in accordance with the surface long radius and a pathological examination was undertaken. Under both the dual- and the triple-freeze protocol, the pathologic phenomena (Fig. 3A) were: (1) central necrosis: many hepatocytes stained uniformly red[2]; (2) inflammation: large amounts of cell necrosis with infiltration of neutrophils and lymphocytes; (3) hyperemia: inflammatory cells gathered in bile duct with a small amount of capillary hyperemia and lymphocyte infiltration; (4) granulation:

[2] For interpretation of color in Figs. 1–4, the reader is referred to the web version of this article.

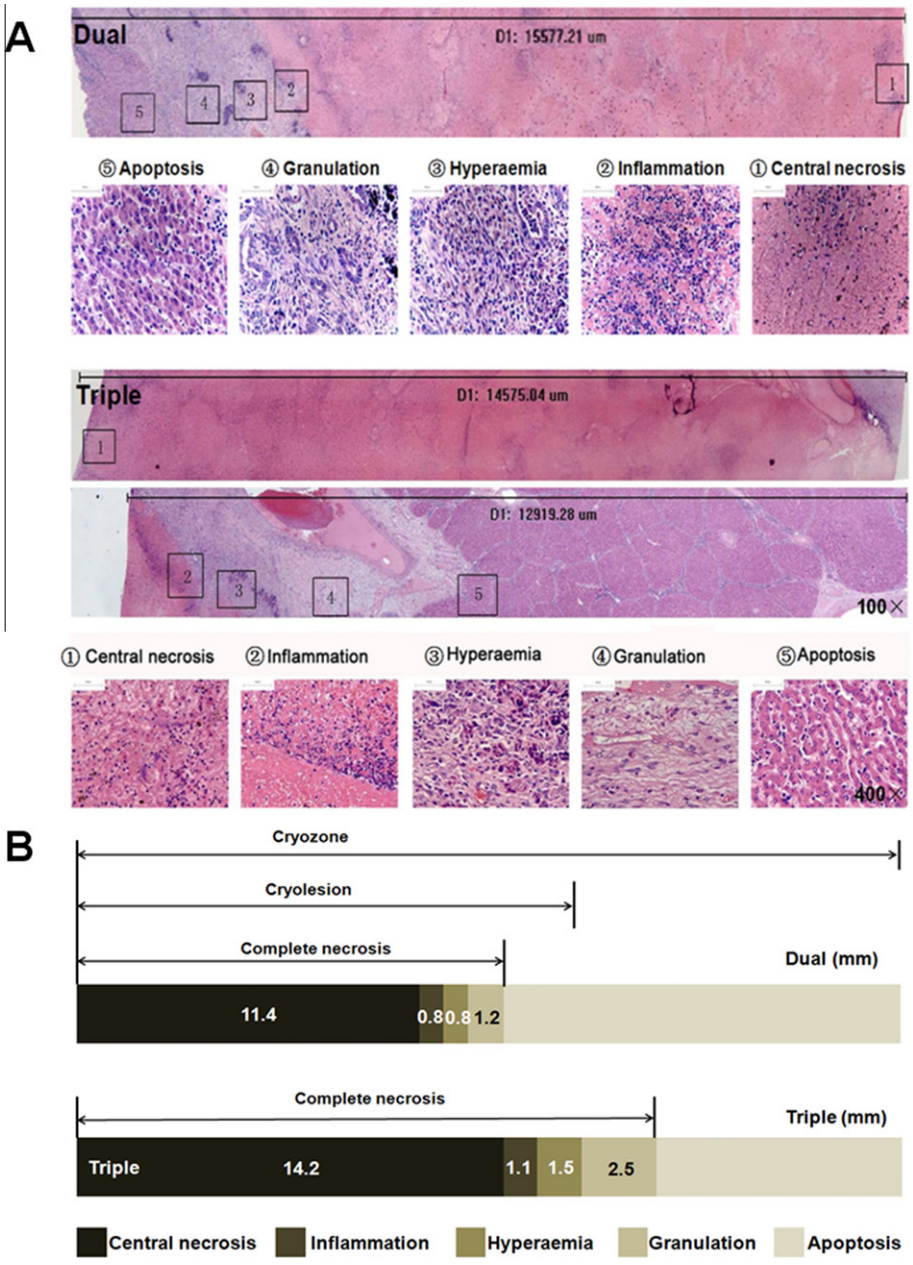

Fig. 3. Pathological changes in cryozones at day 7 post-cryoablation. (A) Cryozones taken from the liver according to the surface long radius were examined pathologically. Five areas of pathologic changes can be seen: a central necrosis zone, an inflammation zone, a hyperemia zone, a granulation zone and an apoptosis zone. (B) Typical pathological changes in cryozones, cryolesions and complete necrosis zones. The two graphs are samples representing nine cryoablations under the dual- and triple-freeze protocols.

more bile duct and fibroblast hyperplasia, a few lymphocytes and plasma cell infiltration, local focal calcification, a few apoptotic cells; and (5) apoptosis: hepatocytes arranged in order, more lymphocytes in the liver sinus and more apoptotic cells. The inner four areas of the cryozones showed complete necrosis; in the apoptotic area, normal tissues could still be observed under a microscope.

To further study the relationship between the surface cryolesions produced under the two ablation protocols and the inner pathologic changes, we combined these two aspects. As Fig. 3B shows, the cryozones were much larger than the cryolesions, and the cryozones (radius was about 3 cm in both groups) and the cryolesions under the dual- and triple-freeze protocols exhibited no statistically significant differences. The radius of the complete necrosis zone under the dual-freeze protocol (1.42 ± 0.7 cm) was less than that under the triple-freeze protocol (1.93 ± 0.6 cm; $P = 0.035$).

Discussion

Since the 1960s, experiments have demonstrated that freezing might be useful as a hemostatic agent in liver surgery [6,19]. Subsequently, interest in liver cryosurgery became focused on the treatment of tumors. By 2009, the cryosurgery protocol of argon–helium cryosurgical system for liver and lung had been established by Littrup et al. as two cycles of a 10 min freeze followed by a 5 min thaw [15]. Although this protocol was widely accepted by most surgeons, protocols for hepatic cryosurgery remain to be optimized. The design of the freeze–thaw cycle involves multiple

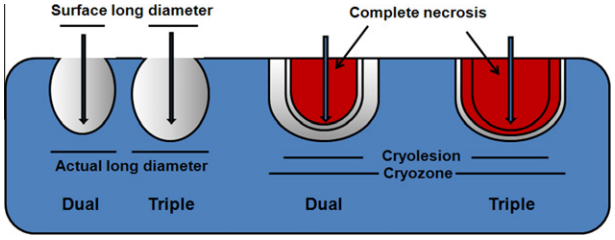

Fig. 4. Schematic representation of the iceball, cryolesion and cryozone. Blue lines in iceballs and cryolesions represent the cryoprobe. Cryolesions reflect zones of destruction observed by the naked eye and cryozones are actual destruction zones on pathology.

factors, including the cooling and thawing rate, tissue temperature, duration of freezing and thawing, cycle number and cycle interval [8]. Experiments have shown that prolongation of the freezing time or an increased number of freezing cycles may produce a greater destructive effect [3,9,10]. Repeated freeze–thaw cycles can extend the lethal effect into the warmer zone at the periphery of the defined target tissue [8]. Slow thawing of the frozen tissue is commonly considered to be another important factor in tissue destruction [20]. Consequently, a "5–5–5–5–10–5" protocol was designed for this study with an increased duration of thawing (5 min).

On pathologic examination, cryozones, cryolesions and complete necrosis zones were observed. Cryoablation using the triple-freeze protocol produced cryozones and cryolesions similar to those seen with the dual-freeze protocol in the same overall freeze time. Because the triple-freeze protocol includes an extra thaw time, the iceball diameter was significantly greater than that in the dual-freeze protocol, and the length of the complete necrosis zone was approximately 19.3 mm compared with about 14.2 mm under the dual-freeze protocol. Only the surface long diameter of the iceball, which does not represent the entire iceball, can be seen; thus, the real diameter of the iceball was significantly greater (Fig. 4). These results showed that the triple-freeze protocol is more effective for tissue destruction in hepatic cryoablation and that the protocol could be exploited for clinical use. Increasing the frequency of freezing and duration of thawing was associated with greater cell lethality and generally larger cytotoxic zones within a larger final iceball.

Earlier visualization of iceball formation on ultrasound or CT can be clinically useful because the cytotoxic isotherm (i.e. approximately −20 to −40 °C, depending on cell sensitivity) is generally 5–10 mm behind the leading edge of the visible iceball, and thus imaging allows intraoperative monitoring of the frozen zone [13,14]. In our study, the cell necrosis isotherms were propelled outward for approximately 5.1 mm under the triple-freeze protocol, and appeared to be approximately 2.3 mm inside (dual-freeze) or 2.2 mm outside (triple-freeze) the edge of the cryolesion.

Under both the dual- and the triple-freeze protocols, five areas of pathological change were observed: a central necrosis zone, an inflammation zone, a hyperemia zone, a granulation zone and an apoptosis zone. This is consistent with typical cryolesions in the liver, which comprise a demarcated volume of coagulation necrosis with a narrow border of partially damaged tissue [8]. After freezing injury, complete necrosis cells can be presented by dentritic cells and cryo-immunology might be activated [18]; inflammatory cell infiltration may contribute to the second development of apoptosis and tissue destruction [7]; most apoptotic cells become necrotic under anoxic conditions in the cryozone [2], while the remaining viable cells in the apoptotic area may recover via the caspase-3 pathway [11] with the help of a macrophage repair effect [5,16,17].

This study demonstrates that, although a triple-freeze protocol produced cryozones and cryolesions similar to those obtained under a dual-freeze protocol, the former induced more complete necrosis than the latter. That is, for the same size of tumor, use of a triple-freeze protocol may reduce the possibility of recurrence of cancer. In summary, for thorough cryoablation, our triple-freeze protocol was better than the dual-freeze protocol, and this finding may help clinicians to improve cryoablation for liver cancer.

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