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Comparison of dual- and triple-freeze protocols for pulmonary 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 pulmonary cryoablation in a porcine lung model. Five dual- (10-5-10-5) and five triple-freeze (5-5-5-5-10-5) cryoablations were performed on an exposed operation field in normal porcine lung. Changes in the temperature of the cryoprobes and the diameter of the iceballs were measured during the ablation and pathologic changes in the cryozones (zones of tissue destruction) were reviewed 7 days after the procedure. The diameter of the iceball surface differed between the two protocols. Pathologically, the triple-freeze protocol was associated with a longer complete necrosis zone than the dual-freeze protocol, though the two protocols produced cryolesions and cryozones of similar length, and in both cases there were five areas of tissue destruction. With the same duration of freezing (20 min), the triple-freeze protocol may be better for pulmonary cryoablation than the dual-freeze protocol.

Introduction

Radiofrequency, microwave and cryoablation are now the principal ablative therapies used clinically in primary and metastatic lung cancer [19]. Cryotherapy has many advantages, including the ability to visualize the iceball [10,11] and to activate cryoimmunology [18], the absence of severe damage to the great blood vessels [9], lack of severe pain to the patient [1], better healing and minimal invasion. Experiments in the 1970s demonstrated that the trachea, bronchus and lung can all be safely subjected to local freezing [14,20]. Cryosurgery can be used for unresectable tumors, to slow tumor growth and reduce pain. Different tissues react differently to the intensity of ablative procedures [4]. These differences are primarily related to the local environment, including blood flow, the insulative properties of the surrounding tissues, electrical resistance and air flow in the organ [22]. As pulmonary fluid fills the alveolar spaces after the first freeze–thaw cycle, the thermal conductivity increases 20-fold (0.024 W/mK for air vs. 0.58 W/mK for water and 0.49–0.5 W/mK for blood). This may result in more rapid formation of the iceball on subsequent freeze cycles, thereby expanding the cytotoxic isotherm for more thorough tumor necrosis throughout the visualized cryolesion [13].

The effect of the temperature, duration and speed of both freeze and thaw cycles has been studied for many tumor and organ types, and the use of a triple-freeze cycle has been suggested to have a greater cytotoxic effect in more fibrous tumors and organs [4]. However, the optimal protocol in an aerated tissue might differ from that in solid organs [13]. In 2010, Hinshaw et al. studied the effects of a triple-freeze protocol on porcine lung in vivo, and found that larger iceballs and areas of necrosis could be achieved intraoperatively under the triple-freeze protocol compared with a dual-freeze protocol [6]. There is no pathological long-term evidence of the effects of cryosurgery. In this study, dual- and triple-freeze protocols for pulmonary cryoablation were compared in a Tibet pig model and pathologic examinations were conducted to assess the zones of necrosis 7 days after the procedures.

Materials and methods

Experimental animal

Five 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

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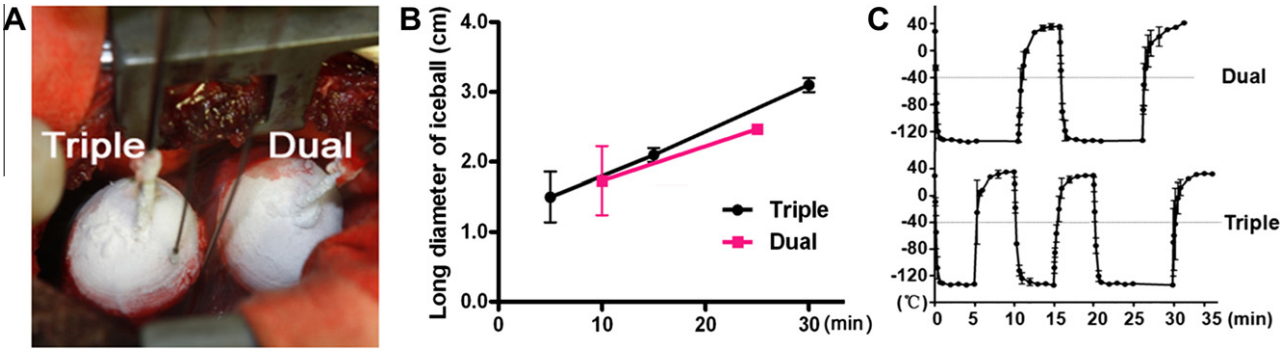

Fig. 1. Iceball formation under dual- and triple-freeze ablation protocols. (A) The iceballs at the end of the final freeze–thaw cycle. (B) Long diameters of the iceballs. (C) Cryoprobe tip temperature changes with time.

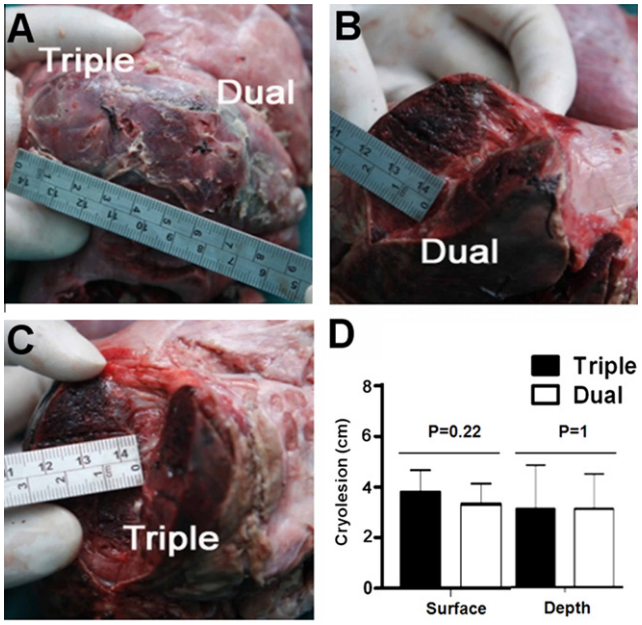

Fig. 2. Surface length and depth of cryolesions in the lung. Seven days after cryoablation, the cryolesions produced under the two protocols were contrasted. (A) Surface cryolesions, the suture needle represents probe-insertion sites. (B) Depth of cryolesion produced under the dual-freeze protocol, the 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.

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.

Protocols and experimental methods

All pigs were anesthetized with isoflurane. Using sterile technique, the thoracic cavity was opened and the lung was exposed. Two cryoprobes of 2 mm in diameter were inserted 2 cm into the right lung, with their freezing points about 5 cm apart. The output power of the two gases was set to 100%. Dual- and triple-freeze protocols (freeze–thaw cycles 10-5-10-5 and 5-5-5-5-10-5, respectively) were applied to the inside and outside freezing points, respectively. The probes were then removed and the pinholes were filled in with thrombin gelatin sponge. The chest 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 diameters of cryolesions were measured 7 days after cryoablation using a ruler and the cryolesions were inspected for their gross anatomic appearance. The cryozones 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 the cryolesion surface length and depth, and cryozone areas were analyzed using the *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 on the central right lung simultaneously, iceballs were seen to form rapidly (Fig. 1A). Their measured diameters under the dual-freeze protocol were 1.7 ± 0.5 and 2.46 ± 0.06 cm, and those under the triple-freeze protocol were 1.5 ± 0.4, 2.1 ± 0.1 and 3.1 ± 0.1 cm (Fig. 1B). The final diameter of the iceball was greater under the triple-freeze protocol than under the dual-freeze protocol ($P = 0.011$). Under both protocols, the probe temperature dropped sharply to below −120 °C and rose sharply to more than 40 °C during the freeze–thaw cycle, with no significant differences between cycles (Fig. 1C).

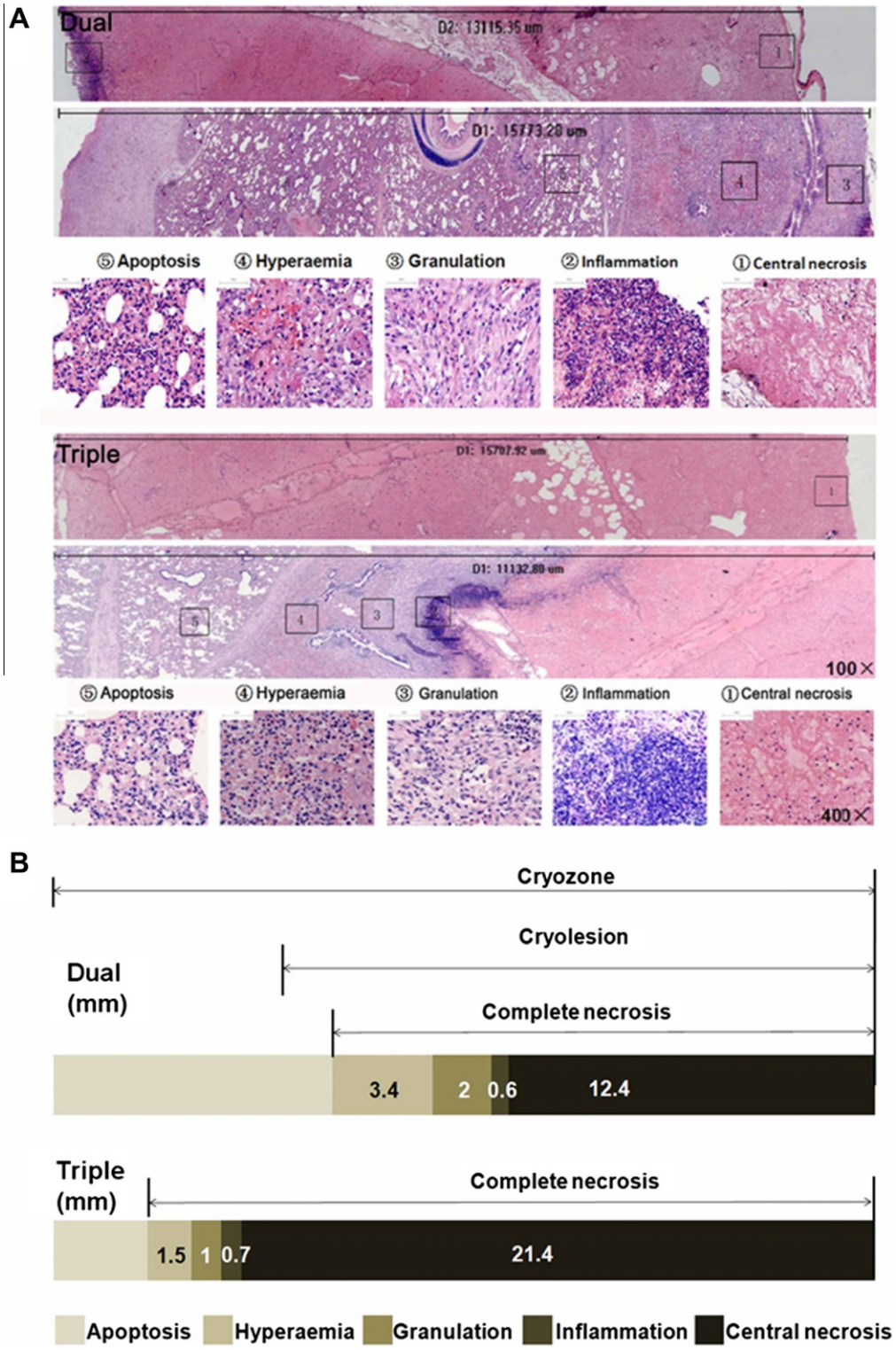

Fig. 3. Pathological changes in cryozones at day 7 after cryoablation. (A) Histology of cryozones produced under the dual- and triple-freeze protocols. (B) Typical pathological changes in cryozones, cryolesions and complete necrosis zones. The two graphs are samples representing five cryoablations under the dual- and triple-freeze protocols.

Surface length and depth of cryolesions

All five animals were sacrificed 7 days after the cryoablation, at which time boundaries were visible between cryolesions and the surrounding tissue. The surface length of the cryolesions produced under the dual- and triple-freeze protocols were similar (3.87 ± 0.64 and 3.95 ± 0.81 cm, respectively). Although the freezing points were 5 cm apart, the two cryolesions were closer after cryoablation (Fig. 2A). The two cryozones were then sectioned vertically, revealing oval cryolesions within. Measured with a ruler in the direction of placement of the needles, the depth of the cryolesions produced under the dual-freeze protocol was 3.57 ± 0.4 cm (Fig. 2B) and that of the triple-freeze protocol was 3.8 ± 0.44 cm (Fig. 2C). No statistically significant differences in the surface

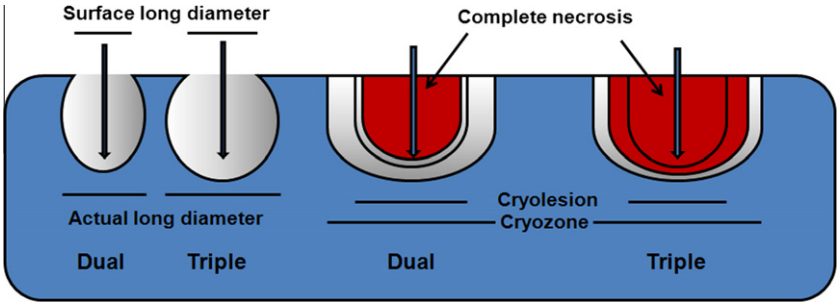

Fig. 4. Schematic representation of the iceball, cryolesion and cryozone. White lines in iceballs and cryolesions represent the cryoprobe. Cryolesions reflect zones of destruction observed by the naked eye and cryozones are the actual destruction zones on pathology.

length or depth of cryolesions were observed between the dual- and triple-freeze protocols (Fig. 2D).

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 pathological phenomena (Fig. 3A) were: (1) central necrosis: a large amount of tissue necrosis uniformly stained red; (2) inflammation: a large amount of cell necrosis with infiltration of neutrophils and lymphocytes; (3) granulation: including much hyperplasia of fibroblasts and capillaries, a few neutrophils; (4) hyperemia: including necrosis with bleeding and red-stained lesions, a little hyperemia and bleeding from capillaries; and (5) apoptosis: normal pulmonary alveolar tissue, wider intervals between alveoli, more fiber hyperplasia and infiltration of lymphocytes and apoptotic cells. The inner four areas of the cryozone constituted a zone of complete necrosis. Normal tissues could still be observed in the apoptotic area.

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 (Fig. 3B). The radii of the cryozones in the dual- and triple-freeze groups were both approximately 3 cm. The radius of the complete necrosis zone under the dual-freeze protocol (1.84 ± 0.6 cm) was smaller than that under the triple-freeze protocol (2.46 ± 0.5 cm, $P = 0.031$). The cryolesion was inside the apoptotic area after the dual-freeze protocol and inside the area of central necrosis after the triple-freeze protocol.

Discussion

In 1974, Neel et al. froze the lungs of dogs and monkeys via thoracotomy, compressing the tissue against the cryoprobe without penetrating the lung. The lesions appeared as hemorrhagic infarctions after thawing, and healing yielded a contracted, fibrous scar [15]. More recently, Izumi et al. investigated the feasibility of transthoracic insertion of a cryoprobe into the parenchyma of swine lung (a 10-5 scheme, one or two cycles). The use of two cycles can increase the bleeding time and decreased the leakage of air [8]. In 2009, Littrup et al. established a pioneering protocol (the 10-5-10-5 scheme with a 1-2 rule) for cryosurgery of liver and lung, which has been widely accepted by most surgeons [12]. In the same year, Gage reported that the design of the freeze–thaw cycle depends on multiple factors, including, the cooling and thawing rate, tissue temperature, duration of freezing and thawing, cycle number and cycle interval [5]. Subsequently, Hinshaw and associates amended the commonly used 10-5-10-5 cryogenic scheme to a 3-3-7-7-5-5 scheme and contrasted the cryogenic effects achieved by these two protocols with the same total freeze–thaw time. They found that a triple–freeze protocol may have several advantages in pulmonary cryoablation, including earlier imaging findings during ablation, a shorter procedure and larger zones of ablation [6].

In general, at tissue temperatures of −50 °C and cooler, the temperature is sufficiently low to destroy the tissue in a single cycle. Because the temperature of tissue around the cryoprobe can drop to −140 °C in 1 min, a reduction of the freezing duration will not influence its effect. The major reason to use a third freeze–thaw cycle is to extend the complete necrosis zone into the warmer zone at the periphery of the tumor after cryosurgery [5]. Meanwhile, slow thawing of the frozen tissue is commonly considered to be another principal factor in tissue destruction (e.g. Whittaker showed that intracellular ice crystals are larger in the second freezing cycle and suggested that this effect was due to a longer thawing time [21]). The actual diameter of the iceball is significantly greater than the surface diameter, and thus the surface diameter cannot act as an index of the freezing effect; the cryozone will be significantly greater than the cryolesion (Fig. 4). The size of the actual complete necrosis zone can be evaluated, not from the surface cryolesion, but by the pathologic changes observed in the cryozone. Our study showed that the triple-freeze protocol produces cryozones and cryolesions similar to those produced with the dual-freeze protocol. With one extra period of thawing, the length of the complete necrosis zone was approximately 24.6 mm, compared with 18.4 mm under the dual-freeze protocol. Thus, the triple-freeze protocol may be more effective for tissue destruction in pulmonary cryoablation. These observations suggest that more complex protocols are justified by their potentially greater cytotoxic efficacy.

Earlier visualization of iceball formation on ultrasound or CT 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 [10,11]. The advantages of pathological evaluation include the fact that the changes are visible with the naked eye, the actual state of the necrosis can be observed, and the examination can be conducted at various times after cryosurgery. In our study, the cell necrosis isotherm under the triple-freeze protocol was propelled outward for approximately 6.4 mm, and appeared to be approximately 2 mm inside (dual-freeze) or 4 mm outside (triple-freeze) the edge of the cryolesion. This demonstrates that the triple-freeze protocol produces cryolesions similar to those of the dual-freeze protocol, but with a larger complete necrosis zone. The ability to monitor the development of the complete necrosis zone should allow more accurate and safer ablation, especially when the target lesion is adjacent to sensitive structures such as the esophagus, recurrent laryngeal nerve and central airway.

After freezing injury, complete necrosis cells can be presented by dentritic cells and cryo-immunology might be activated [18]; inflammatory cell infiltration contributes to the second development of apoptosis and tissue destruction [3]. Most apoptotic cells will become necrotic under the anoxic conditions in cryozone,

while the remaining viable cells may recover via the caspase-3 pathway [7] with the help of a macrophage repair effect [2,16,17]. So trying to reduce apoptosis area is of great importance for complete necrosis of tumor, and it looks like triple-freeze protocol has more obvious advantages in this aspect than dual-freeze protocol.

This study demonstrates that a triple-freeze protocol can induce much more necrosis than a dual-freeze protocol, with similar cryozones and cryolesions. That is, for the same size of tumor, the triple-freeze protocol is associated with an increased probability of complete tumor necrosis and a decreased risk of recurrence. This triple-freeze protocol may be more suitable for pulmonary cryosurgery than dual-freeze protocol, and our results may provide information to help clinicians improve lung cryoablation.

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