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**Number of freeze-thaw cycles**

There is some evidence that a double freeze–thaw cycle induces a higher percentage of tissue destruction within a given lesion, thus improves uniformity of cell death.

Kollman[20] using a porcine model, studied ?intrahepatic cryolesions induced by freezing the hepatic tissue for a total of 15 mins. Additional animals underwent a double freeze–thaw cycle of 7.5 mins each (DF). Seven days after freezing, DF did not change the volume of the cryolesion compared to SF, however, resulted in enhanced destruction of hepatocyte nuclear morphology. He showed that double freezing may improve uniformity of hepatocyte nuclear destruction within the margin of the lesion due to a more pronounced shutdown of microvascular perfusion, resulting in irreversible ischemia [21,22]. In addition, as it is well known that thawing is a more important mechanism of cell death than cooling [23], the application of a double thaw cycle in double freezing may account for the more complete marginal hepatocyte nuclear destruction.

Robinson[24] showed that for bone cryosurgery, the difference of the viability of targeted cells between one freezing cycle and two freezing cycles was highly significant. But the viability following three freezing cycles was similar to that after two freezing cycles (Figure 1-8-10). Also, after a single cycle of freezing, most specimens were necrotic, some were still viable. In contrast, there were no specimens that had viable cells following two freezing cycles (Figure 1-8-11).

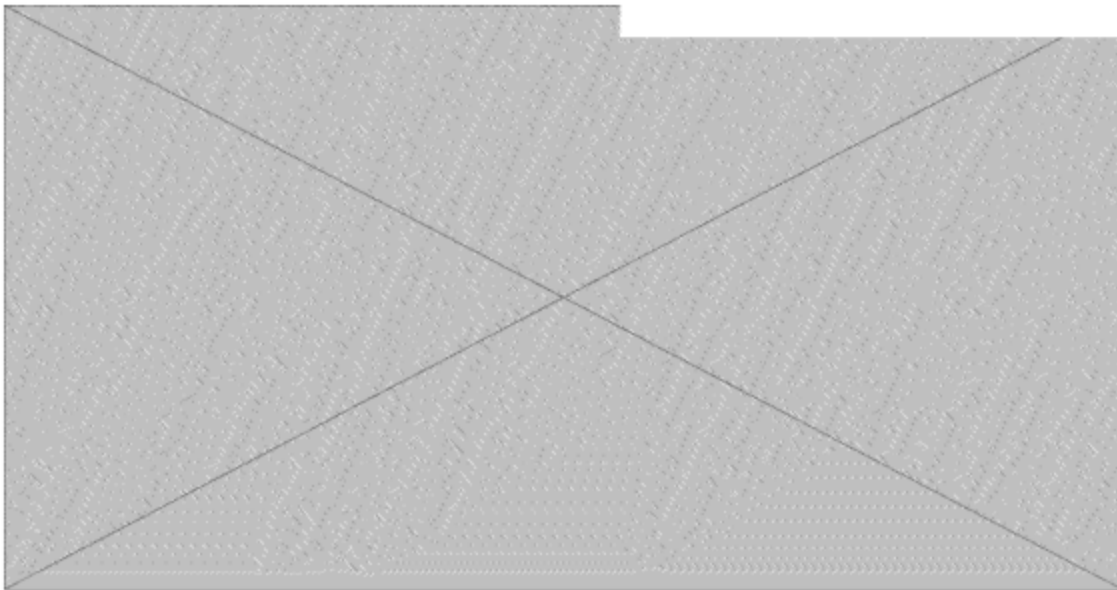
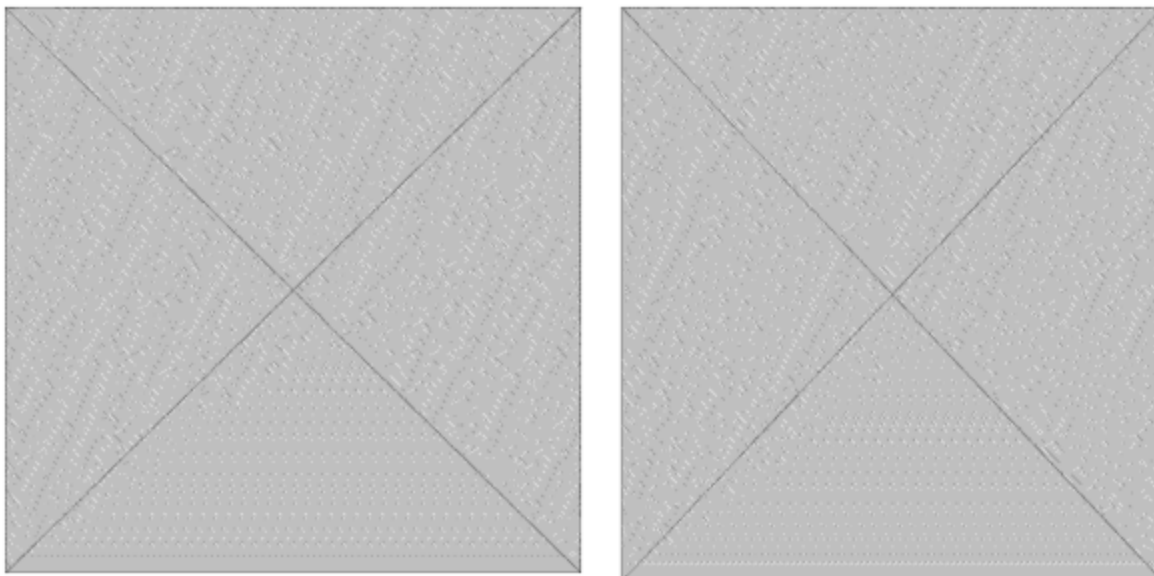


Figure 1-8-10. Average viability  $\pm$  6 standard deviation of tumor mouse  
From Robinson? D, et al. *Cryobiology* 2001;43:4-10



A

Figure 1-8-11.7 A. Cell necrosis following a single freezing cycle is r  
Cell necrosis following two freezing cycles appears uniform (Alcian blue stain, original magnification 3200).  
From Robinson? D, et al. *Cryobiology* 2001;43:4-10

Interestingly, Kollman's study indicates a marked reduction of leukocytic infiltration after double freezing compared to single freezing[20]. This may be due to the fact that the double freeze procedure provides a more distinct microvascular shutdown at the margin of the cryolesion compared to the single freeze procedure[21,25], which can prevent rapid marginal tissue infiltration by leukocytes derived from the blood stream. The distinct eosinophilic infiltration in marginal zone of cryolesion was more pronounced after double freezing compared with single freezing, and thus inverse to leukocyte (mainly neutrophil) infiltration. This may indicate a specific immune response after double freezing, which remains to be elucidated in further studies.

A double freeze thaw cycle was shown to produce significant increases in cell destruction for every set of thermal parameters investigated. This finding is also in agreement with the two factor theory. At high cooling rates a double freeze thaw cycle should increase the statistical probability of intracellular ice formation and cell damage. At lower cooling rates the effect of a double freeze thaw cycle is to increase the amount of time cells exposed to the hypertonic conditions, and thus increase the degree of osmotic injury[8].

#### **Selective vascular inflow occlusion**

Selective vascular inflow occlusion during the application of a single freeze–thaw cycle effectively enhances marginal cell destruction, additionally, significantly increases the overall volume of the lesion. In clinical practice, additional Pringle maneuver (vascular occlusion) was introduced with the idea to increase the volume and effectiveness of the cryosurgical procedure[26,27]. In a sheep model of cryosurgery, Dilley[27] reported that the rate of increase of iceball diameter is significantly greater after vascular inflow occlusion, and that the necrosis as a mean percentage of initial iceball diameter after 1 month is more pronounced after double versus single freezing.

Kollman[20] used a porcine model to study whether selective vascular inflow occlusion can achieve complete hepatic tissue destruction. Results showed that if the single freeze–thaw cycle was applied during selective vascular inflow occlusion, the volume of the cryolesion was significantly increased compared to single freeze–thaw cycle (SF) and double freeze–thaw cycle (DF). More detailed analysis of the transition zones of the cryolesions of each group revealed destruction of the intralobular trabecular architecture in zone 1, which was slightly more pronounced in VO-SF-treated livers compared to DF-livers. Importantly, destruction of hepatocyte nuclear morphology in zone 1 was not complete in SF-treated livers, but was evident in DF- and VO-SF treated hepatic tissue. Accordingly, within the transition zone 1 DF- and VO-SF livers showed a significantly higher score of destruction of hepatocyte nuclear morphology compared to SF-treated tissue. In zone 2, VO-SF resulted in comparable leukocyte infiltration as observed after SF, while DF was associated with a significantly reduced leukocytic infiltrative response. In zone 3, bile duct proliferation was associated with apoptotic cell death and eosinophilic infiltration. Bile duct proliferation was comparable in all three groups. Moreover, VO-SF-treated livers showed more pronounced apoptotic cell death, while DF-treatment increased the infiltration

of eosinophilic cells. The petechial bleedings at the transition to the normal hepatic tissue in zone 4 did not differ in severity between the three different treatment groups(Figure 1-8-12).

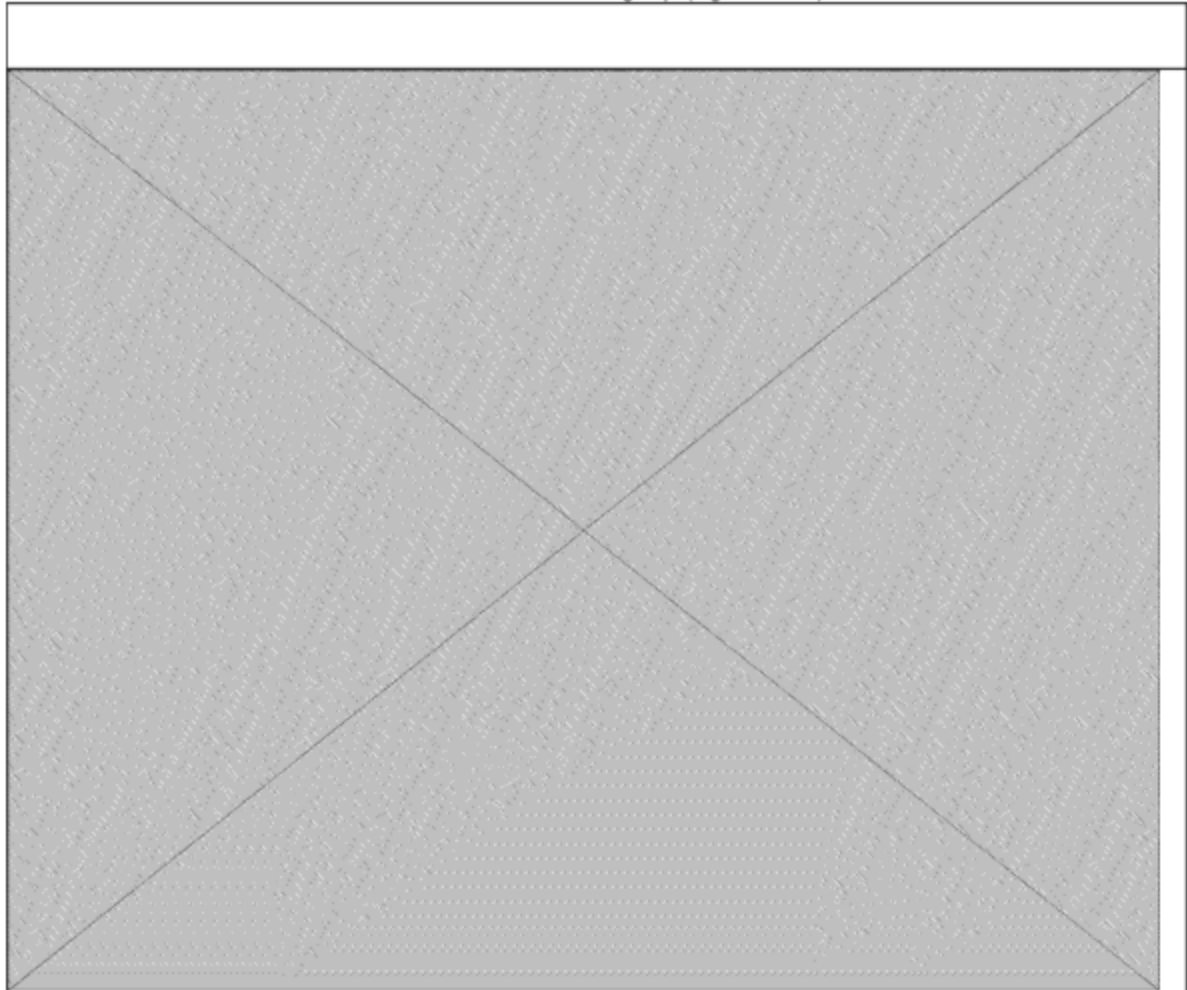


Figure 1-8-12. Histomorphological characteristics 7 days after cryosurgery. Note the bile duct proliferation (A. after single freeze–thaw cycle of 15 mins), the accumulation of apoptotic cells (B. after single freeze–thaw cycle of 15 mins during selective vascular inflow occlusion (VO-SF)), and the infiltration of eosinophilic cells (C. after double freeze–thaw cycle of 2X7.5 mins) in zone 3 of the transitional area within the margin of the cryolesion. In addition, within the transition from zone 3 to the normal hepatic tissue of zone 4, petechial bleedings are observed (D. after VO-SF).

From Kollman O, et al. *Cryobiology* 2004; 48:263–272

Mala[28] studied the effect of vascular inflow occlusion for cryolesions in pigs during hepatic freezing. Ice-ball volume was estimated by intraoperative magnetic resonance imaging. Results showed that the median volume of cryolesions made during inflow occlusion was 195% larger than cryolesions induced without occlusion. The geometry of the iceballs was more regular if produced during inflow occlusion than if not.

Seifert[10,29] showed that using 8 mm-cryoprobes in vivo placed in the pig liver, a 20 mins single freeze cycle with additional Pringle manoeuvre, resulted in a safety margin of about 15–16 mm. However, a significant ischaemia–reperfusion injury may result in addition to the liver injury caused by Pringle manoeuvre. For this reason hepatic inflow occlusion during cryotherapy should be limited to special situations requiring this technique (for example lesions >3 cm in difficult locations close to large vessels, which do not allow the placement of multiple probes) and not recommended as a routine procedure.

The higher effectiveness achieved by inflow vascular occlusion has to be attributed to the abrogation of the “heat sink effect” due to the lack of microvascular perfusion[1,8].

#### **Antifreeze proteins**

It has been shown that antifreeze proteins can enhance the destruction of cells frozen. All the AFPs, including AFP-I, share the ability to depress the freezing point of body fluids noncolligatively. When the fluids eventually freeze, these proteins modify the structure of the ice crystals[31].

A more comprehensive study on antifreeze protein adjuvant cryosurgery for prostate, breast, and liver cancer was performed. Over 30 control studies compared the viability of prostate cancer cells, breast cancer cells, and hepatocytes. To show that antifreeze proteins are effective during cryosurgery in vivo, Pham[31] performed experiments with human prostatic adenocarcinoma grown subcutaneously in nude mice. Prior to cryosurgery, the tumors of test mice were injected interstitially with AFP-I. In control mice, the tumors were injected with PBS in a similar manner or with no injection at all. The results showed (Figure 1-8-13) the tissue frozen with AFPs has completely lost its structural integrity. The cell membranes are not intact, the nuclei have become distorted, and, in particular, the connective tissue surrounding the cells appears to be sheared. There is no continuity between the cells, and numerous lacunae are evident between the cells.



Figure 1-8-13. Typical micrographs of the prostate cancer tumors. a. Obtained from prostate cancer tumors that were injected with phosphate-buffered saline solution prior to freezing; b. Obtained from tumors that were injected with a solution of PBS containing 10 mg/ml AFP-I. The staining shows the cell nuclei; a. Illustrates the round shape of the nuclei in prostate cancer tumors and the normal tissue structure; b. Shows that after freezing some of the nuclei have become distorted and have darkened while others (marked with arrows) appear intact; c. Shows that after freezing with AFP the nuclei are distorted and irregular in size. Furthermore, there are numerous lacunae in the tissue. Scale bar, 10mm  
From Pham L, et al. *Cryobiology* 1999;38:169-175

Muldrew[32] had a similar study. He used subcutaneous tumors of Dunning AT-1 rat prostate cells grown in Copenhagen rats to detect adjuvant effect of AFP I for cryosurgery, and the cryoinjury was assessed with the alama blue indicator of metabolic activity.

Results showed that a double-freeze procedure with AFP was found to give significantly better ablation than a double-freeze without AFP or a single-freeze with or without AFP.

The mechanism by which AFPs destroy cells and tissue was not yet understood. There is a possibility that AFPs modify the structure of ice crystals. The destruction occurs regardless of the thermal conditions during freezing and appears to be related only to the observed formation of the needlelike ice crystals. Histology suggests that this mode of freezing is associated with severe disruption of the cellular and connective structures, including the nuclei membrane. A possible explanation for the damaging effect of the antifreeze proteins is mechanical. The small needle-like ice crystals propagate through the tissue in the direction of the temperature gradients and may shear the cell and nuclei membrane and the connective tissue as they propagate through the tissue. Formation of the spicules is concentration dependent[32].

Regardless of the mechanism by which antifreeze proteins produce their destruction, the demonstration of the effectiveness of the AFPs in cryosurgery in vivo is important for clinical practice.

#### ***Tumor necrosis factor- $\alpha$ (TNF- $\alpha$ )***

The cytokine TNF- $\alpha$ , while systemically toxic, has shown benefit when locally administered to tumors. This adjuvant is known to promote inflammation, endothelial injury, and apoptosis, in addition to being cytotoxic to cancer cells and generally harmful to tumor microvasculature. Because of the role of TNF- $\alpha$  in cellular (apoptotic and necrotic cell death) as well as vascular mechanisms of injury related to endothelial cell activation and inflammation, TNF- $\alpha$  may enhance cryosurgical lesion in vivo[33-35].

Chao[36] examined the effect of TNF- $\alpha$  on cryosurgery of an in vivo microvascular preparation in a nude mouse. A comparison of injury data to a thermal model indicated that the minimum temperature after moderate cooling, thawing, and hold time required for causing necrosis, shows that the local use of TNF- $\alpha$  can dramatically increase the threshold temperature of cryo-destruction by more than 10°C (Figure 1-8-14).

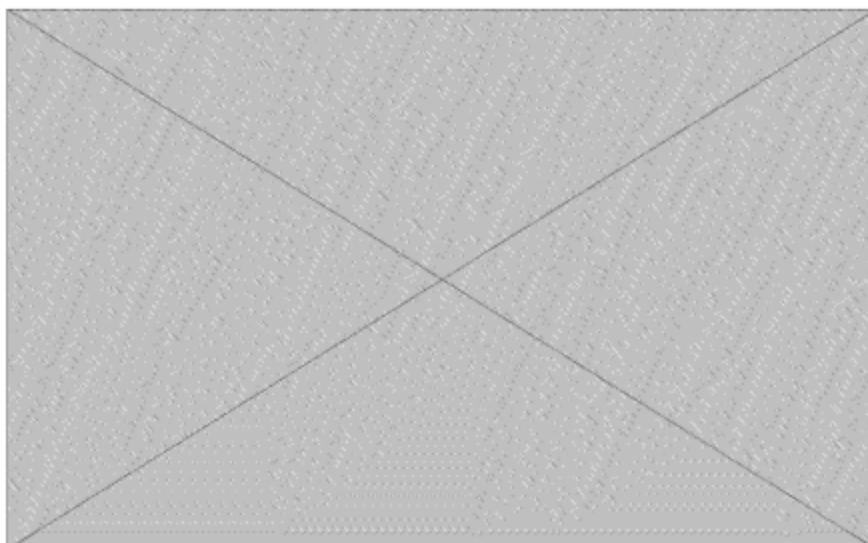


Figure 1-8-14. The minimum temperatures required to cause necrosis in different tissues following moderate cooling, thawing and hold time. "Normal Skin" denotes normal skin tissues from Copenhagen rats ( $n = 6$ ; white column), nude mice ( $n = 9$ ; light gray), or inflamed skin tissues from nude mice after TNF- $\alpha$  treatment ( $n = 4$ ; dark gray). "Tumor Tissues" denotes AT-1 Dunning rat prostate tumor ( $n = 6$ ; white column), LNCaP Pro 5 human prostate cancer ( $n = 8$ ; light gray), or inflamed LNCaP Pro 5 tissues after TNF- $\alpha$  treatment ( $n = 4$ ; dark gray).

A previous results obtained using normal tissue and AT-1 rat prostate tumor in the Copenhagen rat are listed for comparison ( Hoffmann NE, Bischof JC. J Biomech Eng 2001; 123:310–316 ). Error bars are standard deviations  
From Chao BH, et al. Cryobiology 2004;49:10–27

However, neither normal nor tumor tissues showed necrosis after TNF- $\alpha$  treatment without cryosurgery, suggesting local application of TNF- $\alpha$  by itself at this dosage would not damage tissue.

There is the hypothesis that vascular-mediated injury is responsible for defining the edge of the cryolesion in microvascular-perfused tissue, and therefore the inflammation induced by local use of TNF- $\alpha$  augments cryoinjury.

The effect may impact the monitoring of clinical cryosurgery. During cryosurgery of the prostate and other organs such as liver, kidney or brain, ultrasound, CT or MRI can be used to monitor the extent of the cryosurgical iceball. However, this is not optimal with cryosurgery on some special sites such as prostate, since overfreezing into sensitive adjacent structures can cause complications.

On the other hand, if the surgeon underfreezes by keeping the iceball solely within the tumor, cancer existing at the periphery of the cryolesion may not be effectively treated, which may lead to recurrence of disease. Local application of TNF- $\alpha$  to targeted tissue in vivo would decrease its cryoinjury threshold, or increase its thermal threshold, to  $3.5 \pm 6.9^\circ\text{C}$  under moderate freeze/thaw conditions, which are close to the temperature at the edge of the iceball. Therefore, the combination of cryosurgery and local inflammation induced by TNF- $\alpha$  may improve the clinical application of cryosurgery, specifically in the prostate but also in other organ systems, by increasing the ability of ultrasound and other iceball-monitoring technologies to monitor and predict injury, subsequently reducing potential side effects from cryotherapy[37].

#### CONCLUSION

Main causes of tumor persistence or recurrence after cryosurgery at the site of cryoablation are incomplete destruction due to inaccurate procedural monitoring and inadequate criteria for treatment adequacy. The optimization of cryosurgery should consider crucial factors such as the time of freezing, freeze-thaw cycles, number of probes, probes' sizes, the spatial position of the probes, and shape and size of the tumor. The selective occlusion of vascular inflow and addition of molecular adjuvants, such as TNF- $\alpha$ , can dramatically increase the threshold temperature of cryo-destruction.

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