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EVALUATION OF POST CRYOSURGERY INJURY IN A SHEEP BREAST MODEL USING THE VITAL STAIN 2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE

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Summary: Pilot experimental cryosurgery on a recently pregnant sheep breast model is presented. Three tissue fixation protocols have been tested to study the feasibility of preserving the entire breast for both macroscopic and microscopic examination. Viability testing using the 2,3,5-triphenyltetrazolium chloride (TTC) histochemical technique has further been applied. A comparison is presented of the frozen region diameter, measured *in situ* via ultrasound images, with the cryoinjured region diameter, measured during histological examination.

Keywords: Cryosurgery, Breast, Minimally Invasive, 2,3,5-Triphenyltetrazolium Chloride, Animal Model

Introduction

Cryosurgery has been used successfully for more than three decades and is well established in a variety of treatments for benign and malignant neoplasms (1,8,12,13,17). *In situ* breast cryosurgery has recently proved to be feasible and efficacious in small and large animal studies and has been successfully performed in 1 patient with breast cancer (14). The current study arises from efforts to establish a technique to evaluate the extent of injury associated with cryotreatment and especially in the case of breast tissues. The proposed evaluation technique is based on the application of 2,3,5-triphenyltetrazolium chloride (TTC), an oxidation - reduction indicator that has been used effectively for histochemical analysis of infarct volume in ischemically injured tissues (4,6). TTC, as a water soluble salt is not a dye but is reduced by certain mitochondrial respiratory enzymes in normal tissue to a deep red, fat soluble, light sensitive compound (formazan) that turns normal tissue brick red and thereby delineates abnormal areas (5). TTC has been used extensively to stain tissues from human and experimental animals and has been shown to reflect accurately the extent of irreversible ischemic damage (4,6).

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Materials and Methods

A new minimally invasive, liquid nitrogen-based, cryosurgical device has been applied in this study, which is described elsewhere (11). The *in vivo* experiments were performed in the Surgical Oncology Laboratory at the Allegheny University of the Health Sciences. The cryoprocedures were carried out in accordance with the guidelines and standards of the United States Public Health Services for Use and Care of Laboratory Animals and with the approval of the Institutional Animal Care and Use Committee of the Allegheny University of the Health Sciences.

Six pilot experiments were performed on 3 recently pregnant sheep under general anesthesia, one in each breast. All animals were about 3 years old, between 5 to 8 weeks post lambing, and having body weight in the range of 49 and 77 kg, as listed in Table 1. Each cryoprocedure was performed using a single cryoprobe, having an oval-shape cross-section of 1.6 × 3.2 mm and a sharp pointed tip, which was inserted through a small incision of about 3 to 5 mm in the skin. This minimally invasive cryoprobe can easily create its own track in the breast tissues, once the skin has been penetrated. The cryoprobes were placed deep in the breast within a region of dense breast tissue, as far as possible from large breast ducts. The identification of the dense breast fibrous region, the insertion of the cryoprobe, and the formation of the frozen region were all monitored and recorded via Doppler ultrasound imaging device (7 MHz linear array transducer). The ultrasound images were routinely recorded on video tapes for further analyses of the frozen region shape and diameter. Each cryoprotocol included cooling at maximal achievable rate by the new device of about 200°C/min, down to the liquid nitrogen boiling temperature (-196°C), followed by natural thawing with no external assistance (10 to 15 minutes). The animals were sacrificed about 15 minutes after completion of thawing by a standard protocol.

Three different protocols of preparing the breast tissue for histological examination were tested, in order to determine the most practical method to examine the early pathologic effects of the cryotreatment. In the first protocol, the whole specimens were immersed in 10% natural buffered formaldehyde for 48 h. The breasts were then bisected along the cryoneedle track and each half was immersed again in formaldehyde for a few weeks.

In the second protocol, the breasts were bisected prior to fixation. The bisected specimens were subdivided into three parts in parallel as possible to the cryoneedle track. Parts 1 and 2 were separated from each other by dividing along the cryoneedle track. Part 3 represented essentially normal tissue. Each of the parts was immersed in 10% natural buffered formaldehyde for a few weeks.

Perfusion of formaldehyde *in situ* was applied in the third protocol. All the major veins leading from the breasts were exposed and ligated about 15 min after thawing. The two major arteries leading to the breasts were exposed, cannulated and connected to an Harvard syringe pump via a T connector. The breasts were perfused with 150 *ml* of 2,3,5-triphenyltetrazolium chloride (TTC), 2% in phosphate-buffered saline, at a rate of 2.7 *ml*/min, followed by 50 ml of 10% natural buffered formaldehyde at a rate of 3.3 *ml*/min. The animals were maintained at 37°C throughout the procedure for optimal histochemical enzyme reduction in the tissue. The breasts were immediately excised and immersed in the same formaldehyde solution for 24 h. The specimens were then bisected along the cryoneedle track, the color of the cut surface was observed, and the specimens returned to the formaldehyde solution for a few weeks.

Blocks of tissue, from the entire cryoteated region, were submitted for standard histological examination by light microscopy using hematoxylin & eosin stains (H&E). The cryotreated area was identified by decoloration of the tissue in the first two protocols and by staining in the third one.

Results

The specimens of the first fixation protocol (immersion of the whole specimen in fixative) appeared relatively rigid and non-deformed after the first 48 h, and it was easy to trace the cryoneedle track and to cut the partly fixed specimens. The diffusion velocity of the formaldehyde in breast tissues is very slow and therefore only the most outer layer, of about 10 to 15 mm, was well fixed after 48 h; while some signs of early tissue degeneration were observed at the center of one of the specimens. No degeneration was observed in the cryotreated area or nearby. In the second fixation protocol (bisecting the specimens prior to immersion in the fixative), it was very difficult to subdivide the specimen in parallel to the cryoneedle track since the unfixed breast tissue is very elastic and very difficult to cut. After fixation the specimens parts were deformed and it was difficult to trace their original orientation. In the third fixation protocol (perfusion *in situ*), although the breasts were not completely fixed after the first 24 h, they were rigid enough to allow further bisection and tracing of the cryoneedle track. The specimens were not deformed after bisecting and reimmersing in the formaldehyde solution. Furthermore, no degeneration of tissue were observed after 24 h.

The average frozen region diameter at the end of operation for the various cases are listed in Table 1, which was estimated from the recorded ultrasound images.

Microscopic findings in all cases showed hemorrhage in the track of the cryoneedle and, in some case, ducts penetrated by the cryoneedle. Stromal edema in the adjacent connective tissue and vascular congestion with red blood cells were seen within a macroscopic brown discolored area. A few thrombi were observed in small blood vessels. The surface of the epidermis of the cryotreated area was sloughed. Numerous widely dilated ducts were observed in most breast specimens.

The skin showed a brown circular discoloration centered around the cryoprobe. Microscopic examination showed intact skin. Vascular congestion with red blood cells and variable amounts of edema in the dermis of the skin, subcutaneous tissue, and breast tissue, were observed. Compared with the amount of edema in the breast tissue, more edema was observed in the subcutaneous connective tissue.

Table 1: Summary of experimental data.

Animal / Case	Frozen Region Diameter via Ultrasound Image, mm	Cryoinjured Region Diameter × Length via Histological Preparation, mm	Body Weight, kg	Time Between Lambing and Cryosurgery, Weeks
1 / 1	26.5	30 × 70	56	5
1 / 2	38.3	40 × 50		
2 / 1	24.7	25 × 60	77	5
2 / 2	29.0	30 × 55		
3 / 1	24.3	30 × 42*	49	8
3 / 2	21.7	27 × 48*		

* Using 2,3,5-triphenyltetrazolium chloride staining.

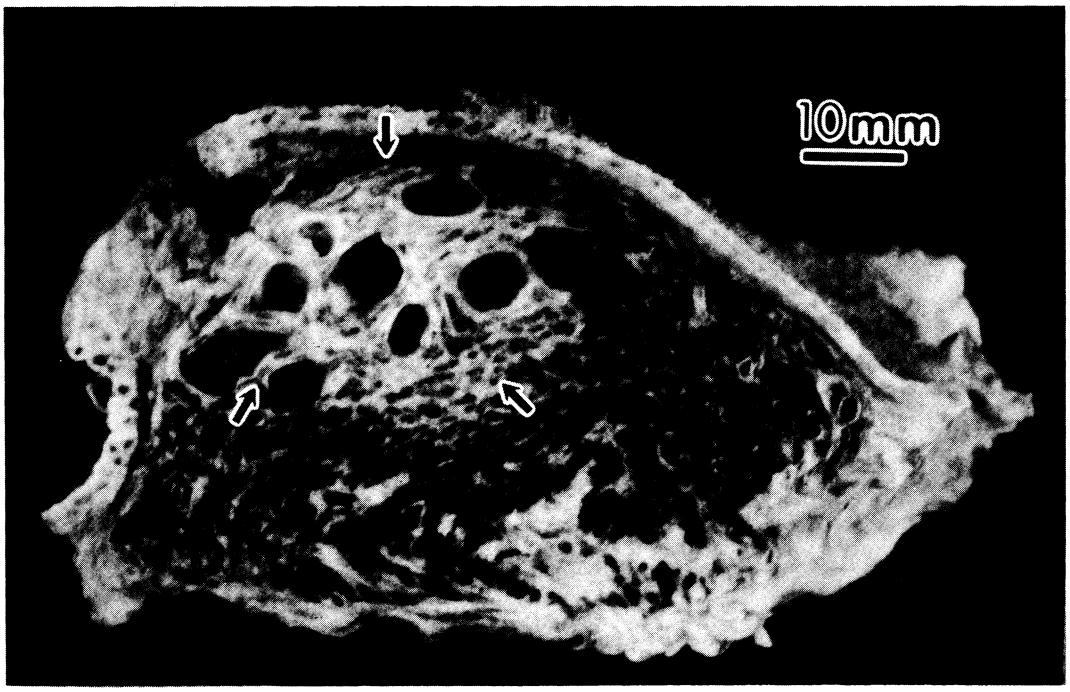

Figure 1: Macro cross-section view of a cryotreated breast specimen, which was perfused by 2,3,5-triphenyltetrazolium chloride (TTC) stain and 10% buffered formaldehyde. The cryotreated region appears pink (pointed by arrows), while the surrounding healthy tissues have a dark-red color due to the staining of viable cells by the TTC. Note the large duct of the recently lactated ducts (8 weeks post lambing). Bar length is 10 mm (top right).

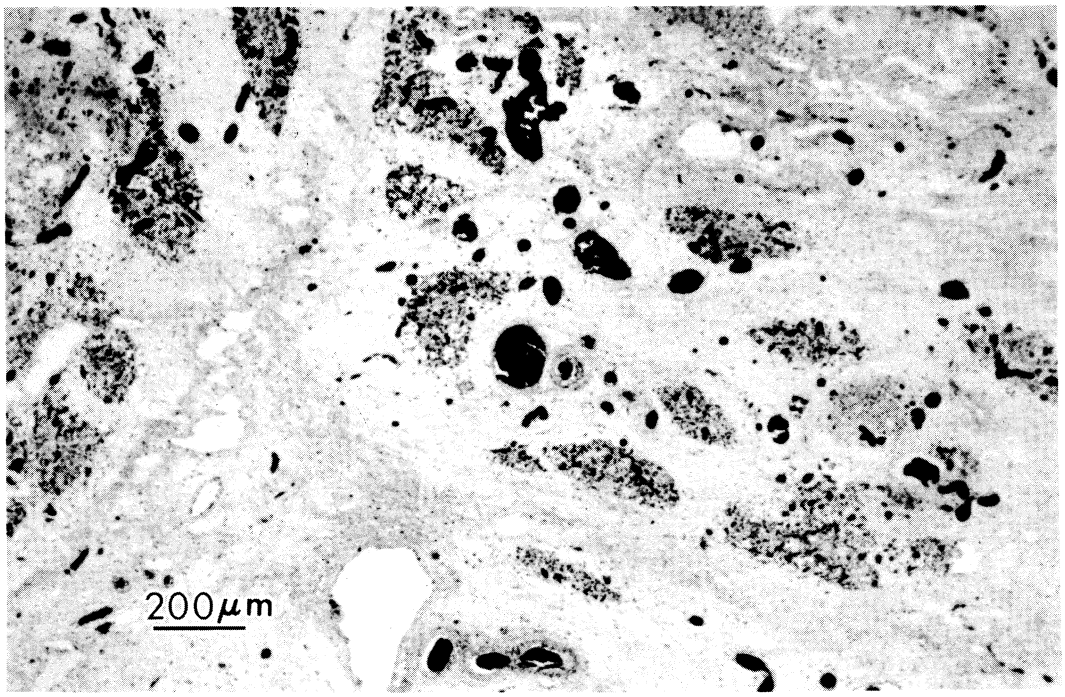

Figure 2: Low power magnification of the cryotreated tissue shows vascular congestion with blood and thrombosis.

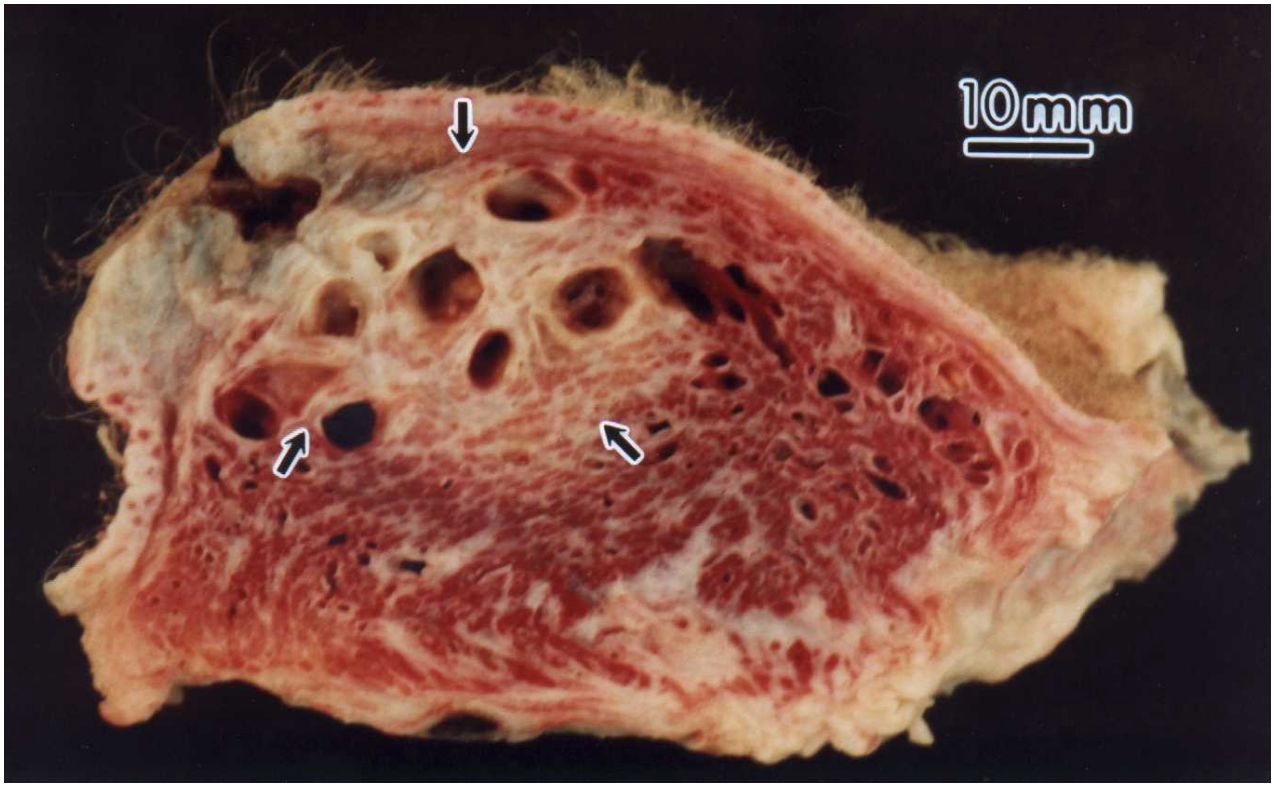

Figure 1: Macro cross-section view of a cryotreated breast specimen, which was perfused by 2,3,5-triphenyltetrazolium chloride (TTC) stain and 10% buffered formaldehyde. The cryotreated region appears white (pointed by arrows), while the surrounding healthy tissues have a dark-red color due to the staining of viable cells by the TTC. Note the large duct of the recently lactated ducts (8 weeks post lambing). Bar length is 10 mm (top right).

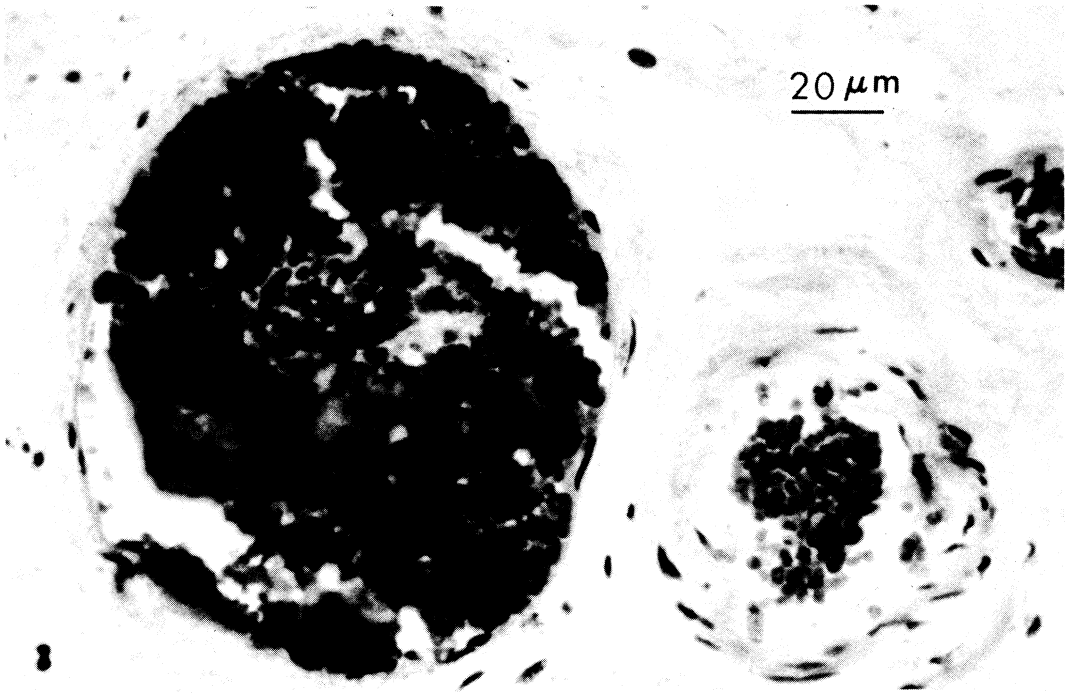

Figure 3: High power magnification shows a vein (left) and an artery (right) congested with blood and thrombosis from the center area of the cryotreated region. The blood vessels appear intact and viable 15 min after thawing.

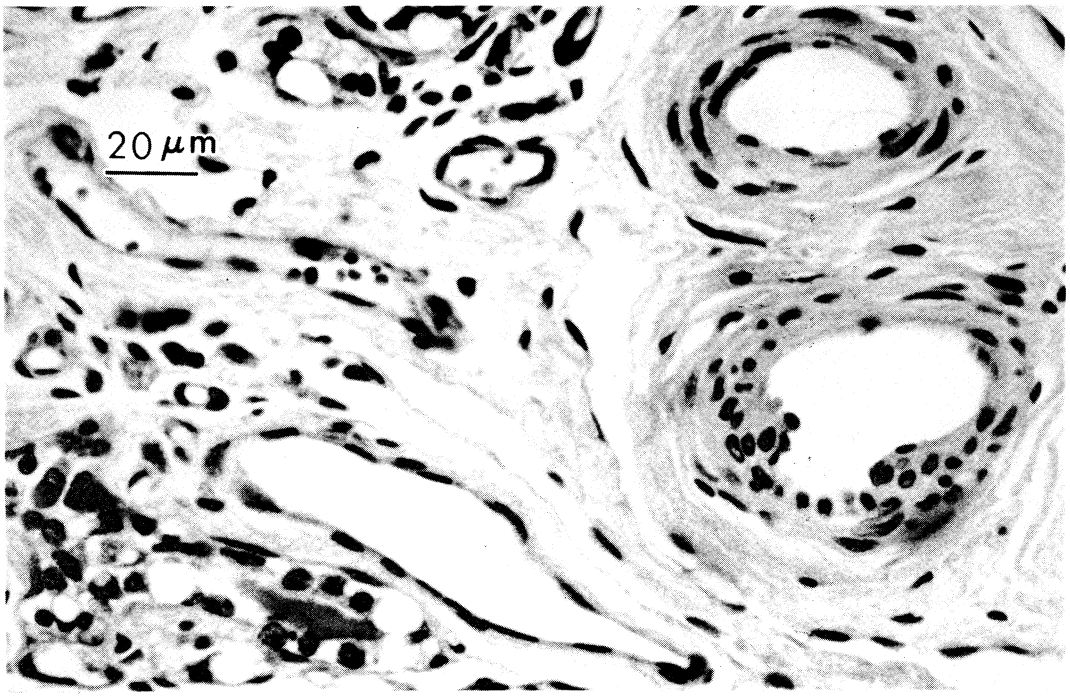

Figure 4: High power magnification of blood vessels (right) from a control tissue which has not been cryotreated.

In the third protocol, in which the TTC was applied, the cut surface showed a pink area around the track while the rest of the specimen appeared dark red, upon bisecting the specimens along the cryoneedle track 24 h after perfusion. The dark red coloration is due to the TTC which stains viable cells (4,6). The cut surface after 3 weeks of fixation became dull and darker compared to the fresh cut surface, and the pink area had turned brown. Macro cross-section view of one of those cases is shown in Fig. 1, where the pink area is pointed by arrows. Vascular congestion with blood and thrombosis in the cryotreated region of the same specimen are shown in Figs. 2 and 3. The blood vessels appear intact 15 min after thawing (Fig. 3). For comparison, Fig. 4 presents control tissue from the same specimen, from an area which had not been cryotreated.

Discussion

Perfusion of formaldehyde *in situ* appeared to produce the best results among the three fixation protocols presented above: the specimen did not deform, no degeneration was observed, it was easy to trace the cryoneedle track, and it was easy to measure the cryotreated region. However, we consider that it may be possible to improve the technique by perfusing a larger quantity of formaldehyde at the same perfusion rate.

Perfusion of a vital stain for viability testing *in situ* is an important technique in order to correlate the temperature field with cellular destruction around the cryoneedle. This technique enables evaluation the extent of destruction in different areas of the ice-ball and avoids the difficulty in tracing the location and orientation of the tissue blocks which are processed for routine histological examination, as is the case in the ordinary procedure of stain diffusion in small tissue blocks. The TTC technique revealed less viable cells in the pink area which indicates the main cryodestructed region (Fig. 1). This means that immediately after thawing some cells appear to be viable in the main cryotreated region. The pink color immediately after thawing is more likely due to the congestion of red blood cells in the area, because this area turned brown as blood does with fixation, while the unfrozen region remained red. When using TTC for viability testing, it is assumed that TTC stains viable cells on the basis of the presence of respiratory enzymes; however, unstained tissue does not necessarily mean all cells are permanently damaged. For example, a study of brain infarction observed intact mitochondria in 5% of cells after 4 h of ischemia (7).

Table 1 lists the frozen region diameter, as measured at the end of the cryoprocedure via ultrasound imaging, and the cryodestructed region diameter, as measured during the histological examination. It was observed that the ultrasound imaging resulted in an under estimation of the cryotreated region in the range of 0.3 to 5.3 mm, in the breast tissue. To put this in perspective, it is emphasized that the accuracy in measurements via enlarged ultrasound images is at least 5 times higher than that using a simple ruler, as was done in the histological study. Furthermore, the frozen region edge appears much sharper via ultrasound image than the edge of the cryotreated region, as it appeared during the histological examination. One should bear in mind that this difference is affected by many factors such as the physical principles of ultrasound imaging, the biological response to low temperatures during thawing, and the tissue volume changes in the fixation process. For example, vasodilatation and congestion with blood are normal mammalian responses to tissue injury, in the injured region and in the periphery around the injured region.

An estimation of tissue destruction is an important component of studies for characterization and development of cryosurgical procedures. The TTC vital staining technique has been widely employed as a convenient, inexpensive and reliable method of detecting and quantifying the size of infarcted areas in ischemic tissues and hence justifies

consideration for application in studies of cryoablation and cryosurgery. This study confirmed that the regions of tissue destruction demarcated by TTC staining correlated well with estimates derived using conventional histological staining with H&E, thus corroborating studies in other tissues such as heart and brain (4,11). Moreover, the preliminary application of this technique to the freezing of breast tissue suggests that the area of tissue destruction, as judged by the criteria of both the absence of TTC staining and H&E histology, was larger than the measured zone of freezing depicted by ultrasonic imaging. Vascular stasis as a widely recognized component of tissue injury after freezing, such as frostbite, may contribute to a larger area of infarction when compared with the estimated size of the frozen region detected by ultrasound. It is also feasible that vasoactive agents released from damaged cells, or cell debris from the frozen region could influence the vascular bed of the tissue and give rise to vascular stasis and secondary hypoxia in the penumbra zone of the frozen tissue. In frostbite, indirect cellular damage secondary to progressive microvascular insult is considered more severe than the direct cellular effect. There is good evidence to suggest that hypoxia is the fundamental cause of damage to frostbitten tissues (16). Tissue changes, even after prolonged freezing, are not manifest unless the tissue is thawed (3). Upon thawing the previously damaged endothelial-lined capillaries, now dilated, leak fluid and protein into the interstitial space (10). As reperfusion continues, edema formation in the tissue worsens. Oxygen free radical generation results in continued endothelial damage exacerbating blood cell aggregation and thrombosis leading to microcirculatory failure. The interruption of microvascular integrity appears to be a significant mechanism for tissue loss in cold-induced injury (2). Continued tissue injury may follow reperfusion as a result of prostaglandin-induced vasoconstriction, leukocyte sticking and platelet aggregation.

This preliminary evaluation of the application of the TTC staining technique in experimental cryosurgery corroborates the findings of others showing that this vital stain offers a useful indicator of ischemic injury, and in this case tissue destruction after cryoablation. As with most methods of viability assessment this index is open to interpretation in terms of absolute cell death, or survival (9,15). For example, the failure of cells to stain red with TTC could be for a variety of reasons: The basic interpretation is that cells that are unstained do not contain the normal complement of mitochondrial respiratory enzymes to reduce the stain to the deep red formazan compound. The assumption is that cells deficient by these criteria are unlikely to survive by many other criteria of cell viability. An alternative explanation for non-staining is from compromised accessibility of the dye to all regions of the tissue following freezing. Certainly, vascular stasis would restrict or inhibit delivery of the stain during perfusion, but in the context of assessing cryoinjury non-perfused tissue would succumb to secondary injury and the component cells would die anyway such that the technique would still reflect tissue viability in an indirect way. As indicated above, this is a preliminary evaluation of the TTC technique without attempt to optimize the conditions to provide the most accurate assessment of cell death in relation to cryoablation. Factors such as perfusion conditions and fixation times are known to influence the precise degree of staining and will require further evaluated in additional studies. Nevertheless, it is demonstrated here that TTC staining provides a convenient vital assay for assessing the size of destructive zones in cryoablated breast tissue.

Animals up to 8 weeks after lambing were selected for this study since recently pregnant sheep have breasts which are similar in size and structure to those of humans. However, the dilated duct system of sheep that have lactated are not as homogeneous as desired. We propose that the sheep breast model can be used to simulate the cryoprocedure in the human's breast, however, a longer waiting period after lambing would be

advantageous. A follow-up study is now being conducted to indicate the long-term effect of breast cryotreatment, which includes regeneration and recovery of the breast tissues.

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