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Long-Term Follow-Up Post-Cryosurgery in a Sheep Breast Model[1]

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This study constitutes the advanced stage of an ongoing project for the development of cryosurgical devices and techniques for breast cryosurgery. The current study focuses on the long-term follow-up post-cryosurgery in a sheep breast model. Results of this study indicate that the cryotreatment site in a sheep breast model cannot be identified up to 5 months post-cryosurgery by means of ultrasound, mammography, or MRI. Histology findings of this study further indicate that there is no gross or microscopic difference between lesions that have been subject to one versus three freeze/thaw cycles. Under either cryosurgical protocol, there is a main cryoinjured region that has uniform destruction of epithelium and healing scar formation and a transition zone of damaged lobules without acini, surrounded by healthy tissues. The cryoinjured region at 5 months post-cryosurgery was found to be about half the diameter of the ultrasound-imaged frozen region during the cryoprocedure. This study shows that, in terms of recovery and regeneration, surgical excision appears to have an advantage over cryosurgery, which results in a more rapid healing process. Based on observations that the cryoinjured region is no smaller than the ultrasound-imaged ice-ball and that the typical thickness of the transition zone is up to 5 mm, a conservative use of the cryosurgical device developed for the current study in an ultrasound-monitored cryoprocedure requires at least 5 mm safety margins of the frozen region radius around the target region.

Key Words: cryosurgery; breast; animal model; multicycle; MRI; mammography; ultrasound.

The treatment of breast cancer has evolved from the time of mutilation and ignorance in the middle ages to one of breast-conserving management and an intense study and understanding of the biological mechanisms driving tumor cells. As the treatment is directed to the cellular and subcellular level, breast-conserving surgical procedures take on a more important role. Recent published results from neoadjuvant trials (6) indicate a decrease in tumor size in 80% of patients and a modest increase in conversion from mastectomy to lumpectomy. By 2010 AD, it is estimated that 50% of all new breast cancers discovered will be less than 10 mm in diameter (4), which represents 90,000 patients. Standard surgical treatment would require an open segment resection, an operating room, anesthesia, cosmetic concerns, and substantial cost. Add to this the number of patients who require segmental resection following complete clinical or pathological response following neoadjuvant chemotherapy and the cost increases.

An alternative method of tumor removal or destruction for small malignancies is needed to complete the biological assault on breast cancer. Cryosurgery may be one of these alternative means. Cryosurgery has been used successfully for more than three decades to treat benign and malignant neoplasms (1, 14, 19, 23). To date there is one reported case of primary breast cancer treatment with cryotherapy (20), which was followed up with ultrasound-guided biopsy and found negative for malignancy 12 weeks post-cryosurgery. Cryotherapy carries many benefits in addition to the attractive concept of

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minimally invasive surgery. Low temperatures generate anesthetic effect. Hemorrhage is reduced due to thrombosis of small blood vessels. Cryotherapy may cause stimulation of the body's immune system, which additionally augments local tumor destruction and may also induce a response in metastatic tumor sites (1, 2, 19, 21, 22).

With multiple treatments such as neoadjuvant therapy, hormone therapy, and radiation, which have the ability to downsize primary cancers and treat small cancers, the use of lumpectomy can increase. Current diagnostic imaging trends are increasingly detecting small cancers ($\leq$1 cm). The minimization of surgical intervention to compliment these trends is a natural progression of technology and understanding of the biological processes involved.

The current study is a part of ongoing research dealing with development of cryosurgical devices and techniques. This study focuses on the long-term follow-up post-cryosurgery in a sheep breast model in an effort to: (a) study the recovery and regeneration processes of breast tissues post-cryoinjury and (b) determine whether the regenerated tissue (the scar tissue) appears like a potential tumor in the long-term, using standard imaging techniques such as ultrasound, mammography, or MRI. All cryotreatments were performed in healthy breast tissues and not on a tumor model, where the underlying assumption is that the recovery and regeneration processes following cryoinjury are not dependent on the preexistence of a breast tumor. Based on the reported results of 100% tumor kill with cryosurgery of breast cancer in small animals (20), it is further assumed that cryodestruction of breast tumors is feasible. The authors are not aware of any tumor model for sheep breast nor of any other tumor model of large animals which are similar in size and structure to the human breast and, therefore, the cryotreatment of healthy breast tissue is a choice of practice. The animal model must be similar in size to the human breast because: (i) there needs to be large enough volume of untreated tissue surrounding the cryoinjured site because the uninjured tissue surrounding the cryotreated site is expected to take a role in the recovery process and (ii) large size is needed to make imaging practical.

Since the initial clinical reports on cryosurgery in 1965, the need for repetitive freeze/thaw cycles is stressed (8). A multi-cycle procedure is expected to enhance cryoinjury by repeatedly exposing the cells to the deleterious physicochemical changes of damaging thermal conditions. Experimental evidence of the increased destructive effect of repeated freeze/thaw cycles is substantial. An up-to-date overview is given by Gage and Baust (8). However, the enhanced lethal effect of repeated freeze/thaw cycles is best seen at the phase transition temperature range and even in somewhat lower temperatures, i.e., in the -30 to 0°C range. Using modern cryoprobes, this temperature range exists only near the edge of the frozen region. Temperatures below this range are widely expected to be sufficient to cause maximal cryodestruction in a single cycle, hence the benefit in repeated cycles is obscured by the severity of the low-temperature injury (7, 9). In the treatment of some malignancies, it is commonly accepted that a procedure consisting of two freeze/thaw cycles has a better outcome than that of a single-cycle procedure, as in the case of prostate malignancies. It is also accepted that the benefit in more than three consecutive freeze/thaw cycles is marginal, if at all. It is noted that, in addition to the effect of multiple freezing, the extent of cryoinjury is known to be influenced by other factors such as the cooling and warming rates, the minimal temperature achieved, etc.

The current study provides a comparison between treatment with a single freeze/thaw cycle, three cycles, and surgical excision. The extent of cryoinjury was evaluated in the short-term post-cryosurgery with the application of the vital stain 2,3,5-triphenyltetrazolium chloride (TTC) (17). The recovery and reprocess of the cryotreated region was evaluated using routine histology with hematolylin and eosin stain (H&E) and with Masson's trichrome stain (13).

MATERIALS AND METHODS

The *in vivo* experiments were performed in the Human 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.

A new minimally invasive, liquid nitrogen-based, cryosurgical device, which is described in detail elsewhere by Rabin *et al.* (16), has been applied in this study. All cryoprocedures were performed using a single cryoprobe constructed of two adjacent tubes having a diameter of 1.15 mm each. The active surface length of the cryoprobe varied within the range of 20 to 30 mm.

The cryoprotocol included maximal cooling power at the cryoprobe active surface during the freezing stage and subsequent natural thawing with no assistance of external heating. Maximal cooling power of this cryoprobe correlates with liquid nitrogen supply at 30 psi, which yields the following typical variation of temperature at the cryoprobe outer surface: 37°C at the initiation of the procedure, −55°C after 15 s, −82.5°C after 30 s, −107.5°C after 45 s, −116.5°C after 90 s, and −140°C in steady state. This cryoprobe generates an average frozen region diameter of 22.3 mm within 5 min of operation in sheep breast tissues ($n = 21$), which was the cooling period in most cases of this study. The following natural thawing lasted about 10 min. It follows that a complete single freeze/thaw cycle lasts about 15 min and a three-cycle procedure lasts about 45 min.

The animal model for this study is a recently pregnant sheep, 8 to 12 weeks post-lambing and at least 4 weeks post-lactation. All animals were about 5 years old, after five deliveries (once a year), having body weight as listed in Table 1; a total of 20 animals in six groups were studied. The first group included 2 animals which were sacrificed immediately after a single-cycle cryoprocedure in each of the two breasts. These experiments were performed to test the cryoprobes, to provide a baseline for the long-term follow-up, and to improve the application of the TTC perfusion and *in situ* fixation as described below. The second group included 3 animals which were followed-up for 7 days post-cryosurgery. Each animal was cryotreated with a single-cycle cryoprocedure in one breast, while the other breast was taken as a control.

Animals of the third, fourth, fifth, and sixth groups were followed-up for 1, 2, 3.5, and 5 months, respectively. A single-cycle procedure was applied to one of the breasts of each animal, which sums up to three animals in the 1-month follow-up group and four animals in each of the consecutive follow-up groups. About one-half of the animals of the 1- to 5-month follow-up groups were used to compare a single-cycle cryoprocedure in one breast with a three-cycle cryoprocedure in the other breast, which included one animal in each of the 1- and 2-month follow-up group, and two animals in each of the 3.5- and 5-month follow-up groups. The rest of the animals were used to compare the scar tissue developed as a result of a surgical excision with that developed after a single-cycle cryoprocedure, which included one animal in the 3.5-month follow-up group, and two animals in each of the 3.5- and 5-month follow-up groups. All in all, given the limited number of available animals and given the possible complications in animal maintenance for long periods post-operation, an increasing number of animals was assigned with the increase in follow-up period. The control specimens were found not necessary in the advanced stage of this study, which left more animals for comparative tests. The above animal plan reflects high priority for single-cycle procedures and long-term follow-up and also somewhat higher priority for the comparison with three-cycle cryoprocedure over the comparison with a single-cycle cryoprocedure.

The surgical excision procedure included incision in the skin to a length of 15 to 20 mm, followed by excision of tissue with an average diameter of 10 to 15 mm. One skin suture was applied to close the incision created for the

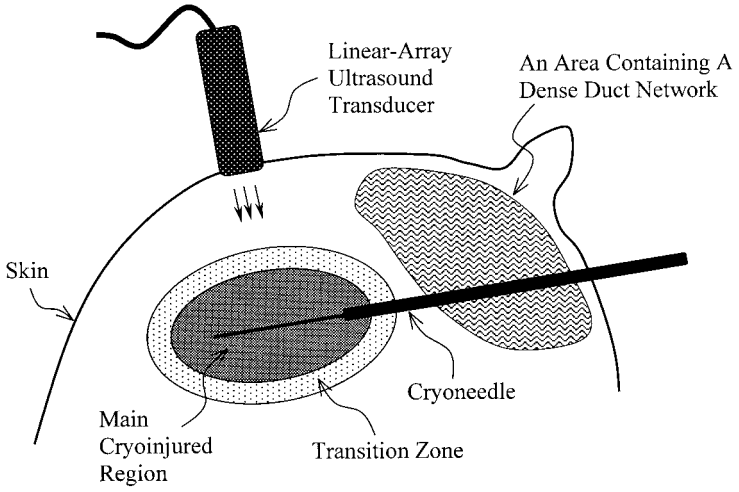


FIG. 1. Schematic illustration of the ultrasound-monitored cryosurgery, identification of the area containing dense breast ducts network, and identification of the different cryoinjured areas for Figs. 2–5.

cryoprobe, and about four sutures were applied for the surgical excision. The animals received antibiotics, 500 mg Keflex B.I.D., for 7 days post-operatively as a preventative measure and 50 mg Banamine S.I.D. immediately after operation.

All cryoprocedures were performed in areas of dense breast tissue fibers and as far as possible from the dilated breast ducts; the location of the dense breast ducts network is schematically shown in Fig. 1. Identification of the different areas of the breast and monitoring the ice-ball formation were performed using a Doppler ultrasound (7-MHz linear-array transducer). The ultrasound transducer was placed perpendicular to the cryoneedle center line and above the middle of its cylindrical active surface for measurements of the frozen diameter (Fig. 1). This is the smallest dimension of the egg-shaped frozen region. The ultrasound images were video tape recorded for further analysis and measurements. Ultrasound was also applied as a routine when the animals were sacrificed, in an effort to identify the site of cryotreatment.

MRI and mammography were applied about every 4 weeks in an effort to identify the sites of cryotreatment. The MRI was performed routinely on animals from the 5-month follow-up group under general anesthesia, for which the animals were laying on their back, in a Seimens MRI unit with 1.5 Tesla intensity. Mammography was performed on a larger number of animals from all follow-up groups, with 26 kV and between 56 and 63 MAS in a Seimens mammography unit. In some cases, the mammography was performed *in vivo* under general anesthesia, for which the animals were laying on their side. In other cases, the mammography was performed on the breast specimens immediately after harvesting.

Breast specimens were prepared for histological analysis by perfusion of the vital stain 2,3,5-triphenyltetrazolium chloride followed by perfusion of formaldehyde *in situ*, as described elsewhere (17) but with different perfusion rates. In brief, all the major veins leading from the breasts were exposed and ligated. The two major arteries leading to the breasts were exposed, cannulated, and connected to a controlled flow rate pump via a T connector. The breasts were perfused with 250 *ml* of TTC, 2% in phosphate-buffered saline, at a rate of 18 *ml*/min, followed by 100 *ml* of 37.2% natural-buffered formaldehyde at a rate of 18 *ml*/min. The TTC and the animal's breasts were maintained at 37°C throughout the procedure for optimal histochemical enzyme reduction in the

tissue using a temperature-controlled thermal blanket. Skin temperature measurements were taken to verify temperature control, using 36-guage hypodermic thermocouples. The breasts were excised immediately after and immersed in 10% natural-buffered formaldehyde solution for up to 24 h. The TTC showed clear demarcation between the cryoinjured region and the healthy surrounding tissues at 1 week post-cryosurgery but somewhat less clear demarcation in the immediately sacrificed group. When comparing the TTC demarcation versus H&E demarcation, the application of TTC became less advantageous with the increase in follow-up period due to the healing process. Hence, TTC was not applied in the 5-month follow-up group (these specimens were perfused with formaldehyde alone).

After harvesting, the specimens were bisected along the cryoneedle track, the color of the cut surface was observed, and the cryoinjured (discolored) region was measured. The cryoinjured region was observed to be dark brown in the absence of TTC, while the surrounding healthy tissues were observed to be light gray/brown. In contrast, the cryoinjured region was observed to be very pale in the presence of TTC, while the surrounding healthy tissues were observed to be dark-red. The penetration point of the cryoneedle was identified by a suture, which has been left in place at the end of the cryoprocedure and until harvesting. The initial bisection plan was made as parallel as possible to the original cryoneedle track. Immediately after, a series of slices were cut, on both sides of the initial bisection plan, in a thickness of about 4 mm, in order to recast the three-dimensional shape of the cryoinjured region. Using elementary trigonometry, one can find the uncertainty in measuring the maximal diameter from slices of this thickness to be no larger than 1.2, 0.8, and 0.6 mm, for maximal injured diameters of 15, 20, and 25 mm, respectively.

Representative blocks of tissue from the cryotreated region were submitted for standard histological examination by light microscopy using hematoxylin and eosin stains (H&E), which stains both collagen and cell cytoplasm red. Some samples of the later groups were alternatively stained with Masson's trichrome stain, which enhances the distinction of collagen fibers from the cytoplasmic processes of fibroblasts and other cells. Masson's trichrome stain depicts collagen as green, cytoplasm as red, and nuclei as black. The pathologic observations included microscopic assessment of cellular and vascular injury in the immediate post-cryosurgery period, as well as necrosis at 1 week. Glandular epithelial changes were assessed in all periods of observation. Reparative changes related to the development of scar tissue and regeneration of epithelium were assessed at 1 month and later.

RESULTS

Table 1 lists the frozen region diameter as measured from ultrasound images, the cryoinjured diameter as measured during histology, and the freezing period for all cryoprocedures. From Table 1 it can be seen that the average imaged frozen region diameter is about 15 mm after 2 min ($n = 2$), and 22.3 mm after 5 min ($n = 21$). It can further be seen that a 10 to 20% increase in the frozen region diameter takes place in the second cycle ($n = 6$). No significant change in the frozen region diameter was observed between the second and third cycles in most cases ($n = 4$). However, a decrease of 6% (equals 1.6 mm) in the fifth case and an increase of 11% (equals 3.2 mm) in the sixth case were observed in the frozen region diameter between the second and the third cycles.

Imaging

Comparison of the frozen region diameter, as estimated from the ultrasound images, with the maximal diameter of cryoinjury (the discolored area), as measured from the bisected specimens, revealed that the cryoinjured region was no less than and up to 2.5 mm larger than the imaged frozen region in the immediate follow-up groups (Table 1). Similar comparison at 1 week post-cryosurgery reveals smaller injured region in two cases (in about 5 mm) and a significant

TABLE 1
Summary of Animal Data, Procedures, and Follow-up Periods

Follow-up group	Animal No.	Body weight (kg)	Left breast: Frozen diameter as measured from the ultrasound image, mm [Cryoinjured region as measured from histological cross-sections, mm] (Freezing period, min:sec)			Left breast: Surgical excision	Left breast: Control specimen	Right breast: Frozen diameter as measured from the ultrasound image, mm [Cryoinjured region as measured from histological cross-sections, mm] (Freezing period, min:sec)			Right breast: Surgical excision	Right breast: Control specimen
			Cycle I	Cycle II	Cycle III			Cycle I	Cycle II	Cycle III		
Immediate	1	53.6	22.0 [22] (5:23)	—	—	—	—	22.0 [22] (6:00)	—	—	—	—
	2	79.1	23.5 [25] (5:00)	—	—	—	—	25.5 [28] (5:00)	—	—	—	—
1 Week	3	54.0	—	—	—	—	Yes	17.0 [25] (5:00)	—	—	—	—
	4	53.6	23.5 [18] (5:16)	—	—	—	—	—	—	—	—	Yes
	5	58.1	22.0 [17] (5:02)	—	—	—	—	—	—	—	—	Yes
1 Month	6	41.8	—	—	—	—	Yes	22.0 [22] (5:00)	—	—	—	—
	7	49.1	25.0 [21] (4:59)	—	—	—	—	—	—	—	—	Yes
	8	58.1	15.0 [20] (2:03)	—	—	—	—	14.3 (2:02)	16.5 (2:00)	16.5 [15] (2:03)	—	—
2 Months	9	47.7	20.1 [12] (5:00)	—	—	—	—	—	—	—	—	Yes
	10	44.5	24.1 [15] (4:58)	—	—	—	—	—	—	—	—	Yes
	11	50.0	15.0 [12] (1:45)	—	—	—	—	13.1 (1:37)	15.1 (1:36)	15.0 [12] (1:42)	—	—
	12	39.5	—	—	—	Yes	—	19.0 [*] (3:00)	—	—	—	—
3.5 Months	13	70.9	19.3 (5:00)	23.5 (5:00)	23.5 [8] (5:02)	—	—	24.0 [8] (5:00)	—	—	—	—
	14	77.3	22.0 (5:00)	25.2 (5:00)	23.6 [26] (5:06)	—	—	20.0 [20] (5:00)	—	—	—	—
	15	79.0	20.0 [*] (5:03)	—	—	—	—	—	—	—	Yes	—
	16	40.9	—	—	—	Yes	—	23.1 [13] (5:01)	—	—	—	—
5 Months	17	52.2	22.0 (5:00)	24.0 (5:02)	24.0 [12] (5:05)	—	—	21.1 [14] (5:00)	—	—	—	—
	18	72.2	24.0 [15] (5:01)	—	—	—	—	26.0 (5:00)	28.3 (5:02)	31.5 [17] (5:00)	—	—
	19	59.0	—	—	—	Yes	—	18.6 [10] (5:00)	—	—	—	—
	20	77.3	25.2 [15] (5:02)	—	—	—	—	—	—	—	Yes	—

Note. The frozen region diameter was measured from video-recorded ultrasound images during cryosurgery. The cryoinjured region was measured from histological cross-sections and is shown in square brackets "[]". These values represent the maximal diameter at the center of the cryoprobe active surface, perpendicular to the cryoprobe centerline. Surgical excision was performed by extracting a tissue sample having an average diameter of 15 to 20 mm. MRI monitoring was performed on the 5-month follow-up group every 4 weeks. Mammography imaging was performed on a few animals from each follow-up group before sacrificing.

* Not well defined.

larger injured region in one case (8 mm), compared with the imaged frozen region.

Starting at 1 month post-cryosurgery, continuous reduction in the cryoinjured region size with respect to the imaged frozen region during cryosurgery can be observed but at different rates. One exception is animal No. 8, in which 5 mm larger injured region is found at 1 month post-cryosurgery but in one breast only. Another exception is observed in both breasts of animal No. 14, in which the injured region at 3.5 months post-cryosurgery is found to be essentially the same diameter as that of the imaged frozen region during cryosurgery. For the purpose of this comparison in the left breast of animal No. 14, one should take into account cycle II, in which maximal frozen region diameter was achieved. Due to the consistency of similar observations for both breasts of animal No. 14, one may conclude that this exception is due to an individual variation. Furthermore, the different rates in reduction of the cryoinjured area are probably due to variation between individuals of the same species. On average, injured regions in the range of 50 to 60% are found at 5 months post-cryosurgery, compared with the imaged frozen region during cryosurgery.

The authors were not able to identify the cryotreatment site using either mammography or MRI, where the first mammogram was taken 3 days post-cryotreatment and where the first MRI image was taken about 2 weeks post-cryotreatment. Attempts to identify the cryotreatment site were repeated every 4 weeks up to 5 months. Mammography *in vivo* in some animals and mammography of the excised breasts in other animals appeared to produce similar results. Mammography of excised breasts was performed to simplify the imaging procedure and to overcome technical difficulties in cases of large animals having small breasts.

Ultrasound was very useful in monitoring the ice-ball formation during the cryoprocedure and the site of cryotreatment in the short-term post-cryosurgery. This was verified in the short-term follow-up groups by ultrasound-guided insertion of an hypodermic needle to the center of the cryolesion, *in vivo,* prior to the bisection of the breast specimens. Ultrasound identification technique of the cryotreatment site became unreliable in follow-up periods longer than 1 month.

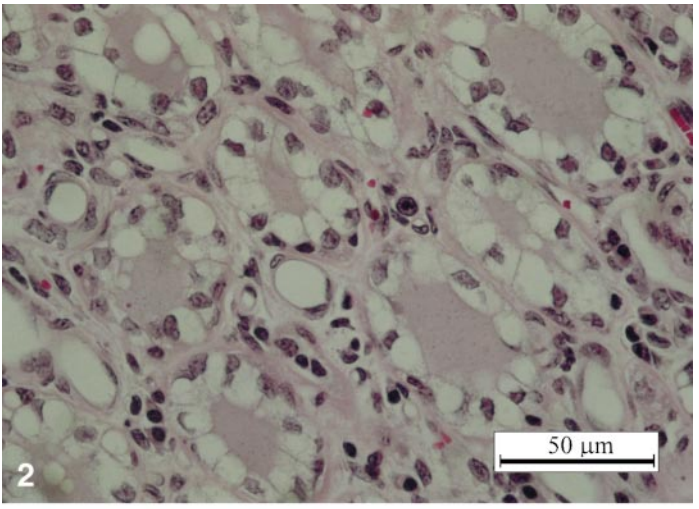
Histology and Histochemistry

Three main areas were identified in the micro-scale, as is also illustrated schematically in Fig. 1: a main cryoinjured region, surrounding healthy tissues, and a transition zone between the main cryoinjured region and the healthy tissues, having a typical thickness of 2 to 3 mm in most cases, and up to 5 mm in some cases. The thickness of the transition region may vary within this range around the main injured region of an individual site of cryosurgery. No significant variation of the extent of injury was observed within the main cryoinjured region. Representative histological cross-sections from these areas are shown in Figs. 2–4. These cross-sections are shown at high magnification to capture the cellular detail. These figures cover relatively small areas of each region and, hence, the orientation of each cross-section is not relevant for analysis. For example, the cross-sectional area shown in Figs. 2–4 is about 0.16×0.23 mm, which is an order of magnitude smaller than the thickness of the transition zone and three orders of magnitude smaller than the main cryoinjured region.

Microscopic findings immediately post-cryo-

FIG. 2. Control tissue, normal glands of the breast stained with H&E.

FIG. 3. Main cryoinjured region stained with H&E: (a) loss of cytoplasmic and nuclear detail immediately post-cryosurgery; (b) necrosis with complete loss of cellular detail at 1 week post-cryosurgery; (c) loosely arranged capillary, fibroblast, and collagen at 1 month post-cryosurgery; (d) capillaries, fibroblasts, and more arranged collagen at 2 months post-cryosurgery; and (e) capillaries, fibroblasts, and bundle of collagen at 5 months post-cryosurgery.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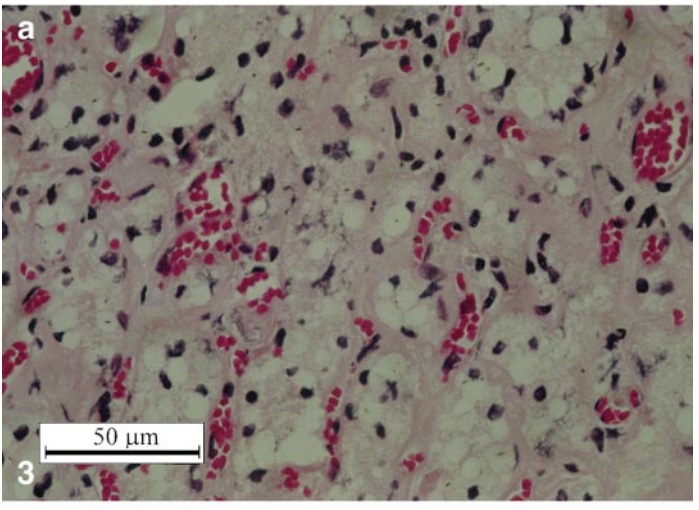
2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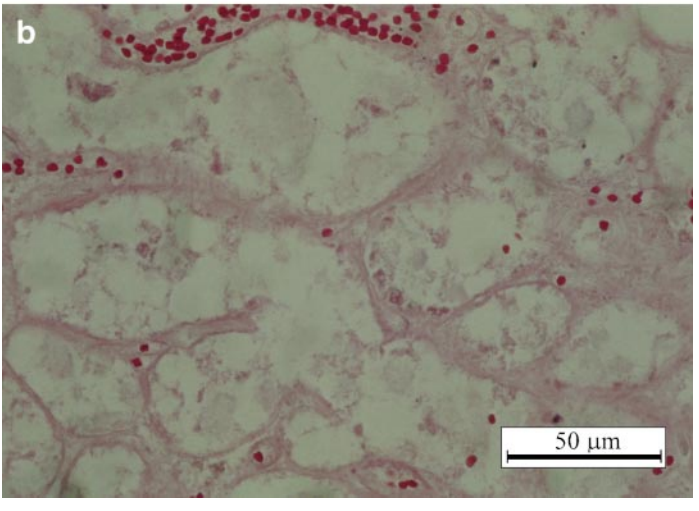
b
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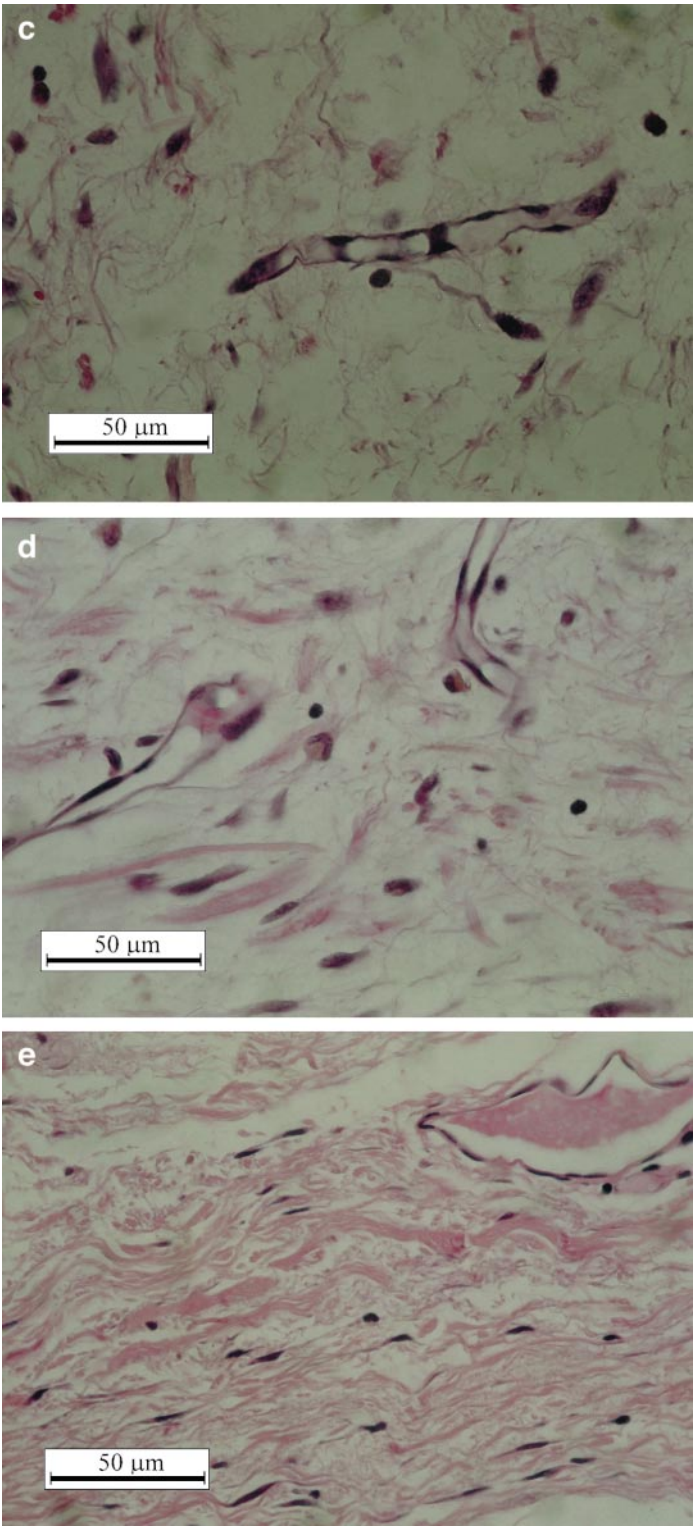

c
50 μm
d
50 μm
e
50 μm

surgery showed edema of the interstitial tissue with vascular congestion by red blood cells (Figs. 3a and 4a). The epithelial cells of glands and ducts show swelling and vacuolation of the cytoplasm and nuclear changes consisting of hyperchromasia and irregular shapes. The normal tissue outside of the frozen region retained good cellular details and did not show edema or vascular congestion (Fig. 2).

Microscopic findings at 1 week post-cryosurgery showed extensive ischemic necrosis, vascular congestion with red blood cells, scattered thrombosed blood vessels of varying size, and extensive interstitial edema. In the areas of ischemic necrosis there were no viable glandular or ductal epithelium (Fig. 3b), which are the sources of human breast cancer (see Discussion). A peripheral rim of damaged breast lobules of varying thickness between 2 and 5 mm (defining the transition zone) was observed (Fig. 4b). The lobules within this zone showed extensive loss of the acinar tissue with greater loss closer to the central region of necrosis (the main cryoinjured region). Within these lobules, a considerable amount of interstitial edema and increasing prominence of fibroblasts and capillaries were observed. Very little inflammation with scattered neutrophils in the necrotic debris and damaged breast lobules were found. The unfrozen breast tissue showed no inflammation, edema, or vascular congestion. The unfrozen breast tissue appeared similar to the tissues of the control breast (the contralateral breast) (Fig. 2).

The TTC allowed a fair identification of the cryotreated region up to 1 month post-cryotreatment on the gross specimen. Microscopic findings at 1 month post-cryosurgery showed that the main cryoinjured region consists of loose edematous connective tissue with scattered fibroblasts and capillaries and a very small amount of collagen (Fig 3c). A few small clusters of lymphocytes and macrophages around some of the blood vessels were observed toward the periphery of the cryoinjured region. The specimens showed a peripheral zone of regenerating breast lobules that retain their general shape (the transition zone); however, they had lost their acinar tissue and showed ducts without the acinar buds. The ducts were lined by enlarged ductal epithelium that varied in thickness from one to two cells (Fig. 4c). The interstitial tissue was edematous and showed increased numbers of fibroblasts and capillaries.

At 2 months post-cryosurgery, the main cryotreated region was found to be pink and not easily distinguished from the red-stained normal tissue (which was stained by the TTC). Compared with 1 month, the main cryoinjured region at 2 months post-cryosurgery is less edematous and has slightly increased numbers of fibroblasts, capillaries, and collagen (Fig. 3d). Some arteries were hyalinized scars and some thrombosed vessels have undergone recanalization. For example, the artery in Fig. 5 shows replacement of the cryotreated lumen by scar tissue and reestablishment of small vascular lumen at the center of that scar tissue. Microscopic findings with regard to the transition zone are inconsistent between animals of the 2-month follow-up group: a well-defined transition zone was found in two cases, as shown in Fig. 4d, while it was not well defined in two other cases (which did not allow the estimation of the diameter of the injured region in the right breast of animal No. 12). The transition zone contained regenerated lobules with elongated ducts but no acinar tissue. Compared with the 1-month follow-up group, the stroma of the lobules contained an increased number of capillaries and fibroblasts.

The cut surface of the surgical excision case at 2 months post-operation showed a scar of white fibrous tissue, which had a firm consistency. The microscopic findings showed dense fibrous connective tissue in the scar surrounded by normal breast tissue. Compared with the cryoinjured region post-cryosurgery, the scar tissue resulting from surgical excision was found to be essentially the same.

The application of TTC at 3.5 months post-cryosurgery was not found to be useful for distinguishing the cryoinjured region from the healthy surrounding tissues. Compared with the 2-month follow-up group, the main cryoinjured region had decreased fibroblasts and edema

with increased collagen. Similar to the findings from the 2-month follow-up group, microscopic findings with regard to the transition zone were inconsistent between animals of the 3.5-month follow-up group: a well-defined transition zone was found in 5 of the 6 cryosurgery cases, and it was not well defined in one case (left breast of animal No. 15). In the cases in which it was well defined, the transition zone thickness was found to vary between 2 and 3 mm. The transition zone contained regenerated breast lobules with branching ducts but no acinar tissue.

The surgical excision procedures left a noticeable thin scar on the skin after 3.5 months. Microscopic findings from the surgical excision cases showed a main injured region consisting of dense fibrous scar tissue that was irregular in shape. An apparent transition zone of 3 mm in thickness was found in one case of surgical excision on the gross specimen but was unable to be identified microscopically. The same animal had enlarged ducts in both breasts, which made the macroscopic evaluation difficult. The transition zone was absent in all other cases of surgical excision.

The cut surface of specimens of the 5-month follow-up group showed a soft white main cryoinjured region surrounded by a light-brown peripheral zone (no TTC applied in this group). These regions were ill defined and sometimes difficult to characterize. Compared with the 3.5-month follow-up group, the central scar (the main cryoinjured region) showed increased fibrous tissue with more collagen (Fig. 3e). Some fibroblast and capillaries were also found in the main cryoinjured region. Regenerating lobules that still had a cellular stroma were found in the transition zone. The ducts showed more complex branching but still no acinar development (Fig. 4e).

The lesions of the surgical excision cases had irregular scars and were difficult to identify in the gross specimen at 5 months post-cryosurgery. No transition zone of regenerating lobules was found microscopically. Microscopic comparison showed denser fibrous scars in the excised lesion than in the cryotreated area, as well as no regenerating lobules.

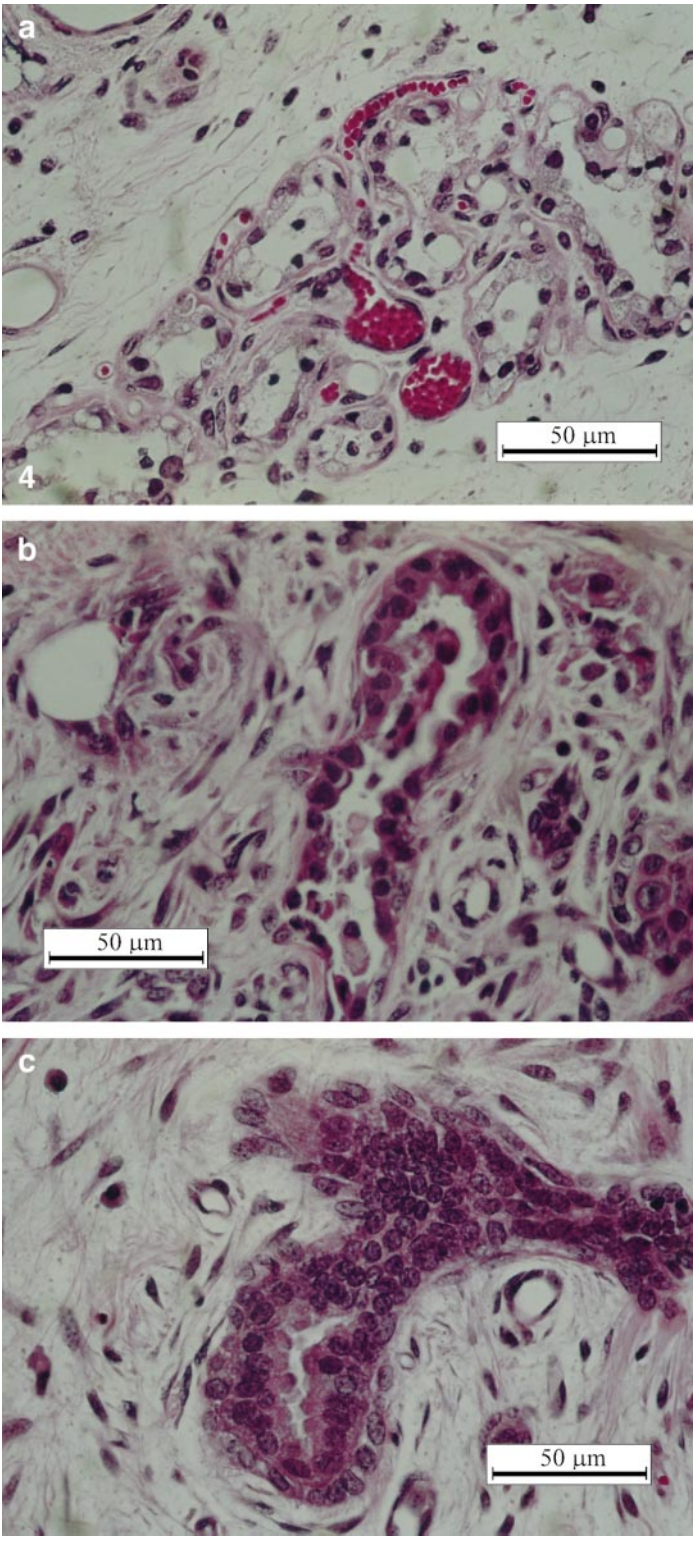
Microscopic findings from one- and three-cycle cryoprocedures were essentially the same and could not be distinguished on the basis of gross or microscopic findings, for follow-up periods between 1 and 5 months.

DISCUSSION

It can be seen from Table 1 that the average frozen region diameter increases by 10 to 20% between the first and second cycles. This can be explained by the fact that the initial temperature distribution of the second cycle is much lower than the uniform initial temperature distribution of the first cycle (about 37°C). The initial temperature distribution of the second cycle varies between about 0°C at the cryoprobe surface to 37°C at far distance from the cryoprobe (further than the freezing front location at the end of freezing). One may assume that blood flow within the cryotreated tissue decreases after thawing of the first cycle due to substantial damage of the blood vessels post-freezing, as is discussed below with regard to the histology findings, and this has also been referred to by others (3, 5). Since the blood flow acts as a heat source interfering with freezing, one may further assume that the decrease in blood flow allows the ice-ball to grow larger during the

FIG. 4. Transition zone between the main cryoinjured region and the surrounding healthy tissues stained with H&E: (a) glands with interstitial edema and vascular congestion with red blood cells immediately post-cryosurgery; (b) duct with loss glands, edema, fibroblasts, and vascular congestion with red blood cells at 1 week post-cryosurgery; (c) regenerating duct in loose stroma at 1 month post-cryosurgery; (d) regenerating duct with branching but no secretory glands at 2 months post-cryosurgery; and (e) regenerating duct, no secretory glands at 5 months post-cryosurgery.

FIG. 5. Recanalization of an artery within the main cryoinjured region stained with Masson's trichrome stain at 2 months post-cryosurgery. The artery at the center shows replacement of the cryotreated lumen by scar tissue and reestablishment of small vascular lumen at the center of that scar tissue.


a
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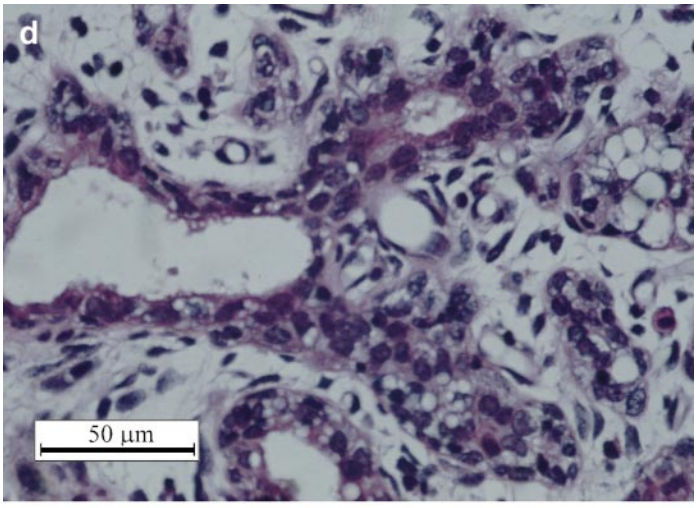
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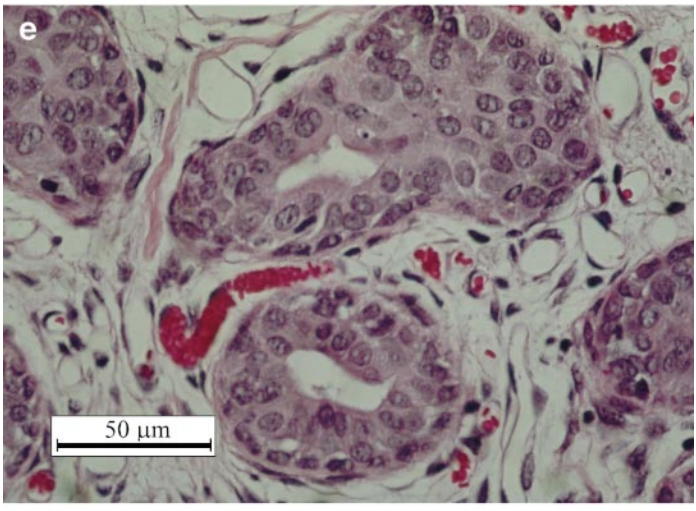
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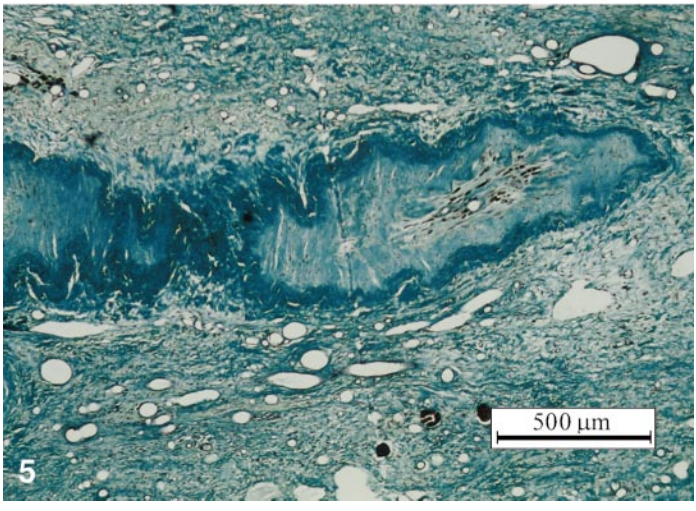
500 μm
5

second cycle. The initial temperature distribution of the third cycle, however, is similar to that of the second cycle, hence the similarity in the final frozen region diameters. It has been suggested that the frozen region diameter should increase in the second cycle and should not increase significantly in the third cycle, based on a theoretical analysis (15). However, a decrease of 6% in one case and an increase of 11% in another case were observed in the frozen region diameter between the second and the third cycles. The decrease of 6% in the first case, which equals 1.6 mm, may be related to uncertainty in experimental measurements. The increase of 11% in the frozen region diameter in the other case, which equals 3.2 mm, may be due to partial blood vessel destruction after the first cycle and more substantial blood vessel destruction after the second cycle.

Comparison of the frozen region diameter, as estimated from the ultrasound imaging, with the maximal diameter of cryoinjury (the discolored area measured in the breast cross-section from the bisected specimen) revealed that the cryoinjured region was up to 2.5 mm larger in diameter than the imaged frozen region immediately post-cryosurgery. It should be noted that the transition zone between the main cryoinjured region and the healthy surrounding tissues is included in the above macro-measurements of the maximal diameter of cryoinjury. This observation coincides with previously reported observations that the cryoinjured region was found to be no smaller and up to about 5 mm larger than the imaged frozen region immediately post-cryosurgery in a sheep breast model (13, 17), which is likely to be related to damage of blood vessels up to the edge of the frozen region, and to accumulation of fluids around them.

One may find the above observation at odds with today's commonly accepted assumption, which is that the frozen region is always larger than the cryoinjured region. However, it has already been reported by others that the visible diameter of the frozen region may, under some conditions, lead to an under-estimation of the cryoinjured region as measured from histology preparations (10–12). For example, Neels *et al.* (12) have performed experimental cryoprocedures on a tumor model of a rat liver and measured cryoinjured region diameters of 22 and 24 mm, following a visible frozen region diameter of 20 mm, at day 1 and day 4 post-cryosurgery, respectively. This observation has been made on an ischemic liver, where a total hepatic ischemia was applied temporarily by clamping the porta hepatis just prior to the cryo-application and by removing the clamp immediately after thawing. However, following a visible frozen region diameter of 19 and 17 mm in the absence of the temporary ischemia effect, the diameter of the cryoinjured region was found to be 16 mm at both day 1 and day 4 post-cryosurgery. The difference in observations between the ischemic and the nonischemic cases may be attributed to either the absence of the heating effect of blood perfusion in the ischemic case or to some damaging effects associated with reperfusion. Unfortunately, comparative results are not available, neither for breast tumor model nor for ischemic breast model.

When comparing the above observation with today's commonly accepted assumption that the frozen region is always larger than the cryoinjured region, special attention should be paid to the means of measurement of the frozen region diameter. The frozen region is measured indirectly via ultrasound images in this study, while it is measured directly in other studies (the visible frozen region surface). The difference between ultrasound-based measurements and histology-related measurements may be partly related to the physical principles of ultrasound imaging, in which the temperature of the imaged front is not actually known. The freezing front temperature is sometimes speculated to be the point at which pure water ice crystals start to form in equilibrium, i.e., 0°C. However, assuming that body solutions behave like an NaCl solution, the ultrasound-imaged front can be somewhere between −22 and 0°C (the phase-transition temperature range). Furthermore, freezing can be suspended down to the homogeneous nucleation point in some cases, i.e., −39.2°C. An error of 20°C in defining the

freezing front temperature can easily lead to an error of 2 mm in estimating the radius of the freezing front location (18).

One may argue that ultrasound imaging and thermocouples could be combined in order to verify the actual temperature of the ultrasound-imaged interface. Unfortunately, this is not feasible due to the high uncertainty in temperature measurements when using thermocouples during cryosurgery, an uncertainty level which can easily reach the typical temperature range of phase transition (18).

After 5 months, the cryoinjured region was found to be between 50 and 60% of the imaged frozen region during the cryoprocedure. The reduction in cryoinjured region size is probably the result of: (a) the contraction of the scar tissue within the area of injury as the scar develops, (b) the healing process of the tissue post-injury, and (c) the natural reduction of the sheep breast with time post-lambing (the breasts were cryotreated 2 to 3 months post-lambing and the evaluation of injury for the last follow-up group was performed 5 months later). We believe that the reasons for the reduction in cryoinjured region size are presented above in the order of significance, although no quantitative data is available.

The cryotreatment site in a sheep breast model cannot be identified by means of ultrasound, mammography, or MRI in the long-term follow-up extending up to 5 months. The scar tissue is in an advanced stage of formation by the end of 5 months. It can be concluded that it is highly unlikely that the scar tissue will be misinterpreted as a potential tumor, using these standard imaging techniques. However, it is emphasized that the scar tissue is not fully developed after 5 months, and we speculate that the scar maturation continues for up to at least 1 year.

The cryoprocedure produces an immediate injury which is characterized by cellular degeneration with vacuolization of the cytoplasm and loss of cellular and nuclear detail. This is associated with vascular congestion with red blood cells and edema. Two major areas were identified within the cryotreated region: the main cryoinjured region at the center (Fig. 3) and an area of gradual lobule damage between the main cryoinjured region and the surrounding healthy tissues, defined as the transition zone in this report (Fig. 4). The cryoprocedure appears not to affect the healthy tissues surrounding the cryotreated region, and all inflammatory processes are limited to the identified cryotreated region. This observation indicates that the cryoprocedure can be applied to a well-defined region (a tumor for example).

While the size of the main cryoinjured region decreases with time, its internal detail changes as follows (as is also presented in Fig. 3). Extensive coagulation necrosis, vascular congestion with red blood cells, interstitial edema, and occasional thrombosed vessels are observed at 1 week. There may be a few fibroblasts after 1 week but all epithelium appears to be necrotic within the main cryoinjured region. The epithelial necrosis is most significant because duct and acinar epithelium are the sources of most human breast cancer. After 1 month, the necrotic area has been replaced by loose connective tissue consisting of scattered fibroblasts and capillaries and, yet, no glandular epithelium was observed. Scar tissue developed within the thrombosed blood vessels as well; however, some recanalization of blood vessels was observed. This means that some vessels, especially arteries, may survive and return to limited function. Progressively increasing collagen in the main cryoinjured region is observed at 2, 3.5, and 5 months post-cryosurgery. A few ducts extending into the main cryoinjured region appear at this later stage. This process of scar formation probably continues up to at least 1 year.

Figure 5 shows a recanalized artery using Masson's trichrome stain. The Masson's trichrome stain is used to distinguish fibers within the scar. In contrast to the standard H&E stain, which gives a pink color to both collagen fibers and cytoplasm of fibroblasts, the trichrome dye stains collagen green, cytoplasm red, and nuclei black. The intensity of the green color is proportional to the amount of collagen, where collagen gives tensile strength to the wound. Thus, it is easier to distinguish which

fibers in a healing wound represent collagen, and it is a useful aid in visual interpretation of the pathologic findings.

While the main cryoinjured region size is determined by the cooling protocol of the cryoprocedure, the thickness of the transition zone appears to be in the range of 2 to 3 mm in most cases and up to 5 mm in some cases. The transition zone was not observed in one case, which most probably represents individual variation in one sheep. One week post-cryosurgery, the transition zone is identified by damaged lobules showing increasing loss of acinar tissue and the stroma is edematous with vascular congestion with red blood cells. Increased fibroblasts and capillaries with time are observed within the transition zone. Ducts are actively being regenerated within the transition zone after 2 months but no acinar tissue had been regenerated up to 5 months. This region could potentially be a safe harbor for surviving cancer, whether the cancer is already present or that ducts are destined to give rise to cancer. Thus in the treatment of a breast tumor, the transition zone ideally should be in healthy tissue surrounding the tumor, and the main cryotreated region should contain the entire tumor and an amount of healthy tissue to insure an adequate margin. If the location of the transition zone is peripheral to the ultrasound-imaged ice-ball, then one should operate the cryoprobe until the ice-ball covers the entire tumor. Unfortunately, the location of the transition zone on the ultrasound image is not known. Based on the observations that the cryoinjured region is no smaller than the ultrasound-imaged ice-ball and that the typical thickness of the transition zone is up to 5 mm, it follows that an ultrasound-imaged ice-ball which is at least 5 mm larger in radius than the tumor will result in tumor inclusion within the main cryoinjured region (a safety margin of 5 mm). This safety margin diameter is a conservative estimate since the cryoinjured region is larger than the imaged ice-ball, as discussed above.

Since no transition zone was observed in surgical excision, one may assume that the presence of the transition zone resulted from the temperature field near the freezing front and is not generated after cryosurgery as a response of the tissue to a lesion of necrosis. Bearing in mind that the temperature distribution within the ice-ball is a function of the cryoprobe cooling power, cooling protocol, cryoprobe diameter, and the thermophysical properties of the tissue, one may further assume that the transition zone thickness is a function of the cryoprobe and the cryodevice as well.

The ducts within the transition zone contain thickened epithelium compared to normal breast. Due to the enlarged epithelial cells with enlarged nuclei, these ducts are lined by epithelium that is two cell layers thick. This increased size appears to be a response of regeneration. As the scar tissue contracts, bringing the breast lobules closer together after several months, there appears to be a few ducts extending into the scar tissue as well (into the main cryoinjured region).

There is no gross or microscopic difference between lesions that have been subject to one versus three cycles of freezing. Under either cryosurgical protocol, there is the main cryoinjured region that has uniform destruction of epithelium and healing scar formation and the transition zone of damaged lobules without acini, which is surrounded by healthy tissue.

All in all, two major differences between the scar tissue resulting from surgical excision and the scar tissue resulting from cryosurgery have been observed. (a) The surgical excision scar at 5 months post-cryosurgery has denser collagen than in the main cryoinjured region. This may reflect a relative delay in healing since the excised tissue has been removed and the wound can fill with fluid which promotes tissue growth. In contrast, all the necrotic lesion has to be removed by the surrounding healthy tissues post-cryosurgery before the repair process can begin. The thrombosed vessels in the cryotreated region also undergo repair, which takes time and may not establish as good circulatory function, which, in turn, can slow down the healing process. (b) The excision wound does not have the damaged blast lobules that is seen in the transition zone of the cryotreated

breast. This is consistent with the temperature distribution within the ice-ball at the end of freezing. Only partial freezing is expected near the ice-ball edge due to the fact that biological tissues freeze over a wide temperature range (typically between 0 and −22°C). Hence, partial freezing is expected to result in partial cell survival.

SUMMARY AND CONCLUSIONS

Ultrasound imaging of the ice-ball formation in a sheep breast model leads to an underestimation of the cryoinjured region in the range up to 2.5 mm in the current study. The cryoinjured region dimensions include a transition zone of partly damaged lobules having a typical thickness of 2 to 5 mm.

A conservative application of the cryosurgical device developed for the current study in breast tissues suggests 5 mm safety margins in an ultrasound-monitored cryoprocedure. It follows that a target tumor diameter of 10 mm requires an ice-ball of 20 mm, which can be easily achieved within less than 5 min using the new cryosurgical device.

The cryoinjured region at 5 months post-cryosurgery is about one-half the diameter of the imaged frozen region during the cryoprocedure. The reduction in cryoinjured region size with time is probably the result of the contraction of the scar tissue within the area of injury as the scar develops, the post-injury healing process of the tissue, and the natural reduction of the sheep breast with time post-lambing.

The cryotreatment site in a sheep breast model cannot be identified up to 5 months post-cryosurgery by means of ultrasound, mammography, or MRI. Using these standard imaging techniques, it is highly unlikely that the scar tissue will be misinterpreted as a potential tumor in the long-term.

The cryoprocedure produces an immediate injury which is characterized by cellular degeneration with vacuolization of the cytoplasm and loss of cellular and nuclear detail. This injury is associated with vascular congestion with red blood cells and edema. There is no gross or microscopic difference between lesions that have been subject to one versus three freeze/thaw cycles. Under either cryosurgical protocol, there is a main cryoinjured region that has uniform destruction of epithelium and healing scar formation, and a transition zone of damaged lobules without acini, which are surrounded by healthy tissue.

In terms of recovery and regeneration, surgical excision appears to have an advantage over cryosurgery, which is a more rapid healing process. The cryosurgical wound will catch up in establishing a fibrous scar in time, perhaps longer than 1 year. As a surgical procedure, however, cryosurgery has the advantages of substantial decrease in the risk of morbidity, simplicity of the procedure, minimal bleeding, anesthetic effect of low temperatures, low cost, minimal scarring, and possible stimulation of the body's immune system.

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