CHAPTER 4

CRYOSURGICAL INJURY: CHANGE OF VASCULAR ANATOMY AND BLOOD FLOW IN A PORCINE KIDNEY
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Abstract

Purpose

We quantified temporal changes in vascular structure and blood flow after cryosurgery of porcine kidney *in vivo*.

Materials and Methods

We studied 5 groups of 4 kidneys each with a survival time of 20 minutes, 4 hours, 2 days, 1 and 2 weeks after cryoablation. Before harvesting the kidneys, fluorescently labelled microspheres were administrated in the descending aorta. After harvest the kidney and its vasculature was casted with fluorescently dyed elastomere, frozen and processed in an imaging cryomicrotome to reveal the 3-dimensional arterial branching structure and microspheres distribution. In regions of interest vessels were segmented by image analysis software and histograms were constructed to reveal the total summed vessel length as function of diameter. A characteristic diameter of the ablated area was measured.

Results

The 20-minute survival group histograms showed a significant shift of the peak to larger diameters (*p*=<0.002), indicating that smaller vessels were destroyed. Microsphere density was decreased to 2% in the ablated region but not in the nonablated border zone, depending on the remaining crater crossing larger vessels. After 2 weeks neither vessels nor microspheres were left in the ablated area, which had shrunk by about 40% in diameter. Study limitations are the lack of histological confirmation and the use of normal rather than cancerous tissue.

Conclusion

Larger vessels remain patent just after ablation and transport blood to the border of the ablation crater but perfusion within the crater is halted instantly. Characteristic crater diameter increases initially but decreases thereafter. Destruction of vessels and tissue is complete 2 weeks after cryoablation.
Introduction

Cryoablation is a valid, alternative minimally invasive treatment for small renal tumors, especially in the elderly and fragile populations who sometimes have compromised renal function. Radical nephrectomy in these patients often results in renal function loss. Also, partial nephrectomy can impact the renal function when prolonged clamping of the renal vasculature is a prerequisite to perform the surgery under good conditions. Renal cryoablation overcomes some shortcomings while maintaining good oncological outcomes.

Vascular stasis after cryosurgery is considered to have a significant role in final cell death. Thus, it is important to understand when vascular stasis is complete. Contrast enhanced imaging, in which local absence of contrast medium in the treated area is considered a sign of a successful ablation, can assess clinical success of renal tumor ablation. However, interpretation of these images must consider that although microvessels are affected by cryoablation immediately, larger vessels can remain patent. Therefore, it was postulated that at the border of the cryosurgical lesion some cells die while others survive and adjunct therapy should be considered to influence the outcome of surviving cells.

Several theories have been put forward of the mechanism of tissue injury in cryosurgery, such as direct cellular damage provoked by the freeze-thaw intervals as well as cryogenic induced apoptosis and vascular injury resulting in a coagulative necrosis. However, understanding the advancement of tissue damage after cryoablation requires a better definition of the time course of vascular damage and related perfusion disturbances. In our earlier study on porcine kidneys particular vessels smaller than 180 µm in diameter were destroyed in the acute phase after cryoablation but not all of them. We also observed that some anatomically patent arteries remained, traversing the ablated area and connecting with the microvasculature of nonablated tissue to allow continuing perfusion of that region. Thus, we quantified the immediate and transient effects of cryoablation on the perfusion and structure of the renal arterial circulation.

Materials and methods

Study design

Ten domestic farm pigs weighing 35 to 40 kg were used in accordance with an approved protocol of the National Institutes of Health Guide for the Care and Use of Laboratory Animals that was registered with the Animal
Research Institute, Academic Medical Center, Amsterdam, the Netherlands. In each pig simultaneous open bilateral renal ablation was performed at the anterior side of the lower or upper pole. Experiments were divided into five groups of 4 kidneys each with different survival times, including group 1 - 20 minutes, group 2 - 4 hours, group 3 - 2 days, group 4 - 1 week and group 5 - 2 weeks. In groups 3-5 the pigs were awakened after abdominal closure and later anesthetized again for nephrectomy.

**Intervention and preparation**

Cryoablation was done using a CryoNeedle triple needle probe configuration (Galil Medical, Yokneam, Israel). The probes were placed in a coaxial and triangular configuration with a 7 mm distance between them. Total freezing time was 20 minutes using a double freeze-thaw cycle. In preparation for nephrectomy 200,000 red fluorescence dyed microspheres (Invitrogen™) with a diameter of 15 µ were suspended in a syringe and rinsed with Tween in 2 cc autologeous heparinized blood. Ten minutes before kidney harvest the solution was injected in the aortic artery at suprarenal level. After nephrectomy the renal arteries were cast with a Batson no. 17 green fluorescence dyed polymer cast (Polysciences, Warrington, Pennsylvania). Subsequently the kidneys were submerged in a container filled with 5% carboxymethylcellulose sodium solvent (Brunschwig chemie, Amsterdam, the Netherlands) and 5% Indian ink (Royal Talens, Apeldoorn, the Netherlands) and frozen to -20°C.

Preparations were sliced in our custom-made imaging cryomicrotome. Using a Megaplus® 4.2i camera equipped with a 70-180 mm lens (Nikon®) digital images were taken from the cutting plane of each slice. The normal white light reflection images were indicated as outline images. Images of the fluorescent cast and microspheres were obtained by applying D440/20x and D560/20x excitation filters, and D505/30m and D635/30m emission filters (Chroma Technology, Bellow Falls, Vermont, USA).

**Generalized data**

The green fluorescent images resulted in a 3-D image of the arterial vasculature of a kidney down to 20 µm vessels. In this stack of images arterial vessels were segmented and vessel diameters were estimated using a combination of filtering and peeling algorithms. Thereafter all vessel segments could be classified by diameter, which was divided by bins of 4 µ wide starting from 40 to 500 µm. The length of all segments of a specific bin diameter was added and histograms of total summed vessel length were plotted as function of diameter. These histograms were quantified by the diameter at the peak of the histogram. In all groups the graphically reported histograms were averaged. Histograms for the ablated area were compared to histograms from a control area in a different pole of the same kidney.
Thus, the effect of cryoablation was quantified as function of the diameter range.

**Microsphere data**

Microsphere positions in the kidney were determined from the appropriated stack of images by dedicated self-developed software. Microsphere density in N/ml in the center of the ablated area, the border zone of the ablated area and a control area were compared to quantify the differences in perfusion through these areas. In group 1 the border zone was determined from the microvascular images, which clearly showed the part perfused by vessels traversing the ablated area. In later groups the border of the ablated area was determined by the intact microvasculature surrounding the ablation area and the outline images showing adjacent scar tissue. The control area was chosen in the same pole but away from the ablated region.

**Ablation zone diameter**

Ablation zone diameter was assessed by selecting the image with the largest ablated area from the stack of outline images of each kidney. A circle was projected over the image to best match the ablated area contours. The diameter of the circle was considered as the characteristic diameter and used to calculate tissue retraction with time.

**Statistical analyses**

Segment length histograms were affected by cryoablation in 2 ways, that is 1) total vessel segment length at certain diameter decreases and 2) the diameter at which the peak occurred shifted to larger diameters. The difference between the sum of the lengths of vessel segments at the peak under control and after acute ablation conditions did not always attain significance in our earlier study, although the shift in the peak diameter did achieve significance. In the current study we determined the histogram of each individual tissue region.

In experimental groups 1 and 2 the shift in peak diameter was determined by the 2-tailed paired t-test. In groups 2 and 3 the difference between peak values was tested by the 2-tailed paired t-test. For groups 4 and 5 no further statistical analysis was done. A one-way ANOVA was used to quantify the retraction of ablation zone diameter. Groups were compared to each other using the Bonferroni multiple comparison test.
Results

In each pig the survival time of the 2 kidneys was equal. No hemodynamic disturbances were noted during the surgical procedures. Kidneys were processed in the imaging cryomicrotome by applying different types of optical filters, resulting in a set of 3 images after each cut of 40 µm. The 3 stacks of images resulted in 1) a 3-D representation of the vascular bed, 2) outline images of the tissue showing ablated area contours and 3) the positions of injected, fluorescently labeled microspheres.

All kidneys had a clearly demarcated cryoablation zone at the capsular surface. One group 1 kidney was not fit for analysis and was excluded due to underexposure of fluorescence images due to a technical problem. Figure 1 shows the development of the ablation zone and the vasculature retained in it in groups 1 to 5. Groups were based on survival time after ablation, including group 1 – 20 minutes, group 2 – 4 hours, group 3 – 2 days, group 4 – 1 week and group 5 – 2 weeks. Ablation zones were clearly visible in all groups due to absent tissue auto-fluorescence, which are seen as red areas. The green fluorescence of the post mortem injected casting agent was already strongly decreased in group 1 but it decreased in the 2 subsequent groups and was practically absent after a week in group 4. Figure 1 shows outline images of groups 1 and 2 in which the transition between the ablated area and its surrounding is sharp while a circular fluorescent band had developed by day 2 and week 1 in groups 3 and 4, respectively, ending in encapsulation after 2 weeks in group 5. The ablated area retracted with time.

Vascular damage in the ablated areas was quantified and compared by the averaged histograms of total segment length, which was determined from 3-D reconstructions of the vascular bed from the casting material, as function of bin diameter (figure 2). In agreement with our earlier observations ablation acutely affected the distribution of total segment length by shifting the diameter of at which the peak occurred significantly to a higher value ($p=0.002$), indicating the disappearance of vessels with diameter smaller than the peak diameter in the ablated area. In group 2 at 4 hours after cryoablation peak diameter had shifted to a larger diameter and the peak value of total segment length was significantly decreased ($p=0.012$ and $p=0.016$, respectively). The strong decrease in total segment length at all diameters at later time points was obvious in subsequent histograms. Some small vessel segments were left after 2 days but after 1 week no detectable structures remained (figure 2, D and E).
Figure 1.

Representative digital images of group 1- acute vascular injury (A), and 2- vascular injury after 4 hours (B), 3- after 2 days (C), 4- after 1 week (D) and 5- after 2 weeks (E) reveal fluorescence cast (green areas) in cryoablation zone vascular structures and autofluorescence of direct surrounding tissue (red areas) achieved by applying D440/20x excitation filter and D505/30m emission filters to single slice cryomicrotome specimen.
**Figure 2.**

Histograms of average total segment length in groups 1 (A), 2 (B), 3 (C), 4 (D) and 5 (E) with total segment length per group based on area used for calculation. Black areas indicate cryoablated zone. Gray areas indicate noncryoablated zone.

**Figure 3** shows a typical ablation crater reconstructed from the autofluorescence images and within it the remaining vasculature in 3 dimensions in group 1. The crater wall was determined by applying a threshold for reflective light, resulting in isoreflectance curves. **Figure 3** supports the conclusions from the histograms that smaller vessels in the crater disappeared but a structure of larger vessels survived ablation.

Fluorescently labeled microspheres were injected in the descending aorta before kidney removal. The regional density distribution of microspheres was representative for perfusion distribution. In group 1 the average relative microsphere density in the ablated crater decreased to 2% that of the control value and it was practically zero in the other groups. **Figure 4** shows microsphere positions with respect to the cast in group 1 in a 3-D visualization in which the surviving vasculature in the ablated area is clearly visible. Microspheres were transported to the perimeter of the vascular structures into nonablated renal parenchyma but were practically absent in
the ablated core. Hence, in group 1 the larger vessels in the ablation zone that were visible by casting remained patent but hardly transported any blood to the ablated tissue. However, by 1 week after ablation the vascular structures in the crater had disappeared completely and microvessels in the border zone were only connected to vascular structures approaching the crater wall from outside.

**Figure 3.**

*Three-D reconstruction from outline images (gray areas) reveal cryoablation crater at lower pole of group 1 kidney with 20-minute survival time. Note cast of surviving vasculature (orange areas) in crater.*
Figure 4.

Three-D visualization shows reconstructed center lines (white areas) of remaining vascular structures of group 1 kidney traversing the cryoablation area and connecting to surrounding noncryoabluted tissue. Vascular structures outside ablation zone are not shown. Color of the originally red dyed microspheres was digitally adjusted. Microspheres (green dots) distributed by traversing vessels were noted particularly in border (arrows) outside the ablation crater (gray areas) (A). Gray dots represent microspheres distributed by vessels outside ablation area. Note detailed side view of same cryoablation area (B).

Figure 5 shows a quantification of the retraction of the ablated area with time (figure 1). Circles were fitted to the borders of the ablated areas by eye and the mean diameter of each group was calculated (figure 5). Diameters at all time points differed from each other \( p<0.0001 \). Hence, 4 hours after ablation the characteristic diameter was larger than at the acute phase but it decreased thereafter by 50%. The increase in lesion diameter from the acute phase to 4 hours probably represents tissue swelling due to the development of edema as the initial response to cellular and vascular tissue injury\(^2\).
Discussion

This study demonstrates that in the target area cryoablation immediately destroys vessels with a diameter of less than 180 µm with a practically complete decrease in local perfusion. In the acute phase the microcirculation in the border zone of the target area remains connected and perfused by the larger remaining structures in the ablated region. With time these larger vascular structures also disappear and the process of secondary vascular stasis is completed within 2 weeks.

The filling with casting material is obviously only possible with an intact structure of the remaining vascular tree in the ablated area. Although occasionally some leakage of casting material was found in the ablated area, this was not the rule. Already in group 1 perfusion in the ablated area was practically decreased to zero. Therefore, filling of the remaining vascular structures with casting material was the result of displacing perfusate into
the surrounding tissue by intact vessels structures or by leakage into damaged tissue.

One may assume that each microsphere passed through the smallest, most proximal detectable vessel in its neighborhood. The result in group 1 clearly showed perfusion of the rim surrounding the ablated area that was perfused by the remaining vascular structures. In our earlier study we reported a halo-like structure surrounding the ablated area \(^7\). A suggested mechanism was hyperemia in this tissue. However, this hyperemia could not be confirmed by the current measurements since the microsphere density at the rim did not differ from nonaffected tissue in either group.

Some of the larger vessels that originally remained patent in the ablated area survived the direct cryoablative conditions. However, their vascular cells were exposed to indirect cell kill mechanisms, such as cryogenically imposed apoptosis and ischemia in surrounding tissue \(^13\). Flow in the remaining vascular structures must have been strongly decreased, as follows from the very low microsphere density in the ablated area and the decreased amount of intact tissue that depended on these vascular structures. Normally decreased flow in a vessel segment results in inward remodeling of these vessels but this requires days \(^14\). Thus, it is unlikely that remodeling alone explains the difference between groups 1 and 2, which had duration difference of only 3.5 hours. Hence, most likely the patency of these remaining vessels was severely affected by acute ablation effects.

Our study method currently restricts the possibility for additional morphological and histological measurements from areas identifiable from the 3-D reconstruction due to the destructive nature or our cryomicrotome method. However, Ames et al. described the histopathological findings of cryoablated porcine normal renal parenchyma after 2 weeks of survival \(^15\). Ablated lesions showed a peripheral rim of fibrosis with chronic inflammation, calcification and foreign body-type giant cells surrounding the central complete coagulative necrosis. These findings correspond to our results showing no vessels and no microspheres in a sharply demarcated zone after 2 weeks.

During the last decades several pathways of cryogenic cell death have been identified that involve direct and indirect mechanisms. Therefore, some investigators have assessed the effects of cellular injury for a period of days to weeks after cryoablation. Our study reveals that the characteristic diameter of the ablation area shrinks from 36mm to 30mm during the period from 20 minutes to 1 week post cryoablation. Consequently this effect can bias results when late cryogenic tissue response is studied in relation to temperature measurements assessed at cryoablation.
A limitation of this study is that it was done in normal porcine renal parenchyma. However, when cryoablation is used to treat cancer, we can only suggest that these cancer tissues will respond to ischemia in the same way.

In the clinical setting perfusion measurements are made soon after ablation to determine its success. However, we noted that flow through the ablated area is still possible for a few days and early perfusion studies should be interpreted with care. However, 2 weeks after ablation secondary vessel destruction should be fully complete and, thus, intravenous contrast measurement can be considered a true indicator of ablation success.

**Conclusion**

In the 2 weeks after cryoablation of porcine normal renal parenchyma gradual vascular anatomical disruption of the arterial architecture develops. At the end of this period without exception anatomical vascular branches and blood flow have fully disappeared from a well-demarcated cryoablation zone.
References


