CHAPTER 3
CRYOSURGICAL INJURY:
ACUTE VASCULAR CHANGES AFTER RENAL CRYOSURGERY IN A PORCINE MODEL
This chapter is published as:

Abstract

Introduction

Injury to blood microvessels has a crucial role in effective cryoablation for renal masses. We visualized the vascular injury induced by a clinically applied cryoablation instrument and established a microvascular diameter threshold for vascular damage.

Material and methods

In five anesthetized pigs one kidney each was exposed and three 17-gauge cryoneedles were inserted in one pole. Tissue was exposed to freezing in for 2 x 10 minutes with 10-minute thaw between freezes. After nephrectomy the arteries were injected with fluorescence dyed casting material and the kidney was frozen to – 20°C and cut in 40 to 60 µ slices in the imaging cryomicrotome, where fluorescent images of the cutting plane of the bulk were obtained. This resulted in a 3-dimensional image of the arterial tree that was segmented, resulting in unbranched vessel segments. Histograms were constructed with the total segment length per diameter bin was plotted as function of diameter.

Results

The ablated zone was sharply demarcated on fluorescent and normal light images. Mean ± SD diameter at the peak of the histogram from the control areas was 152.4 ± 5.3 µ. Compared to control areas the peak diameter of ablated areas was shifted to a larger diameter by an average of 25.4 ± 2.6 µ.

Conclusion

Immediate renal cryoablation injury destroys arteries smaller than 180 µ. Branching structures of larger arteries remain anatomically intact and connected to vascular structures in surrounding tissue.
Introduction

Cryosurgery is gaining interest as a curative or palliative treatment for urological cancer, including renal tumors, prostate cancer, and bone metastasis. Thus, it is important to understand the involved pathophysiological mechanisms that eventually lead to complete, reliable eradication of viable cancerous cells. Freezing induced arterial vascular injury provides an important contribution to tissue destruction. Apart from direct effects of freezing of cells in general, vascular injury induces a cascade of processes that eventually leads to blood flow cessation, causing ischemia, which is considered a therapeutic effect because of further tissue destruction. The vascular effect of freezing tissue has been especially studied in relation to frostbite but not to tumor ablation. In recent studies the focus was on the effect of freezing and thawing on the structure and function of the microcirculation of superficial tissues. Daum et al. reported a corrosion cast study of the acute effects of freezing on circulation of the rat hind limb. They noted that especially microcirculation was destructed without specifying a threshold for the diameter of affected vessels.

More detailed insight into the vascular effects of cryoablation could be useful. Obviously improved insight into the processes involved may result in improved technique but it may also aid in advancing the clinical assessment of cryoablation intervention for cancers. Clinical renal tumor cryoablation success is assessed by intravascular contrast enhanced imaging, such as computerized tomography and magnetic resonance imaging. Thus, it is important to understand the acute changes in the arterial vascular bed induced by cryoablation since these vascular alterations are the basis of what is visualized. We previously reported that the vascular pattern of normal porcine kidneys could be reconstructed by fluorescent cryomicrotome imaging. We applied that method in this study to detect acute changes in the renal circulation induced by ablation and establish a threshold value for vessel diameter sensitive to cryoablation destruction.

Methods

Six domestic farm pigs weighing 35 to 40 kg were used for a laparoscopic training procedure approved by the Animal Research Institute at the Academic Medical Centre of Amsterdam. The pigs were fully anesthetized during the procedure and sacrificed after the procedure was completed. Ablation was done at the anterior side of 1 kidney at 1 pole with a double freeze-thaw cycle cryoablation using a triple needle probe configuration Oncura™ cryoneedle in 5 pigs. In a triangular configuration all needles were placed parallel at a perpendicular angle to the renal capsule using a
template with a 7 mm interneedle distance. Total freezing time was 20 minutes.

Before kidney harvesting it was ensured that all frozen tissue had thawed a minimum of 20 minutes after probe removal. Probing kidney consistency by touching it with a finger was sufficient to test thawing. After kidney removal the arteries were cannulated and cast with a fluorescent dyed, Batson no. 17 plastic replica, consisting of a monomer base solution, a catalyst and a promoter. After the cast hardened the kidneys were entirely frozen again to -20 °C. In our novel developed imaging cryomicrotome the kidneys were cut in slices from anterior to posterior under standard conditions at -20 °C. A Megaplus 4.2i digital camera equipped with a 70 to 80 mm Nikon® lens was used to image the renal surface cutting plane after each slice. Lens settings were adjusted so that the maximal specimen cross-section was imaged, resulting in a pixel size of between 40 and 60 µm. Slice thickness was chosen accordingly so that cubic voxels resulted. Images of the fluorescent cast were obtained by applying a D440/20x excitation filter and a D505/30 m emission filter (Chroma Technology Corp, Vermont, USA). All images were processed using custom developed software written in Delphi (Borland, version 2005). Image analyses resulted in 3-D representations of the branching arterial system of ablated and nonablated renal tissue.

**Arterial tree segmentation and segment diameter estimation**

Vessel diameters were measured from 3-D vascular bed data. A peeling algorithm was used to extract vessel center lines while keeping the topology intact. Spatial location and number of connected center points were determined for all center points along these center lines. When a center point had more than 2 adjacent center points, this was defined as a node and with only 1 adjacent center point it was defined as an end point. Adjacent center points were grouped into segments, resulting in a mathematical vascular tree representation divided into segments spanning between nodes or a node and an end point. Segment length followed from a polynomial fit through the segment points.

Segment diameter was determined. At each segment mid point the local vessel orientation is determined from the 2 adjacent skeletal points. In a plane perpendicular to the local segment direction intensity distributions curves as a function of distance to the center point were calculated over 256 radial lines evenly distributed in the plane. Intensity along a radial line was determined by linear 3-D interpolation over 0.1-pixel intervals, i.e. approximately 5 µ. The vessel border was defined as the full width half maximum of the intensity curve reduced with the estimated background value of the image data.
Maximal intensity projection

Two-dimensional images of the branching nature of the arterial tree were obtained by the technique of Maximal Intensity Projection (MIP). In this technique a stack of data of arbitrary size is compressed to a single image, taking only maximum pixel values in the longitudinal direction of the stack.

Generalized data

All vessel segments were classified in bins by diameter. In a 3-D tissue region of interest the length values of all segments of a certain diameter range were added and histograms of total summed vessel length as function of diameter were plotted. These histograms were quantified by the diameter at the histogram peak. In this way the effect of cryoablation was quantified as a function of diameter range.

Results

All six pigs were hemodynamically stable while under anesthesia. Kidneys in this study appeared to be macroscopically normal without any congenital abnormalities.

From pig P1 a kidney was harvested without cryoablation and processed for arterial reconstruction to test the algorithms needed for histogram construction. Figure 1-A shows a MIP from 554 slices, indicating how the arteries branch from center to periphery and end in vessels of similar diameter distributed in the renal parenchyma. Figure 2-A shows the distribution of total segment length as a function of diameter for the lower pole, the upper pole and the interpolar. These distributions had a similar shape with peak values at the same diameter but peak values were different and, thus, they were not interpreted further. The decrease in total vascular length with decreasing diameter may express in part an anatomical reality but it also resulted from the limitation by which small diameters can be estimated by this technique with its current limitations.

Figure 1-B shows a MIP from more than 300 images, representing slices in which ablation in the lower pole was noticeable from the outline images. A ring of almost small, vessel-free tissue was noted around the frozen area. Some contrast leakage was recognizable as dots of fluorescent material in the ablation zone. After thawing and before harvesting the kidney a typical mark of the ablation is visible as a circular area surrounded by an outer band, appearing as a halo of colour resembling hematoma. In the cutting planes of the cryomicrotome this concentric nature of the ablated area was also noted in the outline image.
Figure 2-B shows histograms of total segment length obtained from ablated and nonablated control poles. In the control area the histogram peak was at 156 µ but in the ablated area the total length of vessels at that diameter was decreased to 30% of the total length at peak diameter in the ablated area.

The table shows overall peak diameter results in ablated and reference histograms. The mean ± SD peak value was 152.5 ± 5.3 µ in reference histograms and 177.9. ± 3.8 µ in ablated areas for a mean 25.4 ± SD 2.6 µ shift to the right in ablated tissue, which was significant (1-tailed paired t test p=<0.0001).

Figure 1.

MIP’s. A, entire stack of images from pig P1 untreated kidney shows no normal vessel distribution. B, images 1 to 300 of lower pole cryoablation in pig P2 kidney. Ablated area (arrow) is particularly noticeable due to surrounding ring or halo in ablated areas. Because of image selection we could not draw conclusions based on visual appearance of vascular structures only. C, 60 images representing 3.3 mm deep layer of pig P2 kidney containing ablation crater cross section (black area). Larger vascular structure is intact in crater and anatomically connected to microcirculation in surrounding tissue.

With the centre of the ablation area as centre line concentric tubes were created with a 6 to 48 mm outer diameter in 6-mm steps. For each 3 mm thick tube wall a histogram was constructed (figure 3). The tube with the largest diameter contained unaffected tissue and was considered the control histogram. Histograms for smaller tube diameters demonstrate revealed a shift of the peak to the right when more ablated tissue was included. When outer tube diameter was smaller than 30 mm, peak diameter did not shift further.
Figure 2.

Filled plot lines of summed length of segments of a class of diameters as function of diameter. Histograms were made for cylindrical regions of 500 pixels or 26.2 mm in diameter. A, lower pole (black area), upper pole (dark grey area) and interpolar region of pig P1 kidney. Histograms cover 250 images in the total image stack. Peak diameters of histograms are rather close and independent of polar region but magnitude of summed lengths at each diameter, including peak, may differ. B, ablated and control area in pig P2 kidney. Histogram covers images 1 to 300 in stack. Two curves can only be compared by peak diameter shape and position. Black area indicates cryoablation. Gray area indicates pole.

Table 1.

Peak values of total segment length as function of diameter in healthy and cryoablated tissue in 5 pigs.

<table>
<thead>
<tr>
<th>Kidney No.</th>
<th>Healthy renal tissue</th>
<th>Ablated area</th>
<th>Shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2</td>
<td>159.5</td>
<td>181.5</td>
<td>22</td>
</tr>
<tr>
<td>P3</td>
<td>156</td>
<td>180</td>
<td>24</td>
</tr>
<tr>
<td>P4</td>
<td>147</td>
<td>172.2</td>
<td>25.2</td>
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<td>P5</td>
<td>148</td>
<td>176</td>
<td>28</td>
</tr>
<tr>
<td>P6</td>
<td>152</td>
<td>180</td>
<td>28</td>
</tr>
</tbody>
</table>
Figure 1-C shows an MIP representing a 3.3 mm thick ablated tissue layer in pig P2, which contains as much intact structure as possible. This image confirmed visually that especially smaller vessels are affected by cryoablation. The remaining larger arterial structures traversed the cryoablated area and connected with vascular units in the surrounding tissue.

Figure 3.

Filled line plot of summed length of segments of class of diameters as function of diameter for concentric tube wall areas around ablation area centre in pig P3 kidney. Two largest tube diameters contained nonablated tissue, serving as control for histograms of ablated area. Peaks of walls of the smaller tubes were about same diameter. To correct for measured volume difference summed segment lengths rings were normalized with respect to largest ring volume.
In this investigation of the immediate effects of cryoablation on vascular injury we used histograms of total summed vessel length as a function of diameter to quantify cryoablation effects. The peak diameter of a histogram of total segment length may shift to a higher value by the disappearance of segments with a diameter less than the original peak diameter or by the addition of segments with a larger diameter. However, the creation of more segments with larger diameters by ablation is mechanistically unlikely. The additional possibility that the ablated area had a larger peak diameter because this diameter was already larger to begin with was excluded since all peak diameter values in the ablated area were larger than in controls. Hence, this study shows that the vessels acutely damaged by freezing were less than about 180 µ in diameter but anatomically intact vessels smaller than this threshold were still found. The position of the peak diameter of the remaining patent vessels in the ablated area was not related to any particular ablation zone and, thus, it was independent of distance to the freezing centre. Intact structures of larger vessels in the ablated area were still connected to the intact microcirculation of the tissue surrounding the ablated area.

The power of the current imaging cryomicrotome technique is that structures of the intact vasculature after ablation may be studied in detail up to a resolution of 40 µ resolution in relation to other images, providing structural information such as outline images. The resolution limitation was the result of the choice to image the kidney as a whole rather than zooming in on only parts of it.

Daum et al. used a corrosion cast technique applied to a rat hind limb submerged in cooled alcohol at temperatures of -10 °C and -20 °C \(^{11}\). In that study the freezing front entered the tissue from outside the tissue while we studied the situation relevant for cryosurgery, for which needles for injecting cold are used. Hence, in our study the freezing front spread from 3 needles had a much lower source temperature (-110 °C) outward over an area that dependent on the balance between the supply of cold from the center and the removal of cold at the outside ablation ring. Also, in the hind limb study Daum et al. concluded that especially smaller vessels disappeared from the cast with freezing but no threshold value for microvascular diameter was provided. The similarity between those results and especially figures 1, C and 3 shows that the effect of freezing is not so much related to the degree of freezing, that is -10 °C is as effective as – 100 °C with respect to vessel obstruction. However, with the needle approach the extensiveness of the frozen area depends on the temperature at the needles. The lower the core temperature, the larger the area.
We used the 3 smaller needles rather than 1 larger, thick needle because the 3-needle technique is used at our clinic and a thicker needle may compromise the vascular structures more than the smaller ones. However, because of the circle symmetry of our freezing lesions, apart from extension of the freezing area, results may not be different using a single but larger cryoprobe.

Without cryoprotection cells are killed in the acute phase after freezing, including endothelial cells. Thus, the structures of a vessel as a tube may still exist after thawing the interface but flowing blood may be damaged and thrombi may form, which contributes to subsequent tissue necrosis. Damage in the venous system may be similar to that in the arterial system but we did not analyze this.

In our earlier analysis of the coronary circulation the casting material filled vessels as small as 10 µm. Hence, filling microvessels was not a limitation of this study. Diameter measurement is also influenced by the point spread function, describing how a small fluorescent object in tissue is blurred in the image because of the optical system. This effect was studied before, showing that diameters may have an error of 15% around 150 µm, which decreases to zero at 250 µm. Hence, the point spread function has no influence on the estimated shift in peak diameter in control versus ablated regions.

By penetrating the vascular lumina the casting material had to push saline out of the vessels that would have passed obstructions in the microvessels, halting the high viscosity casting material. Also, saline may have extruded through the vessel walls because of the long duration of the arterial pressure of the saline solution.

There are a few other study limitations. It is not possible to use the histogram of the ablated area before freezing as a control for the situation after freezing. This limits the possibility of interpreting absolute values of the magnitude of the summed length as a function of diameter. Also, cryoablation is done with to destroy cancerous tissue while we studied the effect of freezing on the arterial vasculature of normal tissue. The effects of destruction via thrombus formation in the smallest vessels may also exist in cancerous tissue but the structure of the vascular bed may be different. Our study does not provide information on the process that further evolves with time after cryoablation and it is likely that the remaining structures disappear completely with time. A follow up study is needed to determine these effects.

Our method is also limited since it restricts the possibility of additional morphological and histological measurement of areas identifiable on the 3-D
reconstruction. Tissue slices are collected normally as waste and not identifiable with respect to images. Thus, using the cryomicrotome technique more detailed correlation among cryolesion size, tissue necrosis and arterial vessel wall destruction requires further development of the technique and cannot currently be provided.

It is not well documented in the literature but after clinical cryoablation we observe an outer ring or halo, which in appearance is different from the frozen and surrounding tissue on the kidney capsule surface. The halo is also visible on the epi-illumination images and on cryomicrotome MIP’s. It is unlikely that this halo is the result of post-ablation hyperemia since it coincides with the disappearance of microvessels on MIP’s. To our knowledge the cause and implication of the structural difference in this halo region in regard to the clinical outcome of cryoablation remains to be established.

The remaining arterial structures in the ablated area warrant further critical analysis in immediate postoperative perfusion studies. Residual vascular structures that conduct contrast medium through the ablated area may remain unnoticed on images because of resolution issues. According to the distribution pattern of the cast material contrast medium passes through the residual larger vessels in the ablated region rapidly but is not distribute in the ablated area, although it is distributed in bordering nonablated tissue.

**Conclusion**

Cryoablation with three 17-gauge needle probes of normal parenchyma in a pig kidney resulted in an ablated area in which especially vessels smaller than 180 µ in diameter are blocked flow in the acute phase. Some anatomically patent arteries remained traversing the ablated area and connecting with nonablated tissue vasculature. The role of these transport vessels in post-ablation distribution of blood flow and malicious cells in a temporal manner warrant further attention.
References


