An evaluation of the protective effect of an infusion of chilled glucose solution on thermal injury of the bile ducts caused by radiofrequency ablation of the liver[1]

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ABSTRACT

PURPOSE: To evaluate the protective effects of chilling the bile ducts with cold (5°C) 5% glucose solution (GS) during radiofrequency (RF) administration.

METHODS: Twenty male pigs (3 mos. old; 25–30 kg) were subjected to RF delivery with chilling (experimental group, N=10) or without chilling (control group, N=10). Half of the animals in each group were euthanized immediately after the operation, and half were euthanized one week later. The following histological variables in relation to the bile ducts were evaluated by a pathologist (blind examiner): degenerative changes to the epithelium; epithelial necrosis; ulceration, regenerative changes of the epithelium; polymorphonuclear neutrophil infiltration; and thermal effects.

RESULTS: The experimental group (88 bile ducts examined) showed reduced thermal damage relative to the control group (86 bile ducts examined) as demonstrated by significant differences in the following histopathological parameters: epithelial detachment of biliary epithelium (84.1% vs. 59.3%; $p<0.006$); elongation/palisade arrangement of nuclei (65.1% vs. 87.5%; $p<0.001$); pseudo-goblet cells (32.9% vs. 56.8%; $p<0.001$).

CONCLUSION: Infusion of 5% glucose solution (5°C) has a protective effect on bile ducts subjected to heat (95–110°C, 12 min) from radiofrequency thermal ablation device.

Key words: Catheter Ablation. Radio Waves. Cold Temperature. Common Bile Duct. Liver. Intraoperative Period. Swine.

Introduction

Radiofrequency thermal ablation (RFTA) is a highly promising technique for treating liver tumors wherein high-frequency currents are used to induce ionic agitation within cells, resulting in sharp increases in temperature through friction and thus tumor destruction[1-3].

A combined use of surgical and RFTA techniques allows the surgeon to remove the principal part of complex tumors and large tumors, while RFTA can be used to destroy small or residual lesions[4,5]. RFTA has been used primarily for liver tumors that cannot be resectioned, with the main advantages being that it can be used many times, is relatively safe, is minimally invasive, and can be applied to invasive tumors and/or those adjacent to major vascular structures, in cases where surgical resection with an adequate safety margin is not possible[6-8].

When RFTA is performed in the liver region, a systemic precautionary technique must be employed to maintain a safe distance from the bile ducts. Using histological and radiological assessments, a prior experimental study performed in pigs showed that the RFTA microelectrode probe can produce contact lesions on the intra-hepatic ducts[9-12]. Elevated risk of bile duct injury is associated with close proximity of the tumor mass to the liver center and portal vein; the incidence of lesions in this situation is significantly greater than in tumors located along the organ's edges.

One way to protect the biliary tree from heat-induced damage is to cool the bile ducts by perfusion of a chilled solution via a catheter inserted in the common liver duct or in the left or right liver duct, depending on the circumstances of the case[13]. To our knowledge, there has not been a controlled study reported thus far with a specific and detailed anatomopathological focus on thermal lesions caused by radiofrequency (RF) application that has examined the injury-protection efficacy of bile duct infusion with a chilled solution. Thus, here we closely evaluated the pathological changes stemming from thermal lesions caused by RF. Our aim was to compare the magnitude of histopathological thermal lesions of the bile ducts between groups of animals that did or did not have their bile ducts chilled with a cold 5% glucose solution (GS) while lesions were induced by the RF microelectrode probe.

Methods

The protocol for this study was approved by the Research Ethics Committee of the UNIFESP and the HIAE. A group of 20 animals (described below) were divided into an experimental group (with chilling) and a control group (without chilling) of ten animals each. To maximize our use of the animals, each animal was given two RF thermal lesions of the liver: one on the right lobe and one on the left lobe. We independently evaluated the intra-hepatic bile duct lesions in the interior of the induced thermal lesions. Five animals from each group were euthanized immediately after the experimental operation, while the remaining five were retained for clinical observation for one week before being euthanized (Figure 1).

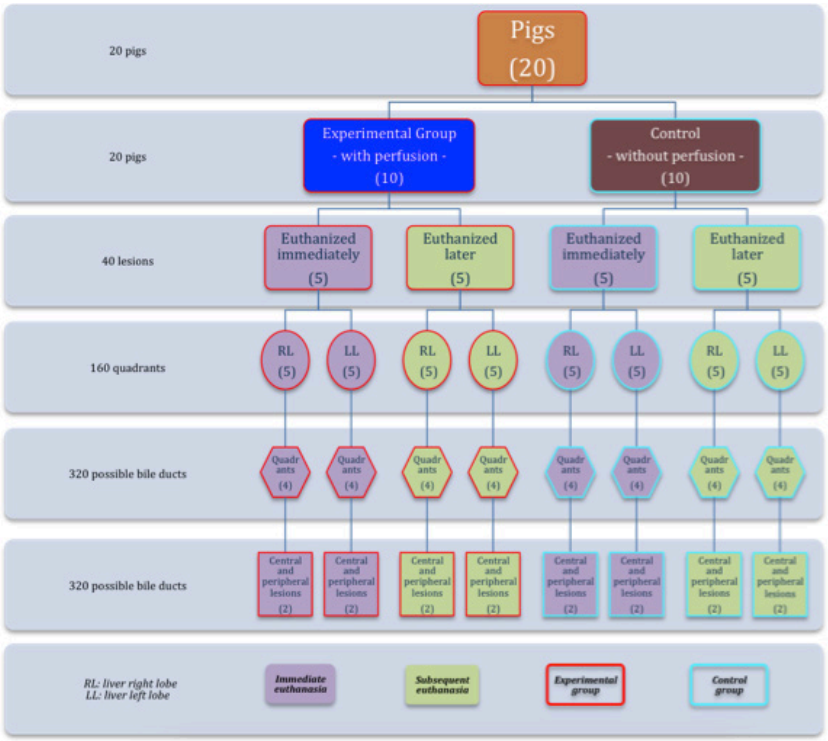

FIGURE 1 - Distribution of animals by group (control and experimental) and time of euthanasia (immediate or subsequent).

Each lesion was divided into four quadrants, and microscopic studies of the bile ducts and the periphery of each quadrant were carried out, constituting a total of 320 possible observations (bile ducts) to be evaluated (Figure 2).

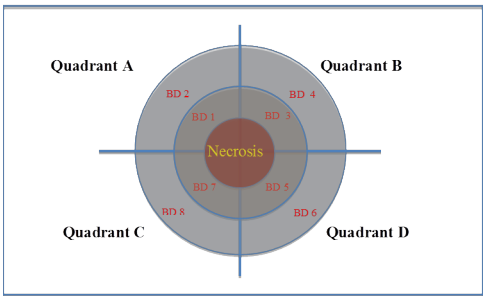

FIGURE 2 - Scheme anatomopathological study. Each lesion was divided into four quadrants and evaluated under a light microscope. In each quadrant, the central and peripheral bile ducts (BDs) were delineated for assessment.

Subjects and pre-operative preparation

A group of 20 large white male pigs (3 mos. old, weighing 30–35kg) were obtained from a specialized and accredited farm. After it was confirmed that the animals appeared to be healthy, they were placed in quarantine for three days in our experimentation center, where the surgical procedures were to be performed. The animals were fed a conventional diet and remained without dietary restrictions up until the time the operations were performed. The surgical procedures were performed under general anesthesia, with endotracheal intubation and mechanical ventilation. A veterinary professional monitored the animals throughout the experiment.

Before being sedated, the animals were given ketamine chlorhydrate (15 mg/kg) together with midozolam maleate (0.2 mg/kg) by deep intramuscular injection in the gluteus region. A nº22 peripheral catheter was placed in the peripheral vein of the ear and the anesthetic etomidate (6–8 mg/kg) was introduced for endovenous delivery of drugs. Next, the animal was intubated with a nº6.5 orotracheal probe, and anesthesia was maintained with inhaled 2.5% isoflurane gas in oxygen (2 L/min) with a constant volume of 10 ml/kg/h. Immediately prior to starting the surgical procedure, the analgesic fentanyl citrate (2.5 µg/kg) and the muscle relaxant pancuronium bromide (0.4 mg/kg/h) were delivered through the intravenous catheter. At the end of the procedure, the animals in the early intervention subgroups received an overdose of the anesthetic potassium chloride intravenously. Animals that were maintained for clinical observation for 1 week received the antibiotic cephalexin (1.0 g), the analgesic dipyrone (500 mg), the spasmolytic adephenine hydrochloride (10 mg), and promethazine hydrochloride (5 mg), mixed with their food daily.

Surgical procedures

When the animals were under general anesthesia, each was placed in horizontal dorsal decubitus and the abdomen was sterilized using 1% Povidine-Iodine®. A right subcostal incision was made to open the peritoneal cavity. The liver was freed from its ligaments to allow ultrasonography (US) transducer access. The principal bile duct was then identified and repaired. The principal bile duct was catheterized with a double-lumen tube via the ductus choledochus to enable intra-hepatic bile duct cooling in the experimental group (Figure 3).

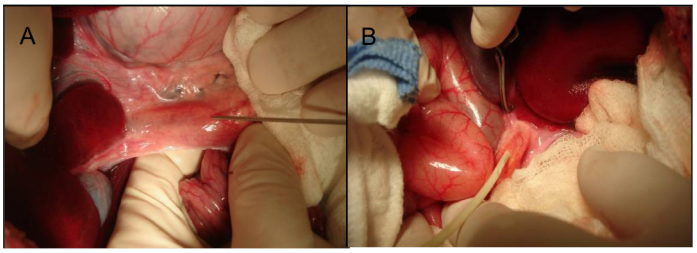

FIGURE 3 - Photographs illustrating ductus choledochus puncture with a rachianesthesia needle for catheterization of the bile ducts (**A**), and catheterization of the ductus choleduchus with a double-lumen tube to enable cooling of the intra-hepatic bile duct (**B**).

A Philips model EnVisor® (Healthcare, The Netherlands) US machine was used to identify the confluence of the right and left intra-hepatic bile ducts. The liver was then punctured with the RF needle, using the Cool-Tip RF Ablation System Valleylab® device with a single-tip needle. The isolated needle tips, which emitted the RF waves, were positioned 1 cm from the right and left intra-hepatic bile ducts. The RF needle was introduced into the hepatic parenchyma at room temperature, then heated to 95–110ºC.

In the experimental (with chilling) group, we began perfusion of the principal bile duct with 5% GS at 5ºC and delivered continuous infusion using an infusion pump. At the same time, the RF device was activated for 12 min, in accordance with the manufacturer's protocol. The GS bottle was submerged in a box with water and ice, with an Incoterm® brand thermometer used to certify the temperature. The control (no chilling) group were subjected to procedures identical to those applied to the experimental group, except that the bile ducts were not perfused with 5% GS.

Euthanasia and collection of samples

In the immediate euthanasia subgroups, the whole liver was removed, including the retro-hepatic vena cava, the hilus with the hepatic artery, the portal vein, and the bile duct. The samples were partially sectioned in parenchymal bands of ≤2 cm

to facilitate fixation, then immediately immersed in formaldehyde solution. All of the livers were injected with 5% toludine blue in the principal bile duct to enable the pathologist to visualize the tissues and identify the intra-hepatic bile ducts. All of the surgical samples were taken collectively to the pathologist for anatomopathological examination. Livers were collected from animals in the subsequent euthanasia subgroups one week later using the same procedures described above.

Anatomopathology

The liver specimens (whole livers) were fixed in a 10% formalin solution and sent to UNIFESP's Pathology Department, where they were weighed. The surgeons used codes to identify the surgical samples, and the pathologist was blinded to the group designations of the samples.

The lesions on the left and right lobes were identified and then measured across their longest diameters. Sequential cuts of the lesions were performed, selecting those whose adjacent parenchyma exhibited the greatest number of bile ducts to ease visualization after toluidine blue staining. The sections were then divided into four quadrants, each containing part of the lesion and the adjacent parenchyma. The fragments were subjected to histological processing, dehydrated in 98% alcohol, diaphanized in xylene, impregnated, and enclosed in paraffin. The histological cuts were colored using the hematoxylin-eosin (H-E) technique.

The septal bile ducts (≥100 μm) located adjacent to the areas of lesion tissue were selected for histological analysis. When there was more than one portal space present on the slide, the ducts closest to and most distant from the lesion were analyzed. When more than one septal duct was present in the same portal space, the one with the most extensive changes was selected for analysis.

The following histological variables were evaluated in relation to the bile ducts: degenerative changes to the epithelium, characterized by disorganization of the nuclear polarity and/or pyknosis and light cytoplasmic vacuolization; epithelial necrosis; exulceration; regenerative changes to the epithelium; permeation of the biliary epithelium by polymorphonuclear neutrophils and the presence of neutrophils in the ductal lumen; and the thermal lesion itself.

The thermal lesion was evaluated in terms of the presence of biliary epithelium detachment (Figure 4A), arrangement of biliary epithelium in a palisade formation (Figure 4C and D), and the presence of pseudo-goblet cells in the ductal septum representing maximum level of cytoplasmic vacuolization (Figure 4E and F). A semi-quantitative analysis of the lesions was performed according to the criteria of ductal epithelium changes represented by degeneration, necrosis, detachment, exulcertation, regeneration, palisade formations of nuclei, and excessive cytoplasmic vacuolization. The lesions were evaluated for the extent to which the ductal epithelium was compromised, with 0 representing an absence of lesion, 1 representing ≤25% compromise, 2 representing a compromise of 26–50%, and 3 representing a compromise of >50%. Compromise was considered to be present if there was any grade of lesion and the scores were aggregated.

Changes in the portal spaces were recorded as either present or absent, with histological parameters being evaluated (Figure 4B). We also evaluated the presence or absence of changes to the adjacent hepatic parenchyma, as characterized by the parenchyma's vitreous appearance, as a result of thermal effects, necrosis, foreign body granulomas, fibrosis, and dystrophic calcification.

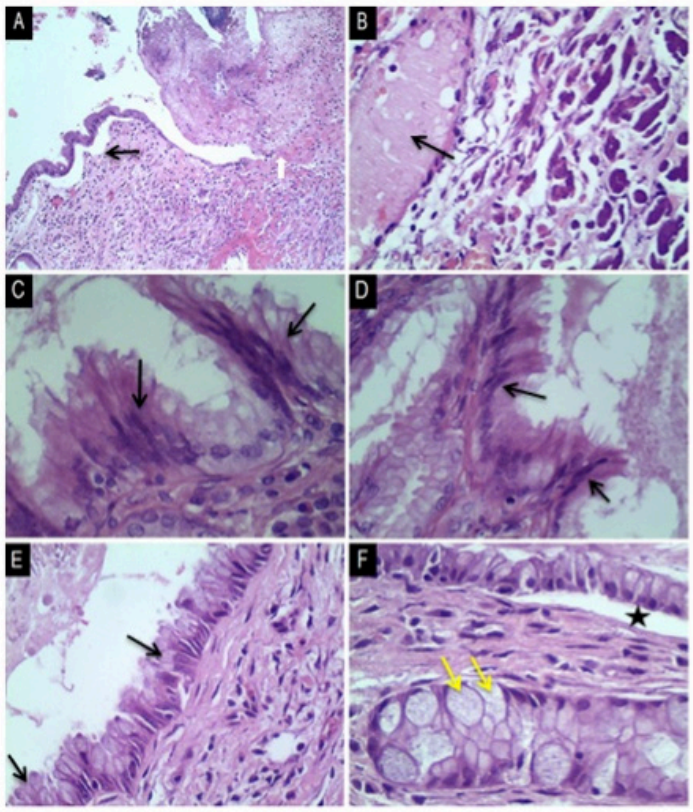

FIGURE 4 - (**A**) H-E stained section of the wall of the bile duct in pig 13 wall showing vesicular detachment of the epithelial coating (**black arrow**) and an area of ulceration (**white arrow**) (x100); (**B**) H-E stained section showing basophilia and necrosis of collagen fibers in pig 4 (**arrow**) (x400); (**C**) and (**D**) H-E stained section of a bile duct in pig 15 showing the thermal effects on epithelial, including cells with elongated nuclei and nuclei arranged in a palisade formation (**arrows**) (x400); (**E**) and (**F**) H-E stained sections showing pseudo-goblet cells in pig 13. Ductal epithelial coating showing a great quantity of mucus, assuming an aspect similar to a goblet (pseudo-goblet cells) (**black arrows**) (x400). Pseudo-goblet cells were also present in the coating of the peribiliary glands (**yellow arrows**). Note the vesicular detachment of the ductal epithelial lining in the upper right corner ( ) (x400).

Statistical analysis

The degree to which the walls of the bile ducts were compromised was evaluated. In making group comparisons with respect to changes to the histology and epithelium, Generalized Estimating Equations (GEEs), which allow co-variant observations made within the same animal to be accounted for, were used. The study's correlation matrix was considered to be of a uniform structure. The results were expressed in odds ratios (ORs) and 95% confidence intervals (CIs). A significance level of 0.05 was adopted. The statistical analyses were performed using SPSS for Windows (version 17.0).

The general goal of the analysis was to compare the two groups with respect to histological variables and changes to the ductal epithelium. Histological changes (i.e., edema, leukostasis, hemorrhage, leukocyte permeation, permeation of the neutrophils, neutrophils in the lumen, vitreous appearance of the parenchyma, homogenization of collagen in the portal space, necrosis, granulation tissue, foreign-body granulomas, fibrosis, and calcification) were analyzed as binary factors: present or absent. Changes to the ductal epithelium—including general histology of the ductal epithelium lesions (degeneration, necrosis, exulceration, regeneration) as well as lesions specifically associated with thermal effects (detachment of the epithelium, palisade formation of nuclei, and excessive cytoplasmic vacuolization or pseudo-goblet cells)—were analyzed as multinominal variables (scored 0–4 as detailed above).

Results

Sample

The animals had an average liver weight of 824.5 g, with a standard deviation (SD) of 162.4g, and a range of 507–1100g. The groups' mean liver weights did not differ significantly (p=0.464). As summarized in Figure 1, each animal had up to 16 observations: two lobes (right and left), and within each of four quadrants, two central ducts and two peripheral bile ducts. However, when no duct was found in the target region (quadrant), no evaluation was performed and, as a consequence, the overall number of observations was not always maximal. Of the 320 possible anatomopathological observations, we did not find bile ducts in the periphery or in the center of some 160 quadrants, ultimately leaving us to observe 174 lesion samples (or bile ducts effectively studied). The average number of observations (bile ducts studied) per animal was 8.7 (SD=2.3), with a minimum of four and a maximum of 13 observations. The number of observations did not differ between the two groups (p=0.852).

Comparisons of biliary lesions between the experimental and control groups

Overall comparisons between the experimental and control groups (combining euthanasia timing subgroups) with respect to non-thermal histological changes did not show significant effects of the cooling treatment on any of histological changes, apparently due to the high variability observed across the time points (Table 1).

TABLE 1 - Comparisons of histological observations between the experimental and control groups, independent of time of euthanasia.

Histological changes	Group		OR (CI 95%)	p-value
	Experimental (with chilling) (n = 88)	Control (without chilling) (n = 86)		
Edema	33 (37.5%)	47 (54.7%)	0.517 (0.166; 1.612)	0.255
Leukostasis	25 (28.4%)	39 (45.3%)	0.455 (0.132; 1.567)	0.212
Hemorrhage	28 (31.8%)	46 (53.5%)	0.380 (0.134; 1.077)	0.069
Leukocyte permeation	55 (62.5%)	54 (62.8%)	0.945 (0.372; 2.400)	0.906
Neutrophil permeation	13 (14.8%)	11 (12.8%)	1.206 (0.268; 5.428)	0.807
Neutrophils in the lumen	6 (6.8%)	5 (5.8%)	1.078 (0.163; 7.131)	0.938
Vitreous appearance of the parenchyma	88 (100.0%)	81 (94.2%)	*	
Portal space collagen homogenization	11 (12.5%)	9 (10.5%)	1.234 (0.418; 3.639)	0.703
Necrosis	48 (54.5%)	48 (55.8%)	1.000 (0.174; 5.733)	>0.999
Granulation tissue	48 (54.5%)	48 (55.8%)	1.000 (0.174; 5.733)	>0.999
Foreign-body granulomas	44 (50.0%)	36 (41.9%)	1.459 (0.273; 7.799)	0.658
Fibrosis	48 (54.5%)	39 (45.3%)	1.500 (0.255; 8.816)	0.654
Calcification	33 (37.5%)	33 (38.4%)	1.122 (0.215; 5.853)	0.892

Comparison of the assessed histological variables for with chilling versus without chilling among pigs that were euthanized immediately after the operation showed that livers from pigs in the experimental chilling group were less likely to show leukostatis and hemorrhage than livers from control pigs (Table 2).

All other variables did not differ significantly between the groups. The same comparisons between with chilling and without chilling for pigs that were euthanized one week later showed that livers in the experimental chilling group were less likely to show edema and leukostasis than livers from pigs in the control group (Table 3). Necrosis, fibrosis, foreign-body granulomas, and areas of calcification were only observed in livers from animals in the subsequent euthanasia (one week) subgroups.

TABLE 2 - Comparisons of histological observations between the experimental and control groups of animals euthanized immediately after the operation.

Histological changes	Group		OR (CI 95%)	p-value
	Experimental (with chilling) (n = 40)	Control (without chilling) (n = 38)		
Edema	27 (67.5%)	22 (57.9%)	1.444 (0.428; 4.870)	0.553
Leukostasis	23 (57.5%)	31 (81.6%)	0.304 (0.099; 0.936)	0.038
Hemorrhage	20 (50.0%)	31 (81.6%)	0.220 (0.064; 0.753)	0.016
Leukocyte permeation	23 (57.5%)	26 (68.4%)	0.628 (0.178; 2.215)	0.469
Neutrophil permeation	-	2 (5.3%)	*	*
Neutrophils in the lumen	1 (2.6%)	2 (5.0%)	1.912 (0.146; 24.994)	0.621
Vitreous appearance of the parenchyma	40 (100%)	34 (89.5%)	*	*
Portal space collagen homogenization	10 (25.0%)	7 (18.4%)	1.484 (0.609; 3.616)	0.385
Necrosis	-	-	-	-
Granulation tissue	-	-	-	-
Foreign-body granulomas	-	-	-	-
Fibrosis	-	-	-	-
Calcification	-	-	-	-

TABLE 3 - Comparisons of histological observations between the experimental and control groups of animals euthanized one week after the operation.

Histological changes	Group		OR (CI 95%)	p-value
	Experimental (with chilling) (n = 48)	Control (without chilling) (n = 48)		
Edema	6 (12.5%)	25 (52.1%)	0.136 (0.023; 0.793)	0.027
Leukostasis	2 (4.2%)	8 (16.7%)	0.240 (0.072; 0.799)	0.020
Hemorrhage	8 (16.7%)	15 (31.3%)	0.433 (0.126; 1.481)	0.182
Leukocyte permeation	32 (66.7%)	28 (58.3%)	1.379 (0.375; 5.063)	0.629
Neutrophil permeation	13 (27.1%)	9 (18.8%)	1.634 (0.343; 7.776)	0.537
Neutrophils in the lumen	4 (8.3%)	4 (8.3%)	0.905 (0.089; 9.228)	0.933
Vitreous appearance of the parenchyma	48 (100%)	47 (97.9%)	*	*
Portal space collagen homogenization	1 (2.1%)	2 (4.2%)	0.434 (0.065; 2.905)	0.389
Necrosis	48 (100%)	48 (100%)	*	*
Granulation tissue	48 (100%)	48 (100%)	*	*
Foreign-body granulomas	44 (91.7%)	36 (75.0%)	4.149 (0.387; 44.537)	0.240
Fibrosis	48 (100%)	39 (81.3%)	*	*
Calcification	33 (68.8%)	33 (68.8%)	1.266 (0.161; 9.964)	0.823

Analysis of the overall frequencies of ductal epithelium changes observed in livers from pigs in the experimental chilling group versus those from controls showed that the chilling treatment was associated with a reduced severity of lesion changes attributable directly to thermal effects, whereas the other secondary changes did not differ between the two groups (Table 4). The results of the group comparisons of ductal epithelium changes among pigs that were euthanized immediately after the operation are shown in Table 5; the same comparisons between with chilling and without chilling for pigs that were euthanized one week later are shown in Table 6.

TABLE 4 - Comparisons between the experimental and control groups in terms of changes to the ductal epithelium.

Changes to the ductal epithelium	Group		OR (CI 95%)	p-value
	Experimental (n = 88)	Control (n = 86)		
LESIONS CAUSED BY HEAT (THERMAL EFFECT)				
EPITHELIAL DETACHMENT				
Absence of lesion	74 (84.1%)	51 (59.3%)	0.276	0.006
Compromise ≥25%	8 (9.1%)	14 (16.3%)	(0.110; 0.695)	
Compromise 26–50%	5 (5.7%)	12 (14.0%)		
Compromise >50%	1 (1.1%)	9 (10.5%)		
NUCLEI IN PALISADE FORMATIONS				
Absence of lesion	77 (87.5%)	56 (65.1%)	0.331	0.001
Compromise ≥25%	8 (9.1%)	27 (31.4%)	(0.178; 0.617)	
Compromise 26–50%	3 (3.4%)	3 (3.5%)		
PSEUDO-GOBLET CELLS				
Absence of lesion	50 (56.8%)	28 (32.9%)	0.127	<0.001
Compromise ≥25%	23 (26.1%)	30 (35.3%)	(0.042; 0.583)	
Compromise 26–50%	5 (5.7%)	17 (20.0%)		
Compromise >50%	10 (11.4%)	10 (11.8%)		
OTHER HISTOLOGICAL LESIONS				
EXULCERATION				
Absence of lesion	77 (87.5%)	76 (88.4%)	1.008	0.985
Compromise ≥25%	7 (8.0%)	8 (9.3%)	(0.438; 2.321)	
Compromise 26–50%	1 (1.1%)	1 (1.2%)		
Compromise >50%	3 (3.4%)	1 (1.2%)		
DEGENERATION				
Absence of lesion	15 (17.0%)	11 (12.8%)	0.640	0.279
Compromise ≥25%	40 (45.5%)	38 (44.2%)	(0.285; 1.437)	
Compromise 26–50%	18 (20.5%)	23 (26.7%)		
Compromise >50%	15 (17.0%)	14 (16.3%)		
REGENERATION				
Absence of lesion	87 (98.9%)	79 (91.9%)	0.195	0.148
Compromise ≥25%	1 (1.1%)	2 (2.3%)	(0.021; 1.790)	
Compromise 26–50%	0 (0%)	5 (5.8%)		
NECROSIS OF THE DUCTAL EPITHELIUM				
Absence of lesion	80 (90.9%)	76 (88.4%)	0.629	0.465
Compromise ≥25%	8 (9.1%)	6 (7.0%)	(0.181; 2.182)	
Compromise 26–50%	0 (0%)	3 (3.5%)		
Compromise >50%	0 (0%)	1 (1.2%)		

TABLE 5 - Comparisons between the experimental and control groups in terms of changes to the ductal epithelium for immediate euthanasia time point.

Changes to the ductal epithelium	Group		OR (CI 95%)	p-value
	Experimental (n = 40)	Control (n = 38)		
LESIONS CAUSED BY HEAT (THERMAL EFFECT)				
EPITHELIAL DETACHMENT				
Absence of lesion	30 (75.0%)	18 (47.4%)	0.313 (0.105; 0.931)	0.037
Compromise ≥25%	5 (12.5%)	8 (21.1%)		
Compromise 26–50%	4 (10.0%)	8 (21.1%)		
Compromise >50%	1 (2.5%)	4 (10.5%)		
NUCLEI IN PALISADE FORMATIONS				
Absence of lesion	32 (80.0%)	23 (60.5%)	0.613 (0.299; 1.255)	0.180
Compromise 26–50%	6 (15.0%)	15 (39.5%)		
Compromise >50%	2 (5.0%)	-		
PSEUDO-GOBLET CELLS				
Absence of lesion	37 (92.5%)	8 (21.1%)	0.023 (0.006; 0.085)	<0.001
Compromise ≥25%	3 (7.5%)	20 (52.6%)		
Compromise 26–50%	-	7 (18.4%)		
Compromise >50%	-	3 (7.9%)		
OTHER HISTOLOGICAL LESIONS				
EXULCERATION				
Absence of lesion	33 (82.5%)	35 (92.1%)	3.586 (0.991; 12.984)	0.052
Compromise ≥25%	3 (7.5%)	3 (7.9%)		
Compromise 26–50%	1 (2.5%)	-		
Compromise >50%	3 (7.5%)	-		
DEGENERATION				
Absence of lesion	12 (30.0%)	4 (10.5%)	0.254 (0.062; 1.039)	0.057
Compromise ≥25%	21 (52.5%)	20 (52.6%)		
Compromise 26–50%	7 (17.5%)	8 (21.1%)		
Compromise >50%	-	6 (15.8%)		
REGENERATION				
Absence of lesion	40 (100%)	37 (97.4%)	*	*
Compromise 26–50%	-	-		
Compromise >50%	-	1 (2.6%)		
NECROSIS OF THE DUCTAL EPITHELIUM				
Absence of lesion	39 (97.5%)	37 (97.4%)	0.968 (0.090; 10.366)	0.978
Compromise ≥25%	1 (2.5%)	1 (2.6%)		
Compromise 26–50%	-	-		
Compromise >50%	-	-		

TABLE 6 - Comparisons between the experimental and control groups in terms of changes to the ductal epithelium for the 7-day subsequent euthanasia time point.

Changes to the ductal epithelium	Group		OR (CI 95%)	p-value
	Experimental (n = 48)	Control (n = 48)		
LESIONS CAUSED BY HEAT (THERMAL EFFECT)				
EPITHELIAL DETACHMENT				
Absence of lesion	44 (91.7%)	33 (68.8%)	0.246 (0.097; 0.626)	0.003
Compromise ≥25%	3 (6.3%)	6 (12.5%)		
Compromise 26–50%	1 (2.1%)	4 (8.3%)		
Compromise >50%	-	5 (10.4%)		
NUCLEI IN PALISADE FORMATIONS				
Absence of lesion	45 (93.8%)	33 (68.8%)	0.157 (0.067; 0.365)	<0.001
Compromise ≥25%	2 (4.2%)	12 (25.0%)		
Compromise 26–50%	1 (2.1%)	3 (6.5%)		
PSEUDO-GOBLET CELLS				
Absence of lesion	13 (27.1%)	21 (43.8%)	0.954 (0.404; 2.251)	0.914
Compromise ≥25%	20 (41.7%)	10 (20.8%)		
Compromise 26–50%	5 (10.4%)	10 (20.8%)		
Compromise >50%	10 (20.8%)	7 (14.6%)		
OTHER HISTOLOGICAL LESIONS				
EXULCERATION				
Absence of lesion	44 (91.7%)	41 (85.4%)	0.566 (0.179; 1.791)	0.333
Compromise ≥25%	4 (8.3%)	5 (10.4%)		
Compromise 26–50%	-	1 (2.1%)		
Compromise >50%	-	1 (2.1%)		
DEGENERATION				
Absence of lesion	3 (6.3%)	7 (14.6%)	1.122 (0.555; 2.266)	0.749
Compromise ≥25%	19 (39.6%)	18 (37.5%)		
Compromise 26–50%	11 (22.9%)	15 (31.3%)		
Compromise >50%	15 (31.3%)	8 (16.7%)		
REGENERATION				
Absence of lesion	47 (91.7%)	42 (87.5%)	0.246 (0.030; 2.032)	0.193
Compromise ≥25%	1 (2.1%)	2 (4.2%)		
Compromise 26–50%	-	4 (8.3%)		
NECROSIS OF THE DUCTAL EPITHELIUM				
Absence of lesion	41 (85.4%)	39 (81.3%)	0.489 (0.151; 1.570)	0.231
Compromise ≥25%	7 (14.6%)	5 (10.4%)		
Compromise 26–50%	-	3 (6.3%)		
Compromise >50%	-	1 (3.1%)		

When the analyses were conducted separately with respect to euthanasia timing, group differences in epithelial detachment and the presence of pseudo-goblet cells persisted at the immediate euthanasia time point, with the experimental group showing tendencies for lower severity scores in these two parameters. Meanwhile, group differences in epithelial detachment and nuclear palisade formation persisted at the one week delay euthanasia time point, with the experimental group showing tendencies for lower severity scores in both of these parameters as well.

Discussion

In this experiment, we successfully reproduced necrotic lesions in the hepatic parenchyma with an RD electrode probe in

laboratory animals, with consequent thermal lesions in the intra-hepatic bile ducts. Furthermore, analyzing observations from a pathologist who was blind to the group designations of the specimens, we demonstrated that the development of such thermal lesions could be attenuated by cooling the ducts. Thus, these new data suggest that complications arising from destruction of liver tumors through RFTA can be significantly abated.

To evaluate the scarring and regenerative effects of thermal lesions in groups with and without perfusion, our study design included groups of animals that were subjected to the same conditions and procedures except for the time of euthanasia. At both the immediate and subsequent euthanasia time points, there were more thermal lesions in the control group without chilling than in the experimental group with chilling. We did not observe scar lesions in either group at either time point, suggesting that the post-operative period was insufficient to allow for the scarring process to occur. Given that the bile ducts of the animals without chilling showed more acute thermal lesions than the animals given the chilling treatment, with a longer healing period, we would expect there to be more scarring and stenoses in the control group, similar to the findings of Marchal *et al*.[14].

Our findings complement previous work investigating the effects of intra-ductal chilling in pigs. Stippel *et al*.[15] reported anatomopathological and cholangiographic observations 28 d after RFTA showing that intra-ductal chilling can reduce the likelihood of biliary system lesions. Their findings suggested that chilling treatment can protect the bile duct from stenoses, necrosis, and other types of lesion.

Our finding that the areas of necrosis were not affected by the chilling treatment (Table 1), with the exception bile duct changes indicates that the chilling treatment did not interfere with tissue destruction. It also underscores the fact that there were minimal lesions of the blood vessels in the portal area. This phenomenon should be associated with a protective blood flow effect, commonly called the "radiator effect"[14]. With a chilling procedure like that tested in this study, the blood flow of the large liver vessels can pull heat away from the RF target site, providing some protection from the development of major lesions on blood vessel walls. Hence, the extent of direct thermal lesions may be influenced by modulating blood flow or administration of a thermosensitiving agent. The processes involved in thermal lesion progression and therapies, and the potential to modulate them, are not well understood[16].

We performed separate analyses of the datasets from the animals euthanized immediately postoperatively and the animals euthanized a week later because animals euthanized immediately after the operation should mainly have acute lesions caused by heat, whereas those euthanized later may have developed scar tissue. Our observations that the immediately euthanized animals showed treatment effects on frequency of leukostasis and hemorrhage (Table 2), while animals euthanized a week later showed treatment effects on the frequency of edema and leukostasis (Table 3), suggest that animals subjected to the chilling treatment were altogether less prone to local inflammation in the area of the injury than control animals.

When we specifically studied changes in the ductal epithelium, it became clear that perfusion of the bile ducts with a chilled solution conferred a protective effect (Tables 4, 5 and 6). Such differences were first observed with respect to changes to the biliary ductal epithelium, such as epithelial detachment, nuclei arranged in a palisade formation, and the presence of pseudo-goblet cells, which are histological findings directly related to the thermal effect of the treatment[17]. These changes are direct consequences of cellular and subcellular damage caused by heat, characterized by destruction of cell membranes (considered the principal cause of cell death), nucleic acids, cytoskeletal components, the Golgi apparatus, lysosomes (lysis and liberation of intra-cellular enzymes) and, above all, irreversible damage to mitochondria. The progression of a thermal lesion, which has been described as proceeding for up to a week, represents a balance of various activating and inhibiting factors, such as apoptosis, Kupffer cell activation, cytokine release, reperfusion ischemia-related tissue damage, and immune system stimulation[16].

Our literature review did not uncover any previous publication studying the thermal effects of RF on bile ducts in animals subjected to hepatic perfusion with a refrigerated solution that involved a sizeable number of histological variables, details, measurements, and details of the possible histological changes to the biliary epithelium, the bile ducts, and the area affected by the liberation of heat. However, detailed histological studies of other organs have provided prior demonstrations of the characteristics of tissue damage related heat exposure. In a pathology study performed on the forensic autopsies of 88 victims of fatal burns and smoke and hot air vapor aspiration, Bohnert *et al*.[17] observed that the typical symptoms of heat exposure were vesicular detachment of the epithelium, pseudo-goblet cells, an increase in mucus secretion, hyperemia, and edema, as well as elongated nuclei arranged in a palisade formation in the epithelial bed of the trachea and bronchi.

Consistent with Bohnert *et al*.[17] observations, we observed more severe lesions in the control group (with no perfusion of the bile ducts) than in the animals whose bile ducts

were protected by way of heat dissipation (Table 4). There were clear signs of thermal lesions in the animals whether they were subjected to immediate euthanasia or euthanized a week later. We did not observe any signs of scar tissue or reparative lesions of a chronic nature in any of the bile ducts studied, suggesting that the additional time beyond a week may have been needed to evaluate definitive established lesions of the bile ducts involving fibrosis and stenoses.

Primary and metastatic liver tumors have been destroyed using RF for many years, and the method has been proven effective and is accepted in the surgical environment[1]. Focal hyperthermia involves complex mechanisms. Experimental evidence suggests that hyperthermal focal lesions occur in two distinct phases, each of which is caused by a distinct subjacent process. The first phase results in wounds caused by direct heat, which are determined by the total amount of thermal energy applied. The second phase involves indirect tissue damage, which produces a progression to tissue lesion after the initial thermal stimulus has ceased. This second phase lesion may involve a progressive equilibrium involving various factors, including apoptosis, microvascular damage, ischemic reperfusion, Kupffer cell activation, changes to cytokine expression, and changes in the immune response.

The main concern with RFTA is that the therapy can cause lesions of the intra-hepatic bile ducts when target tumors are in close proximity to these structures. Bilchik *et al.*[4] have described a case of bile duct stenosis in a patient treated by RF that required an endoscopic stent to be fitted. Similarly, Livraghi *et al*.[20] reported the case of a patient who developed cholecystitis after RFTA of a neoplasia near the bile duct.

Marchal *et al*.[14] suggested that such intrahepatic biliary duct lesions may be avoided by chilling of the liver via infusion of 5% GS (at 5 ºC) through a catheterized cystic duct. It is also possible to chill the intra-hepatic bile ducts with saline solution via a catheter using an US-guided nasobiliary probe[21]. Such techniques are intended to prevent thermal damage and thereby extend the indications for RF ablation to previously excluded tumor lesions[22]. However, Jersenius *et al*.[23] raised questions about the efficacy of chilling intra-hepatic bile ducts for protecting them from RF electrode-emitted heat.

Methodological considerations

We elected to use GS rather than a saline solution because the high osmolarity of saline solution allows it to conduct more heat to the hepatic parenchyma, making intra-hepatic bile duct lesions possible. Thus, in our study, we opted to use 5% GS (at 5ºC), as suggested by Marchal *et al*.[10], in order to avoid biliary lesions caused by electrical conductivity in the liver tissue, as can occur in livers perfused with saline solution.

Although smaller animals are easier to handle and cost less, we chose to use pigs in this study because the pig has a medium-sized portal vein and a liver that is anatomically very similar to that of humans, especially in terms of its dimensions. Similar justifications are used for using the pig is an experimental model of choice for a variety of medical conditions, including cardiovascular, integumentary, urinary, and digestive system models. And the pig is still considered to be the best model for training for open surgery, laparoscopy, endoscopy, and transplants.

The use of US for RF electrode probe guidance is a safe, well-tolerated, and versatile option for positioning the probe within the liver and for effective local control during tumor ablation[7]. Indeed US is particularly important in the treatment of small liver tumors for the application of percutaneous ethanol injection (PEI) as well as a guide for placement of the RF electrode probe[25].

Because we studied lesions in both the right and the left liver lobe, and examined different bile ducts within a single lesion (four quadrants with proximal and distal bile ducts) within each animal, these lesion sites cannot be considered as independent lesions, and thus we could not use the Chi-square method or Fischer's exact test. To resolve this problem, we used the GEE statistical method. GEEs were developed to produce more effective estimates and are not adjusted for the parameters of a regression model when dealing with correlated data, as they consider the structure of the correlation between the observations. This approach provides estimates of regression coefficients and SDs with normal sample distributions, making it possible to test main effects and interactions, and thus allowing for independent or continuous variables to be evaluated. This approach meets the objective of statistical analysis to describe a variable in relation to a conjunction of covariables, considering the correlation between the observations.

Conclusion

Infusion of 5% glucose solution (5°C) has a protective effect on bile ducts subjected to heat (95–110°C, 12 min) from radiofrequency thermal ablation device.

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