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Effects of Freezing and Frozen Storage on Histological Characteristics of Canine Tissues

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ABSTRACT: Frozen tissues were studied histologically to determine what changes were produced by freezing. Samples of brain, lung, liver, small intestine, and kidney from 20 stray dogs were treated in 3 ways: formalin fixation (control), frozen for 2 days plus formalin fixation, or frozen for 7 days plus formalin fixation. Major histological changes caused by freezing were loss of staining, extracellular fluid accumulation, cell shrinkage, fractures, hemolysis, and hematin formation. Lesser changes included loss of bronchial cilia, prominence of collagen in alveolar septa and meninges, and intracellular vacuolization of epithelial cells. Although these changes were annoying, adequate visualization of the tissues was usually possible.

KEYWORDS: pathology and biology, freezing, tissues (biology), microtomy, frozen sections, tissue preservation

Pathologists are sometimes asked to interpret tissue that has been frozen accidentally or has been removed from a frozen cadaver. Everyone intuitively knows that frozen tissue can be examined, but actual reports of controlled studies were not found. Relevant reports [*1-5*] have recommended not freezing tissues intended for histological examination, and others have described freezing artifacts [*2,5-6*].

Therefore, we felt that it would be beneficial to know what statistically significant histopathological changes occur and if increased frozen time mades a difference. In the study reported here, the effect of freezing and frozen storage time on histological parameters for canine lung, liver, kidney, small intestine, and brain have been determined.

Materials and Methods

Subjects

Organ samples were collected from 20 stray dogs presented to Kansas State University for euthanasia. Dogs were necropsied within 8 h of death. They were without gross lesions. The following organs were sampled:

Lung—right apical lobe, approximately 1 cm in thickness
Liver—right lobe, approximately 1 cm in thickness

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Kidney—right, transverse sections, 1 cm in thickness
Jejunum—cranial, approximately 3 cm long
Brain—right hemisphere, transverse sections, 1 cm in thickness

Three adjacent samples were collected from each organ and were assigned randomly to one of the following treatments:

1. Immediate fixation in 10% buffered formalin at room temperature (control).
2. Frozen to −18°C and stored at the same temperature for two days, followed by fixation of still frozen tissue in 10% buffered formalin at room temperature.
3. Frozen to −18°C and stored at the same temperature for seven days, followed by fixation of still frozen tissue in 10% buffered formalin at room temperature.

After fixation the tissues were trimmed, embedded, sectioned, and stained with hematoxylin and eosin. One slide was made for each sample and treatment, 15 slides per dog.

Histological Evaluation

In the different organs studied, the following parameters were evaluated:

Lung—Bronchial transudate; loss of cilia; alveolar transudate; alveolar wall changes; hemolysis; loss of vascular endothelium; pleural and intralobular connective tissue changes; and hematin.
Liver—Hepatocyte color, shape, and nucleus; sinusoidal distension; hemolysis; portal triad cellular and staining characteristics; pigment; and hematin.
Kidney—Renal corpuscle shrinkage, distension, and material in capsular space; tubular epithelial cell vacuolation, detachment, and casts; interstitial alterations; hemolysis; and stain precipitates.
Intestine—Mucosal autolysis; lamina proprial changes; glandular epithelial damage; submucosal, tunica muscularis, and serosal alterations; and hemolysis.
Brain—Gray and white matter fractures; neuronal and meningeal changes; and hemolysis.

For each slide, the above parameters were evaluated using the following scale:

0 = no alterations
1 = mild alterations
2 = mild to moderate
3 = moderate alterations
4 = moderate to severe
5 = severe alterations

Each parameter on each slide was evaluated and given a number from the above scale. This procedure was then repeated at a later time so that there were two independent evaluations of each slide.

Statistical Analysis

Analysis of variance procedures were used to determine the significance of the differences between treatments for each parameter ($P < 0.05$). Comparisons between treatment means were done using a protected Fisher's LSD test [7] by the Statistical Analysis System procedure [8].

Results

Unfrozen Tissue (Controls)

The lung samples showed congestion, a small amount of pink transudate in the bronchi and alveoli, and a mild loss of bronchial cilia. All liver slides had both autolytic and well-preserved

areas. Hepatic changes consisted of variability of staining, presence of small dark nuclei, dilated sinusoids, and yellow intracytoplasmic pigment. Renal changes were mild and consisted of congestion, and vacuolization of tubular epithelial cells. The small intestine showed mucosal autolysis, desquamation of epithelial cells with denuded villi. Intestine from one dog had nematodes in the lamina propria. Brain samples had mild degenerative changes of neurons characterized by increased eosinophilia and occasional loss of nuclei; seven dogs had lipofuscin pigment in neurons.

Frozen Tissue

Lung—Lung parameters are summarized in Table 1. The bronchi contained a homogeneous, noncellular, pink transudate. Although present in the controls, this transudate increased following two days of freezing with further significant increases after seven days of frozen storage. Loss of cilia was more severe than in the controls, but did not increase further with seven days of storage. In some cases cilia remained distinct (Fig. 1).

Alveolar lumens had the same transudate which increased after freezing for two days and increased further by seven days. Moderate septal changes consisted of cell shrinkage; sometimes only thin eosinophilic walls with dark shrunken nuclei remained. Collagen fibers were markedly visible.

In blood vessels, hemolysis increased significantly after freezing, for both two days and seven days. Endothelial sloughing increased significantly with freezing for two days and further after seven-days storage.

The interlobular connective tissue was fragmented; the pleura was shrunken. Further damage was seen after seven-days storage.

Hematin formation was significantly higher in the frozen groups than in the control group. This exogenous, brownish-black pigment was sited largely in interstitial tissue. No difference in amounts of hematin existed between the two frozen groups.

Liver—Liver parameters are summarized in Table 2. Hepatocyte color was paler than in the control group and became even paler after seven days of freezing. Some hepatocytes were shrunken following freezing, and shrunk further with time (Fig. 2). Nuclei were smaller and darker in both frozen groups than in the control.

Sinusoidal distension (Fig. 2) increased by freezing for two days, with further distension after seven days. Sinusoids were dilated, irregular, and filled with a homogeneous, pink,

TABLE 1—*Means for unfrozen and frozen lung samples from 20 dogs.*

Parameter	Unfrozen	Frozen		Standard Deviation
		2 days	7 days	
Bronchi				
Transudate	1.25^{a*}	2.85^{b}	3.37^{c}	0.11
Loss of cilia	1.25^{a}	3.37^{b}	3.57^{b}	0.11
Alveoli				
Transudate	1.25^{a}	3.02^{b}	3.42^{c}	0.10
Wall	1.50^{a}	3.15^{b}	3.37^{b}	0.12
Blood vessels				
Hemolysis	0.87^{a}	4.75^{b}	4.85^{b}	0.04
Endothelium sloughed	0.75^{a}	2.67^{b}	3.07^{c}	0.11
Pleura and connective tissue	0.85^{a}	2.26^{b}	3.27^{c}	0.14
Hematin	0.60^{a}	1.40^{b}	1.75^{b}	0.16

*0 = no alterations, 1 = mild, 2 = mild-moderate, 3 = moderate, 4 = moderate-severe, 5 = severe.

^{a,b,c}Means within the same parameter with different superscripts are significantly different ($P < 0.05$).

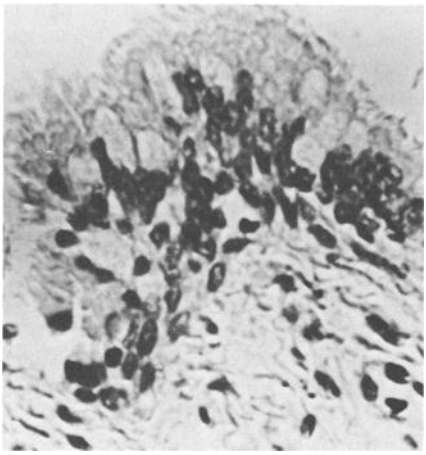

FIG. 1—*Lung bronchiolar cilia are distinct. Frozen seven days: H & E stain (×400).*

TABLE 2—*Means for unfrozen and frozen liver samples from 20 dogs.*

Parameter	Unfrozen	Frozen		Standard Deviation
		2 days	7 days	
Hepatocytes				
Color	2.10^{a*}	3.92^{b}	4.35^{c}	0.09
Shape	2.35^{a}	4.15^{b}	4.60^{c}	0.08
Nuclei	1.62^{a}	3.27^{b}	3.60^{b}	0.12
Sinusoids				
Distended	2.70^{a}	4.02^{b}	4.50^{c}	0.09
Hemolysis	1.15^{a}	4.95^{b}	4.95^{b}	0.04
Portal triads				
Cellular characteristics	1.35^{a}	3.17^{b}	3.42^{b}	0.11
Staining characteristics	1.35^{a}	3.60^{b}	3.75^{b}	0.11
Pigment	1.07^{a}	1.20^{a}	1.20^{a}	0.05
Hematin	1.45^{a}	3.40^{b}	3.52^{b}	0.13

*0 = no alterations, 1 = mild, 2 = mild-moderate, 3 = moderate, 4 = moderate-severe, 5 = severe.

^{a,b,c}Means within the same parameter with different superscripts are significantly different ($P < 0.05$).

non-cellular transudate; occasional erythrocytes were present. With further distension, trabeculae were more compressed, leaving large areas of transudate. Hemolysis was complete by two days.

In portal triads, changes were seen in both frozen groups but not the controls. The portal area itself stained paler. Bile duct epithelium was moderately shrunken and vacuolated.

Brownish-yellow, intracytoplasmic pigment was seen within macrophages and hepatocytes. Hematin increased in both frozen groups.

Kidney—Renal parameters are summarized in Table 3. Although a certain amount of glomerular shrinkage was observed on the external surface of frozen sections, the more common feature was distension of the glomeruli after freezing, for both two and seven days. Although there was distension of glomerular tufts, an eosinophilic, homogenous, noncellular substance

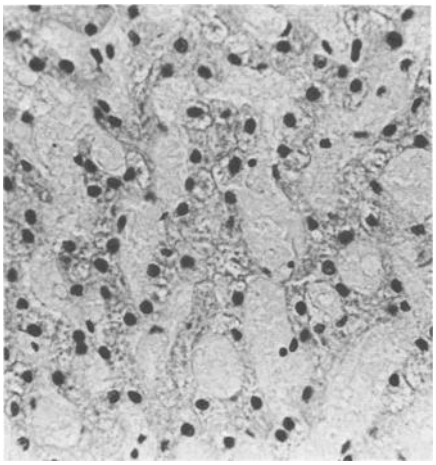

FIG. 2—*Liver with dilated sinusoids and shrunken hepatocytes. Frozen two days: H & E stain (×200).*

TABLE 3—*Means for unfrozen and frozen kidney samples from 20 dogs.*

Parameter	Unfrozen	Frozen		Standard Deviation
		2 days	7 days	
Renal corpuscles				
Shrinkage	0.00^{a*}	0.12^{a}	0.12^{a}	0.06
Distension	1.02^{a}	3.57^{b}	3.77^{b}	0.11
Capsular space material	0.62^{a}	2.72^{b}	3.20^{c}	0.12
Tubules				
Vacuolation	0.95^{a}	3.50^{b}	3.87^{c}	0.09
Epithelial detachment	0.95^{a}	3.45^{b}	3.90^{c}	0.11
Casts	0.95^{a}	3.35^{b}	3.75^{c}	0.09
Interstitium	1.05^{a}	3.72^{b}	4.13^{c}	0.11
Hemolysis	0.47^{a}	5.00^{b}	5.00^{b}	0.02
Hematin	0.75^{a}	1.42^{b}	1.70^{b}	0.22

*0 = no alterations, 1 = mild, 2 = mild-moderate, 3 = moderate, 4 = moderate-severe, 5 = severe.

^{a,b,c}Means within the same parameter with different superscripts are significantly different ($P < 0.05$).

was seen in increased amounts in the capsular space. A further increase was apparent subsequent to seven-days storage.

The tubular epithelium had increased numbers of small, irregular vacuoles after freezing, for both frozen groups. Epithelial separation from their basement membranes were increased by freezing. Separation sometimes consisted of isolated cells, but more frequently the whole epithelial lining was detached from the basement membrane. Seven-day frozen storage further increased separation. Casts, both cellular and hyaline, appeared after freezing and were increased by longer frozen storage.

Interstitial damage consisted of capillary distension, areas filled with a homogeneous, eosinophilic fluid with vacuoles, and fractures. These changes increased with storage time. Hemolysis was complete by two days. A mild increase in hematin was observed in both frozen groups.

Small Intestine—Intestinal parameters are summarized in Table 4. Mucosal autolysis was increased in both frozen groups. More necrotic cells, desquamation, and, in the lamina propria, spaces filled with pink fluid were observed. Additional frozen storage time did not increase these changes. Parasites, observed in the lamina propria of one dog, were not damaged by freezing. Glandular epithelial damage was characterized by shrunken cells and lumens filled with pink fluid. Goblet cells were clearly delimited spaces that did not stain.

The submucosa had fractures that increased in size with storage time. The tunica muscularis also had fractures, but storage did not further increase their incidence. Fractures in both locations followed fiber direction. Serosal detachment increased with freezing and with storage time. Hemolysis was complete by two days.

Brain—Fractures (Fig. 3), which appeared as large, irregular, clear areas, sharply increased in number both in gray and white matter after freezing. Further increases in size were seen with longer storage (Table 5). Degenerating neurons were more numerous after freezing with further increases after storage. They were characterized by increased eosinophilia, loss of nuclei, and loss of cell outlines. Detachment of leptomeninges and prominence of meningeal connective tissue were observed following freezing and increased with storage. Hemolysis was complete by two days.

TABLE 4—*Means for unfrozen and frozen small intenstine samples from 20 dogs.*

Parameter	Unfrozen	Frozen		Standard Deviation
		2 days	7 days	
Mucosal autolysis	1.17^{a*}	3.30^{b}	3.42^{b}	0.09
Lamina propria	1.00^{a}	3.35^{b}	3.57^{b}	0.09
Glandular epithelium	0.82^{a}	3.15^{b}	3.47^{c}	0.08
Submucosa	0.70^{a}	2.62^{b}	2.90^{c}	0.09
Tunica muscularis	0.50^{a}	3.85^{b}	4.07^{b}	0.09
Serosa	0.47^{a}	3.67^{b}	4.00^{c}	0.09
Hemolysis	0.45^{a}	5.00^{b}	5.00^{b}	0.02

*0 = no alterations, 1 = mild, 2 = mild-moderate, 3 = moderate, 4 = moderate-severe, 5 = severe.

^{a,b,c}Means within the same parameter with different superscripts are significantly different ($P < 0.05$).

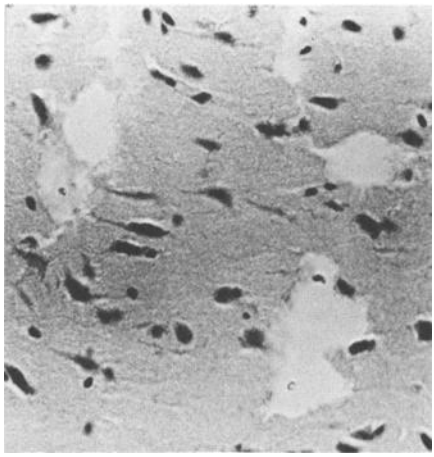

FIG. 3—*Cerebrum with many fractures. Frozen two days; H & E stain (×200).*

TABLE 5—*Means for unfrozen and frozen brain samples from 20 dogs.*

Parameter	Unfrozen	Frozen		Standard Deviation
		2 days	7 days	
Gray matter fractures	0.00^{a}*	3.75^{b}	4.60^{c}	0.11
White matter fractures	0.00^{a}	4.12^{b}	4.72^{c}	0.09
Neurons	0.85^{a}	3.60^{b}	4.37^{c}	0.10
Meninges	0.20^{a}	3.07^{b}	3.90^{c}	0.10
Hemolysis	0.45^{a}	5.00^{b}	5.00^{b}	0.01

*0 = no alterations, 1 = mild, 2 = mild-moderate, 3 = moderate, 4 = moderate-severe, 5 = severe.

^{a,b,c}Means within the same parameter with different superscripts are significantly different ($P < 0.05$).

Discussion

An eosinophilic, homogenous, extracellular fluid accumulation [*6–8*] was a constant feature of our frozen tissues. It may be explained through osmotic mechanisms consequent to increased extracellular solute concentration caused by freezing [*9–14*], or to membrane damage [*11,12,15–20*]. Both mechanisms would allow passage of fluid from the intracellular to the extracellular space. In this study, brownish-yellow pigment (bile) was present in hepatocytes and macrophages following freezing. This finding suggests that cell membrane damage was not extensive.

This loss of cellular fluid was the probable cause of two other major changes seen: cellular shrinkage and loss of staining. Loss of staining was seen in all frozen tissues and was characterized by uniform but lighter color to all structures. This change has been observed by others [*18*]. Cellular shrinkage is the most commonly reported damage as a result of freezing [*6,11–14,18–24*]. In our study, all organs showed cellular shrinkage; however, it also occurred to a mild degree in unfrozen sections.

Fractures were seen commonly in frozen tissues, especially in the brain and in the tunica muscularis of the small intestine. It has been reported that ice crystals form when tissue is frozen. During processing, the ice melts, leaving extracellular fractures in the tissue. Ice crystal formation can also occur within cells [*20,25*]. Intracytoplasmic vacuoles of varied size and number were observed in lung, liver, kidney, and intestinal mucosa. We did not do additional studies to determine if these vacuoles were ice or degenerative changes.

Hemolysis was seen in all organs that were frozen. Hemolysis did not increase with storage because it was complete by two days. This finding has been reported [*20*] as an "all or none" phenomenon, probably triggered by osmotic changes in plasma as a result of extracellular solute concentration [*13,19,23,26–28*]. Hemolysis probably contributed to the eosinophilic extracellular fluid described above.

Hematin was seen in increased amounts in lung, liver, and kidney that had been frozen. It was typical in that it was dark brown, in tiny particles, anisotropic, and concentrated on blood rich areas. Since it was not evident in not frozen tissue, it is probable that the pH of the formalin was altered by the frozen tissues, or the tissues may have been put in too small an amount of buffered formalin after freezing. Although hematin does not alter tissue characteristics, it is annoying to have on a slide.

Bronchial cilia were lost in some areas, but retained in others. Eosinophilic fluid accumulation did not always allow adequate visualization of the epithelial surface, so more cilia may have been present than were seen.

Collagen fibers in the pleura, interlobular connective tissue, and meninges were more distinct after tissues were frozen. This finding is best explained by the fact that collagen is more re-

sistant to deterioration than the surrounding tissues, so shrinkage of surrounding cells makes it appear more prominent.

In addition to the changes seen in individual organs, freezing effects were also observed between organs. The brain was the most affected organ with fractures and neuronal degeneration. The liver was the second most affected with loss of architecture, shrunken hepatocytes, and dilated sinusoids. Small intestinal changes were intermediate. The lung and kidney were affected least by freezing.

Most histological differences were between unfrozen and frozen tissue rather than between two and seven days of freezing. However, parameters that did change quantitatively with additional freezing time included extracellular fluid accumulation, fractures, sinusoidal dilatation, and renal tubular changes.

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