EFFECTS OF FREEZING AND FROZEN STORAGE ON HISTOLOGIC CHARACTERISTICS OF CANINE TISSUES

by

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INTRODUCTION

In clinical practice, necropsy specimens are often not submitted to a diagnostic laboratory because of a lack of appropriate fixative substances. Since most clinicians have a freezer available, those samples could be frozen for later shipment to a diagnostic laboratory. However, the procedure would be beneficial, only if adequate evaluation can be made from frozen specimens.

Although it has been traditionally thought that freezing damages tissue and is a source of artifacts in histological specimens, it is very important to evaluate the real extent of that damage and its significance regarding diagnosis.

The objectives of this study were to determine:

1) a detailed description of the actual damage to the histological characteristics of necropsy specimens, due to freezing and frozen storage
2) if damage to tissue frozen for 7 days is the same as damage to tissues frozen for 2 days, and
3) if a diagnosis can be made from samples submitted to such treatments.

I. REVIEW OF LITERATURE

Freezing Injury

Since all biological structures contain water, it is not surprising that they are disastrously affected by exposure to temperatures below 0 C (Mazur, 1966). When a solution or suspension freezes, ice separates as a pure substance. The dissolved or suspended matter is concentrated in the fluid that remains (Lovelock, 1954). According to Pegg (1976), freezing is the separation of pure water into ice, thus concentrating any remaining solutes. This thought raises the possibility of two different mechanisms for freezing injury: 1) the mechanical action of ice itself and 2) the altered osmotic behavior of the liquid phase. Meryman (1956), stated that freezing represents nothing more than the removal of pure water from solution, into biologically inert ice crystals. All the biochemical, anatomical, and biological sequelae of freezing are consequences of this single event.

When a suspension of live cells is submitted to subfreezing temperatures, their first response is to crowd together (Lovelock, 1954). The biological response of a cell to freezing, depends mostly on how well it reacts to an increase in concentration of the surrounding medium. Even when the cells are completely surrounded by ice, and the environmental temperature is -5 C or -10 C, the cell interior nearly always remains unfrozen (Mazur, 1966). Lovelock (1954) stated that -5 C represents the lower limit to which mammalian cells can be frozen slowly without damage. Between -5 C and -10 C, they will freeze internally. Chambers and Hale (1932) found that living protoplasm could be cooled to an extraordinary extent without being killed.

Freezing occurs either intracellularly or extracellularly and is dependent on cooling speed, cell size, and membrane permeability. Chambers and Hale (1932), worked with fresh water amoebas and muscle fibers from the sartorius of the frog. They found that cell membranes act as a barrier, delaying or preventing invasion of the cell by ice. Once the protoplasm itself is frozen, the cell dies;.plasmolysis occurs on thawing.

Cooling speed is one of the major factors which determines whether a cell will dehydrate (loose its fluid) or freeze intracellularly (Mazur, 1966). Merymann (1956) stated that with slow freezing, ice formation is generally confined to the extracellular spaces and that there is usually no membrane rupture. Even if kept frozen for long periods of time, the water is reimbibed by the living cell after it is thawed. Its histological appearance is often indistinguishible from the unfrozen cell. Luyet and Gehenio (1940) showed that tissue can tolerate the presence of extracellular ice, probably because no mechanical pressure is put on the cells.

Mazur (1966) recorded that the rate of temperature change can affect the size and shape of the ice crystals, both outside and inside the cell. Crystals tend to be smaller with faster cooling rates. Slow cooling produces relatively stable crystals which tend to be large and outside the cell.

Meryman (1970) mentioned that intracellular ice is a special situation which probably does not occur in animal tissues except in laboratories using very high cooling rates. However, in plant tissues, intracellular freezing occurs even at low rates of cooling Lozina-Lozinsky (1963) showed survival of insects at temperatures below 0 C. Deformed cells were not found in insects; these organisms are apprently resistant to intracellular crystallization. Sherman (1962) found that cells of mouse skin and of two parakeet tumors survived

intracellular ice formation. Mazur (1966) stated that extracellular freezing is a prerequisite to injury, but is not lethal by itself. He found that 80% of yeast cells were able to survive cooling to -10 C. Asahina (1961) stated that the main cause that killed sea urchin egg cells, while freezing, was intracellular freezing.

The events occurring in cells at subzero temperatures were summarized by Mazur (1966). He stated that lowered temperatures can affect cells by lowering cell temperature or by ice formation. There are two main consequences: 1) increases in concentration of both intra- and extra-cellular solutes, and 2) deposition of ice crystals. Deposition can occur intracellularly when cooling velocities are high, and extracellularly when cooling velocities are slow. Intracellular crystals are usually not stable and are subject to alterations during storage and thawing. Extracellular ice formation results in dehydration of the cell. The principal cause of injury from slow freezing is not the physical presence of extracellular ice crystals, but the dehydration resulting from the incorporation of the free water into ice (Meryman, 1956).

Influence of Thawing

The freezing process consists of freezing, frozen storage, and thawing. Each step must be properly conducted to obtain optimum results for preserving biological systems (Fennema et al., 1973). Mazur et al. (1970) stated that the final temperature attained, the cooling rate, the storage time, and the warming rate affect the survival of cells. Meryman (1966), stated that the effect of thawing rates has received less attention than freezing rates, because its effects are less striking. Damage from thawing occurs from high extracellular electrolyte concentration and from recrystallization.

Recrystallization, defined by Zaritzky et al. (1982) as an increase in the original crystal size, occurs when frozen products are temporarily subjected to an increase in temperature. Small ice crystals disappear and leave only the larger ones to increase in size. Fennema et al. (1973) defined recrystallization to include any change in the number, size, shape, and orientation of crystals during storage.

Cell damage

There is a wide variability in the response of living tissues to freezing. While epidermal and muscle cells can withstand mild freezing for an hour or more, erythrocytes die almost instantly (Meryman, 1956). Nerve and liver cells from embryonic tissue of rat, mouse, and ox were most sensitive to cold in a range from 0 to -15 C, whereas, heart, lung, and intestine were more resistant to cold (Luyet and Gehenio, 1940). Sato (1958) described various degrees of cell injury. Working with Bacillus megatherium, he found loss of cell shape, cell shrinkage, cell debris, and changes in staining characteristics. He found no remarkable change in the nucleus.

Most of the morphological alterations observed in frozen tissue were described in muscle. Hiner et al. (1945) found that with higher temperatures (18 F) large extracellular ice crystals pushed the fibers together into groups; no intrafibrilar ice was observed. At lower temperatures (0 F), intrafibrilar freezing and some fiber wall damage did occur. Intracellular ice became more extensive, as freezing temperature dropped. Precipitated proteins and nuclear fragments, outside the fibers, became more extensive as freezing temperatures were lowered. Hiner and Hankins (1947) reported an increase in muscle tenderness with lowered temperature. They interpreted this finding as due to

an increased fiber rupture from intrafibrilar ice formation, as well as to the stretching and rupturing of the interstitial connective tissue.

A method for studying the histological structure of frozen poultry products was developed by Koonz and Ramsbottom (1939). Small pieces of frozen sections were dehydrated, fixed, embedded, sectioned at 14 microns and stained with Delafield's hematoxylin. Muscle tissue, which had been slowly frozen, was characterized by larger and fewer ice formations and by fibers in ridge- and band-like formations. They concluded that size, age, and physiological condition of the birds, precooling conditions, and postmortem delay in freezing are all factors that may affect the appearance of frozen tissues.

Freezing and dehydration were studied by Du Bois et al. (1962) in 5 months old New Hampshire Red cockerels. They used different rates of freezing (10 F, 0 F, -8 F and -25 F) and 3 different freezing techniques (still air, blast freezer, and plate freezer at -25 F). Photomicrographs showed that both longitudinal and cross sections of rapidly frozen muscle closely resembled that from unfrozen sections.

Love (1958) studied the effect of intracellular ice in fish muscle cells. He measured DNA phosphorous content from fluid, expressed from thawing fish fillets, as an index of cellular damage resulting from freezing. He also estimated relative quantities of microsomes, as an indicator of cell rupture. Love's results were summarized by Lawrie (1979). He described a cyclic change of cell damage with changes in the freezing rate. A definite pattern of high damage peaks appeared as the freezing rate increased. Differences in size, number, and location of ice crystals were correlated with low and high levels of DNA phosphorous.

Love and Haraldsson (1961) studied the effect of rigor on freezing damage in fish fillets. Patterns of ice-crystal formation, in pre- and post- rigor fish muscle, is different. Intracellular ice crystals, in pre-rigor muscle, are more numerous and smaller than those in post-rigor muscle. Post-rigor muscle freezing starts in the extracellular fluid. Hegarty and Naude (1973) measured fiber diameter in pre- and post-rigor mouse muscle. They found diameter differences between pre- and post-rigor unfrozen muscle. There were no diameter differences in frozen muscle. They also stated that potential shortening, of up to 20% of the muscle fiber, can occur, accompanied by swelling, structural disorganization, and exudation of an acid fluid often amounting to as much as 35% of the original muscle weight.

Blood freezing has been researched extensively as it relates to transfusion use. Doebbler et al. (1966) described mechanisms of freezing injury, such as ice crystal formation, size, location and growth rate, and solute concentration in the unfrozen solution. They concluded that concentration of electrolytes, and ice crystal formation and growth are the important sources of injury to blood components. Cell membrane damage resulting in hemolysis has been measured quantitatively (Nei, 1976).

Shrinkage ratios of lung tissue after freezing, compared to formalin fixation, were studied by Tsunoda and Martin (1972). They found that changes in volume and linear dimension were similar for both techniques.

Zaritky et al. (1982) studied the effect of freezing on surface color in liver. White areas on the liver surface were produced by high freezing rates. Microscopically these areas contained small intracellular crystals. Darker areas occurred with slower freezing rates and contained large extracellular crystals.

Sandberg (1977) observed the same change in muscle. Rapid freezing promoted the formation of small ice crystals, which caused a paler muscle color.

Dietzman et al. (1973) reported functional success following freezing of living canine kidney at -22 C. Renal cell damage was evaluated by means of serum lactic acid dehydrogenase (LDH) and glutamic oxalacetic transaminase (GOT) studies, and tissue adenosine triphosphate (ATP) levels. Formation of large damaging ice crystals was the result of slow freezing and slow thawing rates. In another renal study, Luyet (1970) found that storage of kidneys at temperatures warmer than -62 C, resulted in the formation of large ice crystals.

Freezing of canine digestive tracts for parasitological studies has been reported by Hubert (1980). He found that freezing caused no change in morphology, nor in the number of parasites.

The effects of freezing of feline brain tissue was studied by Walder (1970) by means of electron microscopy. In situ freezing and rewarming, by means of a freezing probe, showed that the principal disturbances are loss of astroglial membranes and swelling of mitochondria of neurons, oligodendrocytes, and capillary endothelial cells.

Morphologic Changes During Storage

Love (1966) stated that ice crystal patterns, formed at freezing, are not necessarily maintained in the tissues during prolonged storage, but that crystals gradually increased in size. Moran and Hale (1932) showed that in frozen beef the small crystals grew into larger ones. They observed rounding of the ice crystals. This change could be prevented by storage below -20 C. Ramsbottom and Koonz (1941), however, reported little apparent change in the ice crystal structure of frozen beef stored at 10 F or at -30 F during one year. Fennema

et al.(1973) stated that when storage continues for long periods, many products, especially those stored near -18 C, undergo more deterioration than what they do during freezing and thawing.

Freezing and Histotechnology

Veit and Walton (1981) and Walton and Liebke (1975) recommended that if tissues cannot be immediately fixed, they should be refrigerated. Tissues should not be frozen, since "freezing causes significant cellular disruption".

Dawson (1972) recommended rapid freezing techniques to avoid gross ice crystal artifacts. He stated that success in freezing techniques and avoidance of gross ice-crystal artifacts is largely dependent on the speed of freezing, slow freezing techniques being useless.

Zappi and Zappi (1980) defined freezing artifacts as "a profound morphological distortion with nuclear shrinkage, effacement of the cytoplasmic boundaries with complete obliteration of cellular details, and formation of clefts and irregular pools filled with amorphous eosinophilic material". Armed Forces Institute of Pathology (1968) stated that cell and tissue distortion, promoted by ice crystal formation, is fixed by formalin. After the tissue is thawed, vacuoles remain as fixed artifacts.

Freezing technique has a special use in laboratory diagnosis when a rapid diagnosis is essential, or when elements such as fats, enzymes, and some radioisotopes would be lost in alcohol or paraffin solvents (Humason, 1972). Although this technique preserves sufficient cell structure for immediate diagnosis of surgical biopsies, it is objected by purists who recommend it only for very limited uses.

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II. EFFECTS OF FREEZING AND FROZEN STORAGE ON HISTOLOGICAL CHARACTERISTICS OF CANINE TISSUES

INTRODUCTION

In clinical practice, necropsy specimens are often not submitted to a diagnostic laboratory, because of a lack of appropriate fixatives. Since most clinicians have a freezer available, those samples could be frozen for a later shipment to a diagnostic laboratory. However, the procedure would be beneficial, only if adequate evaluation can be made from frozen specimens.

Many references [1-4] have recommended not freezing tissues intended for histological examination. However, actual histopathological description was seldom available.[1, 5, 6] In the present study, the effect of freezing and frozen storage time on histological parameters for lung, liver, kidney, small intestine, and brain have been determined. The ability to make a diagnosis from frozen tissue was evaluated.

MATERIALS AND METHODS

Subjects

Organ samples were collected from 20 stray dogs presented to Kansas State University for euthanasia. Dogs were necropsied within 8 hours of death; they were without gross lesions. In addition, the same organ samples were taken from 10 clinical cases submitted to the Small Animal Clinic for evaluation; 2 died, 8 were euthanatized. Routine necropsy techniques were used for the 20 stray dogs and 10 clinic dogs. Organs were sampled;

Lung. Right apical lobe, approximately 1 cm in thickness.

Liver. Right lobe, approximately 1 cm in thickness.

Kidney. Right, transverse sections, 1 cm in thickness.

Jejunum. Cranial, approximately 3 cm long .

Brain. Transverse right hemisphere, 1 cm in thickness.

Three samples were collected from each organ and were randomly assigned 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 2 days, followed by fixation in 10% buffered formalin at room temperature.
3) Frozen to -18 C and stored at the same temperature for 7 days, followed by fixation 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 exudate; loss of cilia; alveolar exudate; alveolar wall changes; hemolysis and vascular endothelium; pleural and intralobular connective tissue shrinkage and eosiniphilia; and stain precipitates.

Liver. Hepatocyte color, shape, and nucleus; sinusoidal distension; hemolysis; portal triad cellular and stain characteristics; pigment; and stain precipitates.

Kidney. Renal corpuscle shrinkage, distension, and material in capsular space; tubular epithelial cell vacuolation, detachment, and casts; interstitial alterations; vascular hemolysis; and stain precipitates.

Intestine. Thickness of mucosal autolysis; other mucosal changes; glandular epithelial damage; submucosal, tunica muscularis, and serosal alterations; and hemolysis.

Brain. Gray and white matter fractures; neuronal and meningeal damage; 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 slide was evaluated twice at different times; each evaluation was independent from the other.

Statistical Analysis

Data from the stray animals were submitted for statistical analysis. Analysis of variance procedures were used, in order 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[8] procedure.

RESULTS

Unfrozen Tissue (Control)

The lung samples had congestion, a mild amount of bronchial and alveolar exudate,and a mild loss of bronchial cilia. All liver slides had both autolytic and well fixed areas. Some hepatocytes had altered color and shape, as well as small dark nuclei. Dilated sinusoids were a typical feature. Yellow intracytoplasmic pigment was observed. Renal changes were mild and consisted of congestion, and vacuolization of tubular epithelial cells. The small intestine had mucosal autolysis, desquamation of epithelial cells, and denuded villi. Intestine from one dog had nematodes in the lamina propria. Brain slides had no fractures and mild degenerative changes of neurons characterized by increased eosinophilia and occasional nuclear loss. Seven dogs had lipofucsin pigment; 6 dogs had mild meningeal detachment and distinct collagen.

Frozen Tissue

All slides from frozen specimens stained paler than unfrozen samples. Damage was usually more evident in the periphery of the section, and decreased in deeper areas.

Lung

Lung parameters are summarized in Table 1. The bronchi contained a homogeneous, non-cellular, pink exudate (Fig 1). Although present in the controls, fluid increased following 2 days of freezing. Further significant increases were seen after 7 days of frozen storage. Loss of cilia was more severe than in the controls, but did not increase further with 7 days of storage. In some cases cilia remained distinct (Fig 2).

Alveolar lumens had the same exudate; it increased by freezing for 2 days with further increases by 7 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 2 days and 7 days. Endothelial sloughing increased significantly with freezing for 2 days and further after 7 days storage.

The interlobular connective tissue had fractures; the pleura was shrunken. Further damage was seen after 7 days storage.

More stain precipitate was present in the frozen groups than in the control group. This exogenous, brownish-black pigment, was sited largely in interstitial tissue; it resembled formalin pigment. No difference 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 7 days of freezing. Hepatocyte shape was distorted by shrinkage following freezing, and shrunk further with time (Fig 3). Nuclei were smaller and darker in both frozen groups than in the control.

Sinusoidal distension (Fig 3) increased by freezing for 2 days, with further distension after 7 days. Sinusoids were dilated, irregular, and filled with a homogeneous, pink, non-cellular exudate; occasional erythrocytes were present. With a more marked distension, trabeculae disappeared leaving large areas of exudate. Hemolysis was complete by 2 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 within macrophages and hepatocytes. Stain precipitates 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 2 and 7 days (Fig 4). Although there was distension of glomerular tufts, an eosinophilic, homogenous, non-cellular substance was seen in increased amounts in the capsular space. A further increase was subsequent to 7 days storage.

The tubular epithelium had increased numbers of small, irregular vacuoles after freezing, for both frozen groups. Epithelial separation from the basement

membrane was 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 increased with freezing and storage time. Changes consisted of capillary distension; areas filled with a homogeneous, eosinophilic fluid with vacuoles, and fractures. Hemolysis was complete by 2 days. A mild increase in stain precipitates was observed in both frozen groups.

Small intestine

Intestinal parameters are summarized in Table 4. Thickness of mucosal autolysis was increased in both frozen groups. More necrotic cells, desquamation, and in the lamina propia spaces filled with pink fluid were observed. Additional frozen storage time did not increase the damage. Parasites, observed in the lamina propia of one dog, were not damaged by freezing. Glandular epithelial damage increased with freezing and with time held. Although nuclear damage was absent, cells were shrunken and lumens contained pink fluid. Mucus disappeared following freezing, leaving goblet cells as clearly delimited spaces.

The submucosa had fractures which 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 2 days.

Brain

Fractures (Fig 5), 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. Damage to neurons increased with freezing; additional damage was seen in those held for 7 days. Degenerating neurons were characterized by increased eosinophilia, loss of nuclei, and loss of cell outlines. Detachment of meninges, as well as markedly visible collagen fibers, were observed following freezing and increased with storage. Hemolysis, was complete by 2 days.

Clinic Cases

Results from 10 clinical cases are summarized in Table 6 and illustrated in Fig 6 - 12. Histopathological changes are recorded under unfrozen tissue. Hitopathologic and freezing changes are listed under Frozen Tissue.

DISCUSSION

Major changes observed after freezing were loss of tissue staining, fluid accumulation, cellular shrinkage, fractures, and hemolysis. Less commonly found changes were loss of bronchial cilia, prominence of collagen in alveolar walls and meninges, intestinal mucosal sloughing, and intracellular vacuolization of epithelial cells.

The freezing process has been reported [9] to affect the staining characteristics of tissue. In this study all the frozen tissues stained paler than the control samples

An eosinophilic, non-cellular fluid accumulation[5,10] was a constant feature of frozen tissues. It may be explained through osmotic mechanisms consequent to increased extracellular solute concentration during freezing[11-16], or to membrane damage[9,14,17-24]. Both mechanisms would promote transport of fluid from the intracellular to the extracellular space. In this study, membrane damage was not extensive, in that the brownish-yellow pigment present in hepatocytes and macrophages remained intracelluar following freezing. This finding suggests that there was little or no leakage of cell membranes.

Cellular shrinkage, probably due to dehydration, was the most commonly reported damage due to freezing.[5,9,10,13-15,23-28] In this study all organs had cellular shrinkage; however, it also occurred to a mild degree in unfrozen sections.

Fractures were a constant feature in frozen tissues, especially the brain. Ice crystals form when tissue is frozen. During processing, the ice melts, leaving fractures in the tissue.[5,16-19]

In most samples, hemolysis was complete by 2 days. It has been reported[24] as an "all or none" phenomenon, probably triggered by osmotic changes in plasma due to extracellular solute concentration.[15,16,23,29-31] Hemolysis probably contributed to the pale staining of tissue mentioned above

Bronchial cilia were lost in some areas, but present in others. Furthermore, eosinophilic fluid accumulation did not always allow adequate visualization of cilia, so more cilia may have been present than seen. This is in agreement with a report[32] of the recovery of ciliar movement following freezing.

Collagen fibers in the pleura, interlobular connective tissue, and meninges were more distinct after freezing. This finding is best explained, in that

collagen is more resistant to deterioration than the surrounding tissues and the shrinkage of surrounding cells makes it appear more prominent.

Sloughed mucosa and denuded villi, following freezing of the intestine, have been described[33] and were seen here. Although mucosal changes are of more diagnostic value, damage was more visible in the muscular layer than in the mucosa. As noted by others[16,34], ice crystals followed the fiber axis direction.

Cytoplasmic vacuoles of varied size and number were observed in lung, liver, kidney, and intestinal mucosa. Intracytoplasmic crystal formation[24,35] might be the cause of this alteration.

Important variations in freezing effects were observed between organs. Brain was the most affected organ; fractures and neuronal damage were observed. Liver was the second most affected. Loss of architecture, shrunken hepatocytes, and dilated sinusoids were seen; however, in some areas large numbers of well preserved cells were present. A possible explanation of this variability could be differences in solute concentrations as a response to cell metabolic status. Small intestinal damage was intermediate. Lung and kidney were least affected by freezing

Most histological changes seen were between unfrozen and frozen tissue. Parameters that did change quantitatively with additional freezing time included: extracellular fluid accumulation, fractures, sinusoidal dilatation, and renal tubular changes. Recrystallization, with growth of ice crystals and increased osmotic gradients, is probably responsible for these changes[15,36,37]

In the 10 clinical cases evaluated, viral inclusion bodies, parasites, and neoplastic cells were well preserved. Diagnoses included canine distemper, Dirofilariasis, chemodectoma, and chronic glomerulonephritis. Since the first 10

dogs available for necropsy were used, all dogs did not have interesting diseases nor morphological changes. For example, in dogs 6 and 7, diagnoses were made from tissues not evaluated in this study; prostate and spinal cord. In conclusion, although freezing damages occurred, they did not interfere with making a histopathological diagnosis.

SUMMARY

Canine brain, lung, liver, small intestine, and kidney tissues that were either unfrozen, or frozen for 2 or 7 days were evaluated histologically. Major changes caused by freezing were loss of staining, fluid accumulation, cell shrinkage, fractures, and hemolysis. Lesser changes included loss of bronchial cilia, prominence of collagen in alveolar septa and meninges, sloughing of intestinal mucosa, and intracellular vacuolization of epithelial cells.

In addition, 10 canine clinic cases were evaluated. Histopathological changes seen in frozen tissue included viral inclusions, parasites, neoplastic cells, and fibrosis. Freezing caused tissue changes, but did not prevent diagnosis.

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TABLE 1 - Parameters for Unfrozen and Frozen Lung from Dogs.

Parameter	Unfrozen	Frozen		Standard deviation
		2 days	7 days	
Bronchi				
Exudate	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				
Exudate	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
Stain precipitates	0.60^{a}	1.40^{b}	1.75^{b}	0.16

* Means within the same parameter with different superscript are significantly different

Data expressed as mean of twenty samples

TABLE 2 - Parameters for Unfrozen and Frozen Liver from Dogs.

Parameter	Unfrozen	Frozen		Standard deviation
		2 days	7 days	
Hepatocytes				
Color	2.10[a]*	3.92[b]	4.35[c]	.09
Shape	2.35[a]	4.15[b]	4.60[c]	.08
Nuclei	1.62[a]	3.27[b]	3.60[b]	.12
Sinusoids				
Distended	2.70[a]	4.02[b]	4.5[c]	.09
Hemolysis	1.15[a]	4.95[b]	4.95[b]	.04
Portal triads				
Cellular characteristics	1.35[a]	3.17[b]	3.42[b]	.11
Staining characteristics	1.35[a]	3.60[b]	3.75[b]	.11
Pigment	1.07[a]	1.20[a]	1.20[a]	.05
Stain precipitates	1.45[a]	3.40[b]	3.52[b]	.13

* Means in the same parameter with different superscripts are significantly different.

Data expressed as mean of twenty samples

TABLE 3 - Parameters for Unfrozen and Frozen Kidney from Dogs.

Parameter	Unfrozen	Frozen		Standard deviation
		2 days	7 days	
Renal corpuscles				
Shrinkage	0.00^{a*}	0.12^{a}	0.12^{a}	.06
Distension	1.02^{a}	3.57^{b}	3.77^{b}	.11
Capsular Space material	0.62^{a}	2.72^{b}	3.20^{c}	.12
Tubules				
Vacuolation	0.95^{a}	3.50^{b}	3.87^{c}	.09
Epithelial detachment	0.95^{a}	3.45^{b}	3.90^{c}	.11
Casts	0.95^{a}	3.35^{b}	3.75^{c}	.09
Interstitium	1.05^{a}	3.72^{b}	4.125^{c}	.11
Hemolysis	0.47^{a}	5.00^{b}	5.00^{b}	.02
Stain precipitates	0.75^{a}	1.42^{b}	1.70^{b}	.22

* Means within the same parameter with different superscripts are significantly different.

Data expressed as mean of twenty samples

TABLE 4 -Parameters for Unfrozen and Frozen Small Intestine from Dogs.

Parameter	Unfrozen	Frozen		Standard deviation
		2 days	7 days	
Thickness of mucosal autolysis	1.17[a]*	3.30[b]	3.42[b]	.09
Mucosa	1.00[a]	3.35[b]	3.57[b]	.09
Glandular epithelium	0.82[a]	3.15[b]	3.47[c]	.08
Submucosa	0.70[a]	2.62[b]	2.90[c]	.09
Hemolysis	0.45[a]	5.00[b]	5.00[b]	.02
Tunica muscularis	0.50[a]	3.85[b]	4.07[b]	.09
Serosa	0.47[a]	3.67[b]	4.00[c]	.09

* Means within the same parameter with different superscript are significantly different.

Data expressed as mean of twenty samples

TABLE 5 - Parameters for Unfrozen and Frozen Brain from Dogs.

Parameter	Unfrozen	Frozen		Standard deviation
		2 days	7 days	
Gray matter fractures	0.00^{a*}	3.75^{b}	4.60^{c}	.11
White matter fractures	0.00^{a}	4.12^{b}	4.72^{c}	.09
Neurons	0.85^{a}	3.60^{b}	4.37^{c}	.10
Meninges	0.20^{a}	3.07^{b}	3.90^{c}	.10
Hemolysis	0.45^{a}	5.00^{b}	5.00^{b}	.01

* Means for the same parameter with the same superscript letter are not significantly different ($p<.05$)
Data expressed as mean of twenty samples

TABLE 6. Ten Clinic Cases. Morphological Lesions of Unfrozen and Frozen Tissues are Recorded.

No.	Tissue	Unfrozen	Frozen 2 days	Frozen 7 days
1	Lung	Bronchopneumonia Inclusion bodies in bronchiolar epithelium	Cellular shrinkage Inclusions	Shrinkage Inclusions
	Kidney	Interstitial nephritis Inclusion bodies in renal pelvis	Moderate shrinkage Inclusions	Shrinkage Inclusions
	Brain	Inclusion bodies within astrocytes	Inclusions not observed	Inclusions not observed
2	Tumor	Chemodectoma	Chemodectoma	Chemodectoma
	Liver	Fat emboli	Fat emboli	Fat emboli
	Kidney	Severe fibrosis	Fibrosis Fractures	Fibrosis Larger fractures
	Small Intestine	Dipylidium caninum	Dipilydium caninum	Dipylidium caninum
3	Lung	Microfilaria in alveolar wall	Microfilaria	Microfilaria
	Liver	Microfilaria	Microfilaria	Microfilaria
	Kidney	Microfilaria	Microfilaria	Microfilaria
4	Lung	Chronic-active interstitial pneumonia	Interstitial fluid increases	Increased fluid
	Liver	Intrahepatic cholestasis	Intrahepatic cholestasis	Intrahepatic cholestasis
	Kidney	Severe interstitial fibrosis Mononuclear cells Renal corpuscule atrophy Thickened basement membranes Tubular epithelial desquamation	Fibrosis Fractures Moderate shrinkage Renal corpuscle atrophy Fractures Tubular epithelial desquamation	Fibrosis Larger fractures Moderate shrinkage Renal corpuscle atrophy Larger fractures Tubular epithelial desquamation
5	Liver	Severe vacuolization	Loss of trabeculae	Loss of trabeculae
6	Liver	Bile duct proliferation	Bile duct proliferation	Bile duct proliferation
7	Lung Liver Small Intestine Kidney Brain	No diagnosis	No diagnosis	No diagnosis
8	Spleen Lung	Hemangiosarcoma Arterial thrombosis (Dirofilaria-adults)	Hemangiosarcoma Thrombosis	Hemangiosarcoma Thrombosis
9	Small Intestine	Enteritis, bacteria	Enteritis, bacteria Severe muscular fractures	Enteritis, bacteria Severe muscular fractures
10	Heart	Endocardiosis	Endocardiosis	Endocardiosis

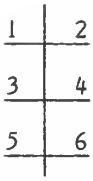


Fig 1 - Lung bronchioles and alveoli contain pink homogeneous fluid. Frozen 7 days; H & E stain; 200X.

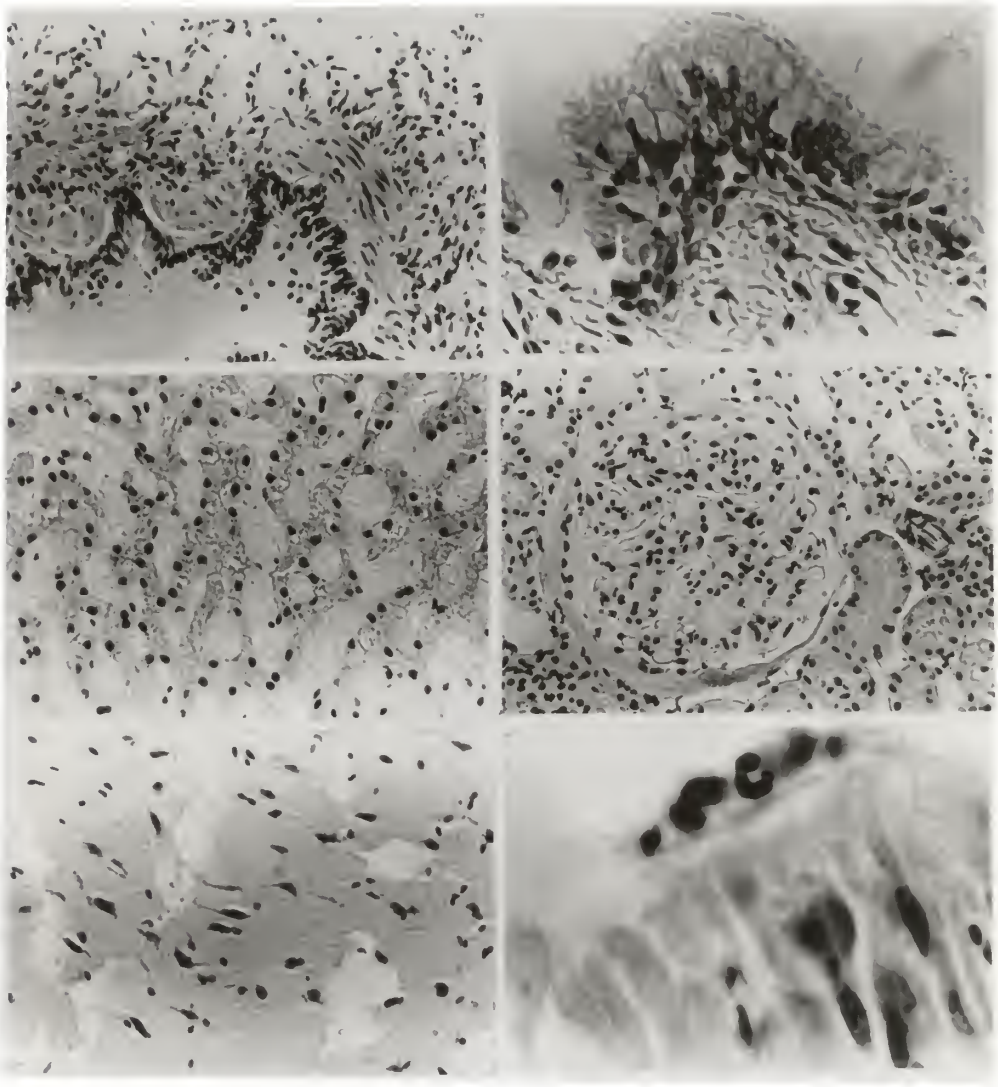
Fig 2 - Lung bronchiolar cilia are distinct. Frozen 7 days; H & E stain; 400X.

Fig 3 - Liver with dilated sinusoids and shrunken hepatocytes. Frozen 2 days; H & E stain; 200X.

Fig 4 - Kidney with enlarged glomerular tuft and fluid in the capsular space. Frozen 7 days; H & E stain; 200X.

Fig 5 - Cerebrum with many fractures. Frozen 2 days; H & E stain; 200X.

Fig 6 - Lung with intracytoplasmic inclusion bodies in bronchial epithelium (Canine Distemper). Frozen 7 days; H & E stain; 1000X



7	8
9	10
11	12

Fig 7 - Renal pelvis with intracytoplasmic inclusion bodies (Canine Distemper). Frozen 7 days; H & E stain; 1000X.

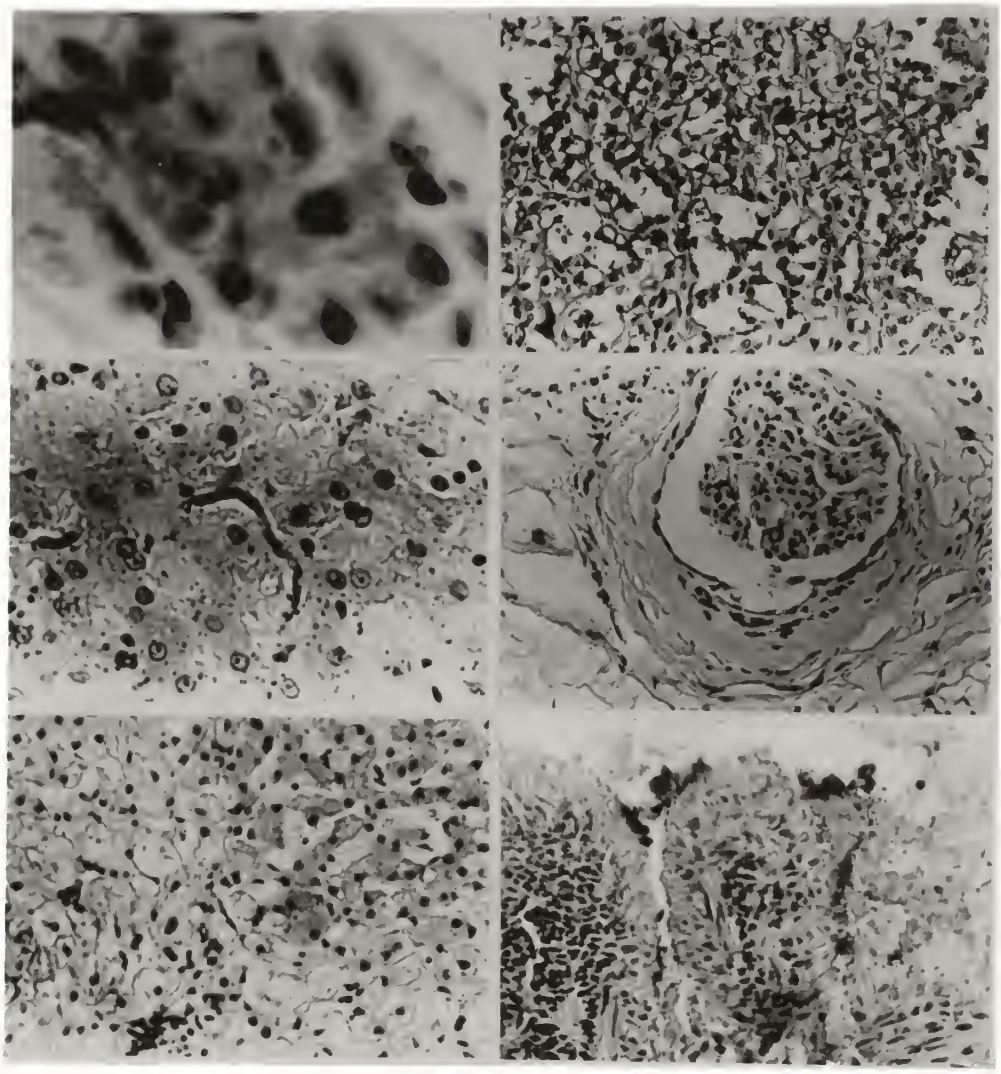
Fig 8 - Chemodectoma. Note several well-preserved cells lining capillaries. Frozen 7 days; H & E stain; 200X.

Fig 9 - Liver with microfilaria (Dirofilaria immitis) and large amount of stain precipitate. Frozen 7 days; H & E stain; 400X.

Fig 10 - Renal corpuscule with a severely thickened capsule. Frozen 7 days; H & E stain; 400X.

Fig 11 - Liver with markedly vacuolated hepatocytes. Frozen 7 days; H & E stain; 200X.

Fig 12 - Small intestine with numerous bacteria lining the mucosal surface. Frozen 7 days, H & E stain; 200X.



EFFECTS OF FREEZING AND FROZEN STORAGE ON HISTOLOGICAL CHARACTERISTICS OF CANINE TISSUES

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The purpose of this study was to determine histological changes caused by freezing for 2 days, and the effects produced by additional frozen storage for 7 days. Studies were also done with 10 clinic cases to determine if a diagnosis can be made from frozen tissue.

Lung, liver, kidney, brain and small intestine samples from 20 stray dogs were collected, within 8 hrs of death, and assigned to one of the following treatments: 1) Immediate fixation in 10% buffered formalin at room temperature; 2) Frozen and stored for 2 days at -18 C; and 3) frozen and stored for 7 days at -18 C. Both frozen treatments were followed by fixation in 10% buffered formalin at room temperature.

Sections were made and stained with hematoxylin and eosin (H & E). Several parameters for each organ were evaluated using a scale of 1 - 5. Analysis of variance was done to determine differences between frozen and unfrozen tissue. The 10 clinical cases were frozen and fixed in the same manner. These tissues were examined histologically and described; they were not analyzed statistically.

Several changes were seen in all organs. Frozen specimens stained paler than unfrozen controls. Hemolysis was complete in all tissues. Damage was generally more severe in the periphery of the organ, and decreased in deeper areas. Accumulation of a non-cellular, eosinophilic exudate was found in frozen tissues, as well as increased cell shrinkage.

Other results were limited to a particular organ. Lung slides had moderate loss of bronchiolar cilia. Liver had hepatocyte shrinkage and sinusoidal distension. The kidney had distension of glomerular tufts and vacuolation of the tubular epithelium. The small intestine was characterized by an increase of

post-mortem autolysis and fractures in the tunica muscularis. Brain had fractures in both the gray and white matter.

Major changes occurred between frozen and unfrozen groups. However, additional frozen storage time affected quantitatively the following parameters: extracellular fluid accumulation, fractures, sinusoidal dilatation, and renal tubular changes.

Diagnoses made from frozen tissue taken from clinical case dogs included Canine Distemper, Chemodectoma, Dirofilariasis, and chronic glomerulonephritis. Lesions were well preserved and allowed histopathological diagnosis. In conclusion, although freezing damage occurred, a histopathological diagnosis could still be made from frozen tissue.