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Summary: Ten anesthetized hairless guinea pigs [CrI:IAF(HA)BR] were exposed to 10 μ l of neat sulfur mustard (HD) in a vapor cup on their skin for 7 min. At 24 h postexposure, the guinea pigs were euthanatized and skin sections taken for histologic evaluation. The skin was fixed using either 10% neutral buffered formalin (NBF), McDowell Trump fixative (4CF-1G), Zenker's formal-saline (Helly's fluid), or Zenker's fluid. Fixed skin sections were cut in half: one half was embedded in paraffin and the other half in plastic (glycol methacrylate). Paraffin-embedded tissue was stained with hematoxylin and eosin; plastic-embedded tissue was stained with Lee's methylene blue basic fuchsin. Skin was also frozen unfixed, sectioned by cryostat, and stained with pinacyanole. HD-exposed skin was evaluated histologically for the presence of epidermal and follicular necrosis, microblister formation, epidermitis, and intracellular edema to determine the optimal fixation and embedding method for lesion preservation. The percentage of histologic sections with lesions varied little between fixatives and was similar for both paraffin and plastic embedding material. Plastic-embedded sections were thinner, allowing better histologic evaluation, but were more difficult to stain. Plastic embedding material did not infiltrate tissue fixed in Zenker's fluid or Zenker's formal-saline. Frozen tissue sections were prepared in the least processing time and lesion preservation was comparable to fixed tissue. It was concluded that standard histologic processing using formalin fixation and paraffin embedding is adequate for routine histopathological evaluation of HD skin lesions in the hairless guinea pig. **Key Words:** Sulfur mustard—Vesicants—Cutaneous toxins—Hairless guinea pig—Tissue fixation and embedding—Lesion preservation.

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Comparison of Fixation and Processing Methods for Hairless Guinea Pig Skin Following Sulfur Mustard Exposure

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Summary: Ten anesthetized hairless guinea pigs [CrI:IAF(HA)BR] were exposed to 10 μ l of neat sulfur mustard (HD) in a vapor cup on their skin for 7 min. At 24 h postexposure, the guinea pigs were euthanatized and skin sections taken for histologic evaluation. The skin was fixed using either 10% neutral buffered formalin (NBF), McDowell Trump fixative (4CF-1G), Zenker's formol-saline (Helly's fluid), or Zenker's fluid. Fixed skin sections were cut in half; one half was embedded in paraffin and the other half in plastic (glycol methacrylate). Paraffin-embedded tissue was stained with hematoxylin and eosin; plastic-embedded tissue was stained with Lee's methylene blue basic fuchsin. Skin was also frozen unfixed, sectioned by cryostat, and stained with pinacyanole. HD-exposed skin was evaluated histologically for the presence of epidermal and follicular necrosis, microblister formation, epidermitis, and intracellular edema to determine the optimal fixation and embedding method for lesion preservation. The percentage of histologic sections with lesions varied little between fixatives and was similar for both paraffin and plastic embedding material. Plastic-embedded sections were thinner, allowing better histologic evaluation, but were more difficult to stain. Plastic embedding material did not infiltrate tissue fixed in Zenker's fluid or Zenker's formol-saline. Frozen tissue sections were prepared in the least processing time and lesion preservation was comparable to fixed tissue. It was concluded that standard histologic processing using formalin fixation and paraffin embedding is adequate for routine histopathological evaluation of HD skin lesions in the hairless guinea pig. **Key Words:** Sulfur mustard—Vesicants—Cutaneous toxins—Hairless guinea pig—Tissue fixation and embedding—Lesion preservation.

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The recent conflict in the Middle East has shown that the potential use of chemical weapons in a military operation is a continuing threat. Sulfur mustard (HD) is a likely component of this threat. Because this threat exists, medical countermeasures are continually being evaluated. The hairless guinea pig has been shown to be a reliable animal model for evaluating cutaneous HD exposure. (1,2). The microblister is the histologically determinable end point that is being used in this laboratory to assess the efficacy of topical protectants and treatment compounds. In previous studies (1-3), cutaneous HD lesions were processed using standard histologic methods (i.e., neutral buffered formalin fixation and paraffin tissue embedding) and characterized by light microscopy. Different methods of tissue fixation, tissue embedding, and cryostat tissue processing were not evaluated. This study was designed to evaluate preservation of microblisters and other lesions that develop after HD exposure to see if they are changed because of, or were induced as an artifact by, different fixation and embedding methods, compared to the standard methods of histologic processing used in this laboratory.

METHODS

Male euthymic hairless guinea pigs [Cr:IAF/HA(hr/hr)BR] were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA, U.S.A.) and quarantined for 7 days before use. Commercial guinea pig chow and water were available ad libitum. All hairless guinea pigs were maintained in an AAALAC-accredited animal care facility. Animals were housed singly on corn cob bedding in plastic cages (Lab Products, Inc., Maywood, NJ, U.S.A.). Animal holding rooms were maintained at $21 \pm 2^\circ\text{C}$ with $50 \pm 10\%$ relative humidity with at least 10 complete air changes per hour of 100% conditioned fresh air. A 12-h light/dark full-spectrum lighting cycle with no twilight was used.

Initially, eight hairless guinea pigs anesthetized with ketamine HCl (30 mg/kg i.m.) and xylazine (6 mg/kg i.m.) were exposed on the skin to HD for 7 min via the vapor cup method (2). Filter paper lining each vapor cup was wet with 10 μl of neat HD. Each animal had eight HD-exposure sites, four sites contralateral to the dorsal midline, with two non-HD-exposure control sites taken from the dorsal midline. After HD exposure, each animal was placed in an individual cage within a charcoal-filtered fume hood. Guinea pigs were euthanatized 24 h post-HD exposure with an overdose of inhaled halothane, USP (Halocarbon Laboratories, Inc., Hackensack, NJ, U.S.A.). Dermal punch (8 mm) specimens were immediately taken from the center of all sites. On the first animal, the cranial pair of exposure sites was fixed in 10% neutral buffered formalin for 24 h. The second pair was fixed in 4% formalin and 1% glutaraldehyde (4CF-1G, McDowell-Trump) (4) fixative for 24 h. The third pair was fixed in Zenker's fluid (Harleco, E M Diagnostic Systems Inc., Gibbstown, NJ, U.S.A.) for 22 h, postfixed in potassium permanganate solution for 2 h, rinsed in running tap water for 24 h, and then stored in 70% alcohol. The last pair was frozen unfixed. The control sites were always prepared in the same manner as the first (cranial) pair of HD-exposure sites for a given animal. On subsequent animals, the method of fixation of the paired exposure sites as well as the control sites were rotated cranial to caudal to

TABLE 1. Fixation and embedding methods with percentage of lesions

Fixation/embedding material	(no. of sites)	Percent incidence of lesions				
		Epidermal necrosis	Follicular necrosis	Microblister formation	Epidermitis	Intracellular edema
Frozen	(14)	100.0%	100.0%	85.7%	78.6%	50.0%
Formalin/paraffin	(16)	100.0%	100.0%	100.0%	87.5%	100.0%
Formalin/plastic	(16)	100.0%	100.0%	100.0%	93.8%	100.0%
4CF-1G/paraffin	(16)	100.0%	100.0%	68.8%	75.0%	100.0%
4CF-1G/plastic	(16)	100.0%	93.8%	75.0%	81.3%	100.0%
Zenker's/paraffin	(24)	100.0%	95.8%	79.2%	75.0%	83.3%
Zenker's formol/paraffin	(8)	100.0%	100.0%	100.0%	100.0%	100.0%

account for any variability due to anatomical locations. After tissue fixation, each punch specimen was cut in half: one half was embedded in paraffin (5) and the other half in plastic (Historesin embedding kit, Reichert-Jung, Heidelberg, Germany) (6). Four serial sections of fixed tissue per paraffin block were cut parallel to the skin surface at a thickness of 6 μ m. The plastic blocks were cut similarly at a thickness of 2 μ m. Paraffin sections were stained with hematoxylin and eosin (H&E, Gill's N° 3, Fisher Diagnostics, Fisher Scientific, Orangeburg, NY, U.S.A.) and plastic sections were stained by Lee's methylene blue–basic fuchsin (7). Unfixed HD-exposed and nonexposed punch sections, to be frozen, were cut into two pieces slightly off center. The outside round surface of the larger section was trimmed parallel to the opposite cut surface, leaving a tissue section approximately 4 mm wide. A black dot of India ink was placed on the shorter side for orientation. The sections were individually wrapped in gauze and, by immersion, snap frozen in liquid freon N° 22 (E. I. DuPont De Nemours & Co., Inc., Wilmington, DE, U.S.A.). After the freon had stabilized (45 to 60 s), the tissue was removed, wrapped in aluminum foil, and placed on dry ice for transport to the cryostat (2800 Frigocut E, Reichert-Jung). The frozen tissue was held in the cryostat at -29°C for 12 h prior to sectioning. The tissue blocks were placed in Tissue-Tek® O.C.T. Compound, Miles Inc., Elkhart, IN, matrix, ink side down. Three replicate sections, 10 μ m thick, were placed on each slide and three slides were made from every block. Sections were stained with pinacyanole (Eastman Kodak Co., Rochester, NY, U.S.A.) for 10 s (8). Two additional guinea pigs were exposed to HD vapor (for a total of 10 guinea pigs used in the study), and the skin punch sections were taken as described above and fixed in either Zenker's or Zenker's formol–saline (Helly's fluid), and then postfixes as described above. All histologic sections were evaluated by light microscopy for each of the following features: epidermal necrosis, follicular necrosis, microblister formation, epidermitis, and intracellular edema. The lesions present in the histologic sections were tabulated for comparison as a percentage of the total number of sections evaluated for each tissue fixative and embedding method.

In conducting the research described in this report, the investigators adhered to the NIH "Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council."



FIG. 1. Skin, guinea pig. A: Normal. B: HD exposure with microblisters (arrowhead). Frozen section (cryostat) stained with pinacyanole. Frozen tissue was processed in less time than fixed tissue and served as a control for comparison to fixed tissue. The increased section thickness required for processing made some inflammatory changes and edema more difficult to evaluate.

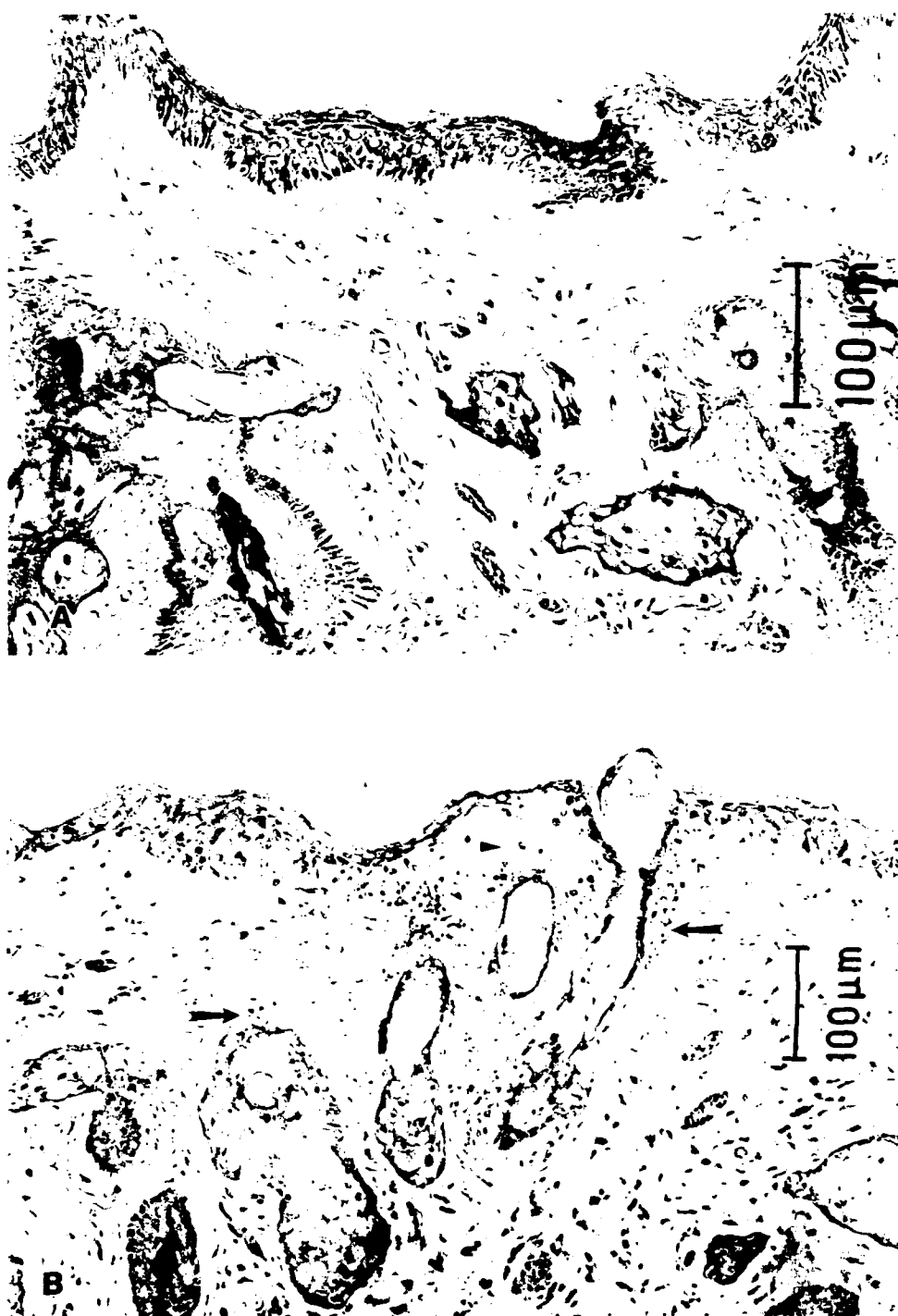


FIG. 2. Skin, guinea pig. **A:** Normal. **B:** HD exposure with microblister (arrowhead) and follicular necrosis (arrows). Formalin-fixed, plastic-embedded section with Lee's stain. Plastic-embedded tissue is sectioned thinner than paraffin-embedded tissue, allowing more sharply focused images with light microscopy. Also, lesion detection was at or near 100% in all areas evaluated in plastic-embedded sections.

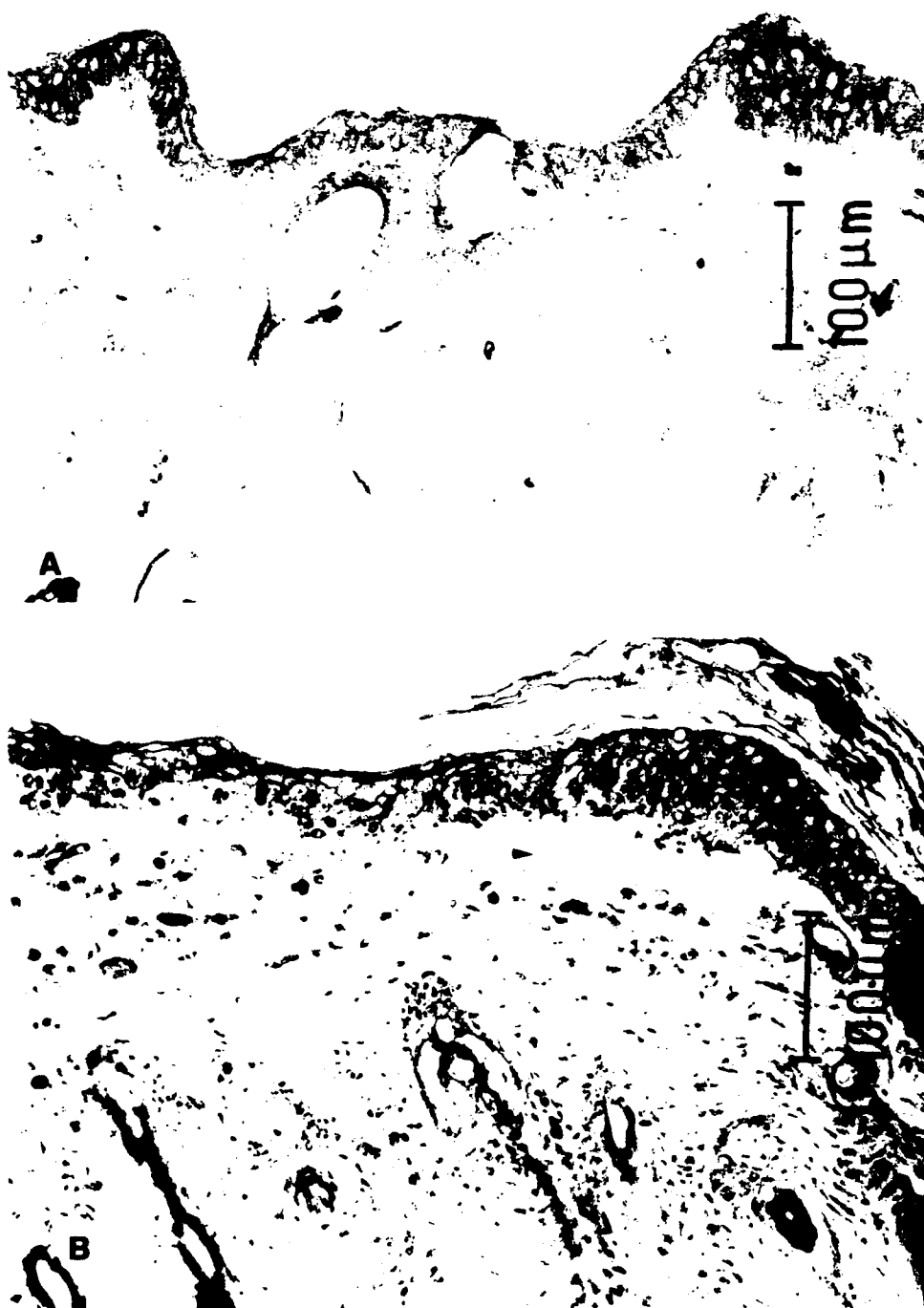


FIG. 3. Skin, guinea pig. A: Normal. B: HD exposure with microblister (arrowhead). 4CF-1G-fixed, plastic-embedded section with Lee's stain. 4CF-1G-fixed tissue can be evaluated with either light microscopy or transmission electron microscopy. Lesion detection rates were not as high as with formalin fixation and paraffin embedding.



FIG. 4. Skin, guinea pig. **A:** Normal. **B:** HD exposure with microblister (arrowheads) and follicular necrosis (arrow). Formalin-fixed, paraffin-embedded section with H&E stain. This is the standard method used in most histology laboratories for light microscopy. No substantial difference in lesion detection was noted between paraffin- or plastic-embedded, formalin-fixed tissue.



FIG. 5. Skin, guinea pig. A: Normal. B: HD exposure with microblister (arrowhead). 4CF-1G-fixed, paraffin-embedded section with H&E stain. This method detected less lesions than formalin-fixed, paraffin embedding.



FIG. 6. Skin, guinea pig. **A:** Normal. **B:** HD exposure with microblister (arrowhead) and follicular necrosis (arrow). Zenker's-fixed, paraffin-embedded section with H&E stain. This fixative must be activated just prior to use and requires additional processing time to remove heavy metals from fixed tissue. Zenker's-fixed tissue cannot be plastic embedded. Lesion detection was no more frequent than with 4CF-1G-fixed tissue.

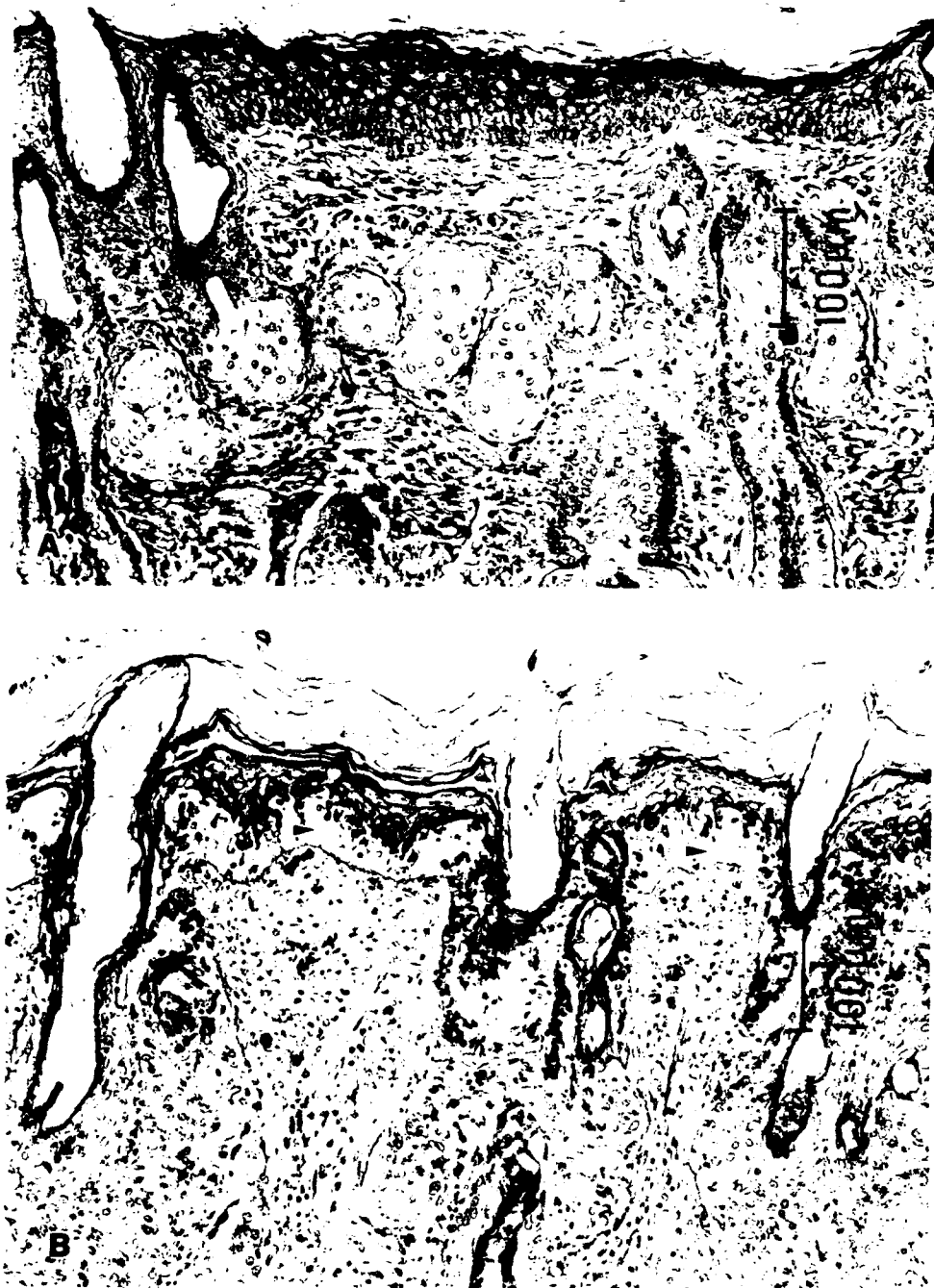


FIG. 7. Skin, guinea pig. A: Normal. B: HD exposure with microblister (arrowheads). Zenker's formol-fixed, paraffin-embedded section with H&E stain. This method has similar processing requirements to Zenker's-fixed tissue. Lesion detection was at 100% in all categories evaluated.

RESULTS

The percentage of lesions observed with each tissue fixative and embedding method are shown in Table 1. Epidermal and follicular necrosis was visible in all frozen sections (Fig. 1), with microblisters and epidermitis being less frequent and intracellular edema present in only 50% of the sections. Similar lesion percentages were present when formalin-fixed, paraffin-embedded (Fig. 2) and formalin-fixed, plastic-embedded (Fig. 3) sections were compared to each other. Small differences in the percentages of lesions present were seen between 4CF-1G-fixed, paraffin-embedded (Fig. 4) sections and 4CF-1G-fixed, plastic-embedded (Fig. 5) sections. Zenker's-fixed, paraffin-embedded (Fig. 6) sections had some variation in the percentages of lesions present, whereas all lesions were 100% present in the Zenker's formol-saline-fixed, paraffin-embedded (Fig. 7) sections. Epithelial and follicular necrosis was present at the highest percentage with all methods of tissue processing; however, the presence of microblister formation and epidermitis was more variable with the method of tissue processing. The HD-induced microblisters that were frozen or fixed were clearly apparent with light microscopy and were associated with intracellular edema, inflammation, and necrosis in the same sections. Although not listed in the table, retention of dermal edema in the section was more evident with Zenker's and Zenker's formol-saline fixatives (Fig. 8) than with other fixatives. The manufacturer of the plastic embedding material and others (5) indicated that plastic could be used with a wide range of fixatives. Based on this, we attempted to embed Zenker's and Zenker's formol-saline-fixed tissue with plastic. However, our skin sections fixed in Zenker's and Zenker's formol-saline did not allow plastic infiltration of the tissue. This resulted in no sections for light microscopic evaluation for Zenker's and Zenker's formol-saline-fixed, plastic-embedded tissue. The 4CF-1G-fixed, plastic-embedded tissue sectioned with less shattering of connective tissue and stained more intensely than the formalin-fixed, plastic-embedded tissue. Plastic-embedded sections were thinner, reducing the depth of field and clarifying the morphologic detail; however, they were more difficult to stain. The control sections evaluated with all of the fixation and embedding methods did not contain any histologically observable lesions as seen in the HD-exposed sections.

DISCUSSION

Proper and adequate fixation and tissue processing is essential for light microscopic evaluation. Fixatives preserve tissue, preventing deterioration over time. Embedding material infiltrates tissue and makes it firm for sectioning with the microtome. Frozen tissue is not fixed or infiltrated by embedding material, but is stabilized by freezing and is sectioned frozen. An important concern in tissue processing is lesion preservation, making sure that fixation and embedding methods do not change the lesions or cause artifacts. This is of particular interest in studies on the HD-induced cutaneous microblister since the microblister is the histologic end point in our screening process.

The present study evaluated HD lesions with light microscopy using one frozen method and four fixatives in conjunction with two embedding methods. Excluding the variations of fixation, embedding, and staining, all fixed tissue sites were pro-



FIG. 8. Skin, dermis, guinea pig, HD exposure with connective tissue separated by edema (arrows). **A:** Zenker's-fixed, paraffin-embedded section with H&E stain. **B:** Zenker's formol-fixed, paraffin-embedded section with H&E stain. These fixatives preserve edema fluid better in the tissue during fixation than other fixatives.

cessed and evaluated in a similar manner. The method of site fixation was randomized to equilibrate any anatomical variations due to site location.

Frozen tissue was processed in the least amount of time when compared to fixed tissue processing time. Also, frozen tissue is not altered by fixatives or embedding material; thus, a near normal tissue state should be maintained. As such, percentages of lesions present in frozen tissue should be accurate. However, comparisons between frozen and fixed tissue should be made with caution. Frozen tissue must be sectioned thicker since it is not embedded. This increases the tissue depth of field, which hinders morphologic evaluation with the light microscope. The stains for frozen tissue are different from fixed tissue as well. Also, ice crystals can form in the tissue if not properly frozen initially or if it thaws and is refrozen. Therefore, processing steps for frozen tissue must be carefully followed not to induce artifacts in the tissue.

Formalin is the most commonly used tissue fixative, because it can be prepared in advance, is stable at room temperature, is easy to use, and is readily available. As such, formalin is the standard by which the other fixatives are normally measured. The percentages of lesions present in 10% neutral buffered formalin, whether embedded in paraffin or plastic, were at or near 100% with all lesions evaluated in this study. The slight increased detection of epidermitis in the plastic-embedded sections may be due to the improved morphological detail. However, for tissue processing laboratories that are set up for paraffin embedding, this slight improvement might not justify changing to plastic embedding with formalin-fixed tissue.

4CF-1G is a standard electron microscopy fixative that must be prepared fresh and requires refrigeration until used. 4CF-1G-fixed tissue, whether paraffin or plastic embedded, had high percentages of lesions in most categories. However, microblister formation and epidermitis in 4CF-1G-fixed tissue were detected less frequently than in formalin-fixed tissue. Thus, routine use of this method offers no clear advantage for light microscopy and should be reserved for use with electron microscopy.

Zenker's and Zenker's formol-saline fixatives must be activated just before use. Tissues preserved in these fixatives required more time, materials, and labor for processing and cannot be embedded with plastic. Also, lack of plastic infiltration for tissues fixed in Zenker's and Zenker's formol-saline has been noted before (9). Regardless, these fixatives are better at holding in tissue edema fluid and fibrin during processing, compared to formalin or 4CF-1G. Zenker's formol-saline-fixed, paraffin-embedded tissue had high percentages of lesions in all areas of evaluation, but due to the increased processing requirements and the limitation of paraffin embedding, this fixative may not be acceptable in some laboratories.

Whether the tissue was embedded with paraffin or plastic, the percentage of lesions present was similar. Plastic sections are thinner than paraffin sections, which allows improved resolution with the light microscope. As such, the plastic skin sections permit a slightly better evaluation of epidermitis, intracellular edema, and microblisters. However, plastic sections do not readily take up routine histologic stains and can require more labor to embed tissues than paraffin.

In this study, HD-induced microblisters were readily visible by light microscopy in both frozen and fixed tissues. The microblisters were not an artifact of processing, since they were substantiated by the presence of intracellular edema, inflammation, and necrosis in the same sections. Also, there was no substantial variation in the

detection of lesions between the different methods used for the preservation of lesions in this study. Therefore, when comparing the percentages of lesions present with the methods utilized in this study and the technical requirements for processing tissue, as well as knowing that lesions such as the microblister were not artifacts, there is no convincing evidence to justify a change from the standard method of formalin fixation and paraffin embedding when evaluating HD-induced skin lesions in the hairless guinea pig.

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