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Comparisons of a Formaline Substitution with Routine Chemical Fixatives on Cryosections

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Abstract: This study was designed to compare tissue preservation efficiency of formaldehyde substitution (FS) on cryosections with acetone (AC) and formaldehyde (FA). Brain, kidney, heart and liver had obtained from adult Dutch rabbit and were fixed with either FA or FS and AC prior to cryosectioning. Hematoxylin and eosin staining showed that AC is a poor fixative in preserving the general tissue and cellular organization. FS and to a lesser extent FA, produced significantly better results. All of cryosections were stained by hematoxylin and eosin and were studied by light microscopy. Results demonstrated that, overall, FS is the best fixative tested. However, FA fixation gave poor results in preserving neuronal tissues. Overall, the data suggest that AC fixation is unacceptable for preservation of most samples, whereas FA and FS fixation should be chosen according to the tissues and proteins to be studied.

Key words: Fixation · Cryosections · Acetone · Formaldehyde · Substitution

INTRODUCTION

The chief aim of any fixation of biological materials is an optimal and long term preservation of those structures which are relevant for the demonstration of morphological details under the microscope. The structure of an animal tissue is determined largely by the configuration of its proteins. Formaldehyde in aqueous solution (formalin) is the most frequently used for the propose [1]. Although formalin has some major disadvantages due to toxic effects for human health [2] and production of methylene cross links [3], which are removable only by applying vigorous techniques [4], scientists are attempting to replace formalin with precipitating or protein-denaturing fixatives [5]. The main contributors to cell structure are the lipoproteins, which are major components of the plasma membranes and membranous organelles of cells; the fibrous glycoproteins of extracellular elements, such as collagen and basal laminae; and the globular proteins, which are dissolved in the cytoplasm and extracellular fluid. For histopathology, all these substances must be stabilized by fixation. This is achieved either by denaturing or protein-modifying compounds [6] such as acids, metallic salts, alcohols, ketons, other organic solvents and combinations of these compounds, or by applying protein-cross linking compounds such as formaldehyde [7]. In the last three decades, cryosectioning has gained prominence in comparison to classical fixation, processing and paraffin wax embedding of tissue blocks, since many histochemistric procedures, as well as routine hematoxylin and eosin (H and E) stains, can be performed on cryosections [8]. However, relatively few reports have appeared in the literature describing a systematic analysis of the advantages and disadvantages of different chemicals on tissue preservation of cryosections [9-12]. In this study, we compared formalin substitution (FS) with two commonly used fixatives such as acetone (AC) and formaldehyde (FA), in order to evaluate FS efficiency in preserving cryosections.

MATERIALS AND METHODS

Four organs with different embryonic origins and with different proportions of epithelial, connective, neural and muscular tissues were selected in this study [13]. These organs were obtained from a Dutch rabbit that had been sacrificed for other studies in which the heart was excised under general anesthesia of halothane (2.5 liters/min) administered via a ventilator. Organs were immediately removed. Three blocks of each tissue with

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5mm thickness were prepared. One block of the tissues were immersed into 10% buffered formaldehyde (Merck Co., Darmstadt, Germany) and the second one, immersed into formaldehyde substitution (Milestone Srl. Italy). The third block was immersed into acetone (Applichem Co., Darmstadt, Germany) for 48 hours in room temperature. All blocks were immersed in 20% (0.58M) and 30% (0.88M) (w/v in distilled water) sucrose solutions (Merck Co., Darmstadt, Germany), for 12 h each, at 4°C for cryoprotection [14]. Sections stored at 4°C were initially stained with Harris's hematoxylin and eosin (Merck Co., Darmstadt, Germany) using routine protocol for general orientation of the sections. Bright-field colored images were recorded from 36 different samples taken from the four organs. Scoring was performed based on preserving of general structure and staining quality of tissues, cells and their components (nuclei and cytoplasm of cells) from (+) poor to (++++) well. All data were analyzed by SPSS ver.16 software.

RESULTS

Acetone: In brain, a loss of tissue organization and cytoarchitecture, artificial widening and swelling throughout the brain cortex and in the medulla (not shown) predominated. Similar findings were also seen in kidney and heart and to a lesser extent in liver preparations. AC fixation resulted in an extreme loss of tubules'' epithelial and peripheral interstitial tissues in kidney and in interfibrillar connective tissue in the heart. Hepatic plates and capillary sinusoids were relatively poorly preserved by AC fixation but nuclei was well stained.

Formaldehyde: Brain and liver sections were relatively well preserved compared to kidney and heart sections. Giant pyramidal cells of brain cortex and intercellular matrices were clearly identified. In contrast, interstitial and interfibrillar tissues were severely disrupted in kidney and heart sections, respectively. More striking features were noted in liver samples.

Formaldehyde Substitution: This fixative provided cell and tissue preservation as well as formaldehyde, particularly in brain, liver and heart sections this effect was better shown. In kidney samples, glomeruli and tubules were moderately maintained with a minor disorganization of interlobular arteries and veins. Staining had better quality in compare with Acetone and Formaldehyde fixatives.

The differences among each group were separately tested utilizing one-way ANOVA and post hoc Dunn tests, with a level of significance set at $p<0.05$.

DISCUSSION

In last decades, a limited number of studies have focused on the comparison of fixatives and fixation methods aimed at preserving vital tissue organization as much as possible [15]. Frozen sectioning has achieved popularity because provides an invaluable tool in diagnostic pathology; as the tissue processing procedure is extremely fast, it can be undertaken while a surgical operation is ongoing. In recent years, frozen sections have been considered as the preferred method to preparation of tissues for immunoenzymatic and immunofluorescent labeling techniques, particularly for preserving delicate protein molecules [16]. Although it is not often adequate for preserving general tissue organization [17]. In this sense, the effectiveness of tissue preservation of several fixation approaches still remains questionable. Like methanol and ethanol, AC is a simple organic coagulant that displaces water from proteinaceous materials, thereby breaking hydrogen bonds and disturbing tertiary structure to produce denaturation. Soluble proteins in the cytoplasm are coagulated and organelles are destroyed. Nucleic acids are not precipitated. Precipitated protein that has not been denatured retains enzymatic or other biological properties and remains soluble in aqueous solution. AC extracts lipid from tissues but carbohydrate containing components are largely unaffected. AC is used alone for fixing films and cellular smears and previously for otherwise unfixed cryostat sections. It is not suitable for blocks of tissue. Unless they are very small, because it causes considerable shrinkage and hardening of tissue [18]. This was seen in our AC-fixed samples, particularly of brain, kidney and heart sections. In contrast, swelling in hepatocytes and enlargement of sinosoids was observed. AC fixation has been reported to have severe detrimental effects in cell surface protein molecules and major cytoskeletal elements [19-21]. Reported that microtubules were poorly preserved in AC-fixed cells [22], while, immunohistochemistry using a monoclonal anti-cytokeratin antibody produced an

incomplete immunolabelling pattern against a diffuse background after FA fixation [23]. FS and FA form both intra and intermolecular cross-links with protein molecules, which result in the formation of more rigid heteropolymers [24]. The monoaldehyde FA is the simplest member of aldehyde's family of fixatives and has been commonly used for both light and electron microscopy [25]. The reactions of FA with proteins are numerous and well understood. FS is preferred over FA because of somewhat better fixation results. A study demonstrated that AC fixation resulted in poor results in preserving the cytological properties [26]. Similarly, in other studies have found that AC was superior to aldehyde fixatives when the aim was to visualize the details of the nucleus. Taken together, AC fixation, particularly for frozen sections, is not a suitable method at least for general microscopic [27]. Scientists compared the cell-protective and staining effects of FA, ethanol and a fixative cocktail (a combination of ethanol, formaldehyde and glacial acetic acid) on dermal tissues after cryosectioning. They concluded that FA fixation was characterized by a significant increase in the staining intensity of basal cells of epidermis [28]. All studies cited above aimed to analyze the relationship between fixation and frozen sectioning. The aim of the present study was to focus on fixatives' effects regarding general morphology on tissues derived from diverse embryonic layers, which have different cellular and extracellular matrix organization and to address the overall efficiency of selected fixatives on four different organs of different embryonic origin and including different amounts of epithelial, connective tissue, neuronal cells and extracellular matrices. FS fixation was partly superior to either FA or AC for subsequent H and E staining at the light microscope level. Further observations using higher resolution imaging systems may reveal the mechanisms by which tissue protective efficiency is achieved by these fixatives. Regarding the type of fixative, FS is likely to be the first choice among tested fixatives for solid tissue components.

REFERENCES

1. Bussolati, G., L. Annaratone, E. Medico, G. D'Armento and A. Sapino, 2011. Formalin fixation at low temperature better preserves nucleic Acid integrity. PLoS One., 6(6): e 21043.
2. Golden, R., 2011. Identifying an indoor air exposure limit for formaldehyde considering both irritation and cancer hazards.Crit Rev Toxicol.
3. Lu, K., W. Ye, L. Zhou, L.B. Collins, X. Chen, A. Gold, L.M. Ball and J.A. Swenberg, 2010. Structural characterization of formaldehyde-induced cross-links between amino acids and deoxynucleosides and their oligomers. J. Am. Chem. Soc., 17;132(10): 3388-99.
4. Heck, H. and M. Casanova, 1999. Pharmacodynamics of formaldehyde: applications of a model for the arrest of DNA replication by DNA-protein cross-links. Toxicol. Appl. Pharmacol. Oct., 1;160(1): 86-100.
5. Benchekroun, M., J. DeGraw, J. Gao, L. Sun, K. Von Boguslawsky, A. Leminen, L.C. Andersson and M. Heiskala, 2004. Impact of fixative on recovery of mRNA from paraffin-embedded tissue. Diagn Mol. Pathol. Jun., 13(2): 116-25.
6. Mura, U., M. Cappiello, P.G. Vilardo, I. Cecconi, M. Dal Monte and A. Del Corso, 2003. Signalling potential and protein modifying ability of physiological thiols. Biofactors., 17(1-4): 279-85.
7. Collins, J.E., A. Kirk, S.K. Campbell, J. Mason and S.J. Wilson, 2011. Enhanced immunohistochemical resolution of claudin proteins in glycolmethacrylate-embedded tissue biopsies. Methods Mol. Biol., 762: 371-82.
8. Chang, K.H., D.T. Finn, D. Lee, J. Bhawan, G.E. Dallal and G.S. Rogers, 2011. Novel 16-minute technique for evaluating melanoma resection margins during Mohs surgery. J. Am. Acad Dermatol. Jan., 64(1): 107-12.
9. Rolbiecki, L., 2007. [The application of acetic acid and benzyl alcohol in parasitological preparations--advantages and disadvantages]. Wiad Parazytol., 53(4): 347-9. Polish.
10. Hall, P.A., P.M. Stearn, M.G. Butler and A.J. D'Ardenne, 1987. Acetone periodate-lysine-paraformaldehyde (PLP) fixation and improved morphology of cryostat sections for immunohistochemistry. Histopathol., 11: 93-101.
11. Onodera, Y., H. Shimizu, S. Yamashita and T. Nishikawa, 1992. Cryofixed, freeze-dried and paraffin-embedded skin enables successful immunohistochemical staining of skin basement membrane antigens. Histochemistry, 98: 87-91.
12. Richter, T., J. Nahrig, P. Komminoth, J. Kowolik and M. Werner, 1999. Protocol for ultrarapid immunostaining of frozen sections. J. Clin. Pathol., 52: 461-4.
13. Cinar, O., O. Semiz and A. Can, 2006. A microscopic survey on the efficiency of well-known routine chemical fixatives on cryosections. Acta Histochem., 108(6): 487-96.

14. Romijn, H.J., J.F.M. Van Uum, I. Breedijk, J. Emmering, I. Radu and C.W. Pool, 1999. Double immunolabeling of neuropeptides in the human hypothalamus as analyzed by confocal laser scanning fluorescence microscopy. J. Histochem. Cytochem., pp: 229-35.
15. Gokhale, S., O. Ololade and P.A. Adegboyega, 2002. Effect of tissue fixatives on the immunohistochemical expression of ABH blood group isoantigens. Appl. Immunohistochem. Mol. Morphol., 10: 282-8.
16. Prento, P. and H. Lyon, 1997. Commercial formaldehyde substitutes for histopathology. Biotech. Histochem., 72: 273-82.
17. Hoetelmans, R.W., H.J. van Slooten, R. Keijzer, C.J. van de Velde and J.H. van Dierendonck, 2002. Comparison of the effects of microwave heating and high pressure cooking for antigen retrieval of human and rat Bc1-2 protein in formaldehyde-fixed, paraffin-embedded sections. Biotech. Histochem., 77: 137-44.
18. Kiernan, J.A., 1990. Fixation. Histological histochemical methods, 2nd ed. Oxford: Pergamon Press, pp: 10-32.
19. Miyamura, Y., C. Park, K. Kinbara, F.A. Leibfarth, C.J. Hawker and T. Aida, 2011. Controlling volume shrinkage in soft lithography through heat-induced cross-linking of patterned nanofibers. J. Am. Chem. Soc., 133(9): 2840-3.
20. Cheong, C., I. Matos, J.H. Choi, J.D. Schauer, D.B. Dandamudi, E. Shrestha, J.A. Makeyeva, X. Li, P. Li, R.M. Steinman and C.G. Park, 2010. New monoclonal anti-mouse DC-SIGN antibodies reactive with acetone-fixed cells. J. Immunol. Methods, 360(1-2): 66-75.
21. Giddings, T.H., 2003. Freeze-substitution protocols for improved visualization of membranes in high-pressure frozen samples. J. Microsc., 212(Pt 1): 53-61.
22. Vielkind, U. and S.H. Swierenga, 1989. A simple fixation procedure for immunofluorescent detection of different cytoskeletal components within the same cell. Histochemistry, 91: 81-9.
23. Mukaratirwa, S., E. de Witte, A.M. van Ederen and H. Nederbragt, 2003. Tenascin expression in relation to stromal tumour cells in canine gastrointestinal epithelial tumours. J. Comp. Pathol., 129(2-3): 137-46.
24. Peterson, J.J., M.M. Young and L.J. Takemoto, 2004. Probing alpha-crystallin structure using chemical cross-linkers and mass spectrometry. Mol. Vis., 10: 857-66.
25. Lighezan, R., F. Baderca, A. Alexa, M. Iacovliev, D. Bonþe, E.D. Murãrescu and A. Nebunu, 2009. The value of the reprocessing method of paraffin-embedded biopsies for transmission electron microscopy. Rom. J. Morphol. Embryol., 50(4): 613-7.
26. Haas, G.G., L.E. Jr, DeBault, O. D'Cruz and R. Shuey, 1988. The effect of fixatives and/or air-drying on the plasma and acrosomal membranes of human sperm. Fertil Steril., 50(3): 487-92.
27. Hosaka, H., S. Ohtoshi, T. Nakada and M. Iijima, 2010. Erythema multiforme, Stevens-Johnson syndrome and toxic epidermal necrolysis: frozen-section diagnosis. J. Dermatol., 37(5): 407-12.
28. Rustad, O.J., V. Kaye, R. Cerio and C.B. Zachary, 1989. Postfixation of cryostat sections improves tumor definition in Mohs surgery. J. Dermatol. Surg. Oncol., 15: 1262-7.