ORIGINAL RESEARCH

Evaluation of a Novel Formaldehyde-Free Fixation Solution for the Fixation of Mouse Organs

Huiqiu Zhang, PhD; Rongquan Huang, PhD; Huyu Zhang, PhD; Zhiyan Gao, MD

ABSTRACT

Objective • A new aldehyde-free fixative has been developed and its effect has been compared to traditional formaldehyde fixative in terms of the fixation effect and HE staining of the heart, liver, lung, and kidney. The air in the experimental area was examined to evaluate its impact on the environment and human health.

Methods • The organs from mice of groups 1-6 were taken respectively (thickness of liver and lung was 3 mm). After the heart and kidney capsule were removed, the organs were longitudinally cut along their maximum surface, and half was taken. Thereafter, the tissue fixation effect was observed by Hematein and Eosin (H&E) staining and the total protein content of tissue was examined by the ultramicro spectrophotometer. Additionally, the volatility ratio of the new fixative and the traditional formaldehyde is compared.

Result • The results showed that there was no significant difference between the fixation effect of the new aldehyde-free fixation and the traditional formaldehyde fixative on mouse organs and the air quality in the experimental area was found to be significantly better when the new aldehyde-free fixative is used than when the traditional formaldehyde fixative is used.

Conclusion • Traditional formaldehyde fixative in HE staining can be replaced by the new environment-friendly formaldehyde-free fixative, however further special staining of fixed tissue and immunohistochemical studies are needed. (*Altern Ther Health Med.* [E-pub ahead of print.])

Huiqiu Zhang, PhD; Zhiyan Gao, MD; Experimental Center of Morphology, School of Basic Medical Sciences, Guangzhou Medical University. **Rongquan Huang, PhD**; **Huyu Zhang, PhD;** Department of Pathology, School of Basic Medical Sciences, Guangzhou Medical University.

Corresponding author: Zhiyan Gao, MD
E-mail: gzgylxm2@126.com

INTRODUCTION

Tissue fixation is an important aspect of pathological technology work, and its effectiveness directly affects the quality of subsequent routine slides and the results of related molecular pathological tests such as immunohistochemistry.[1] For a long time, 4% neutral buffered formaldehyde has been used as the preferred fixative for pathological laboratories due to its reliable fixation and good economic practicality.[2] Its fixation of pathological specimens, routine H&E staining, and molecular biology detection effects have also been recognized by pathological experts.[3] However, considering the toxic side effects of formaldehyde and its potentially harmful effects on the environment and human health, finding an environment-friendly fixing solution with the same effect and no toxicity was much required.

Through experimental exploration, our center has discovered a new type of formaldehyde-free fixing solution, which is environment-friendly, novel, and does not pose any harm to the environment and human health.[4,5] This new formaldehyde-free fixative was compared with traditional formaldehyde fixative, and the fixation effect along with the HE staining of mouse hearts, liver, lungs, and kidneys was compared and analyzed.[6-9]

MATERIALS AND METHODS

Materials

Fixative. (1) A new type of aldehyde-free fixing solution (composed of natural atomic compounds and 70%-90% pH 4.3-6.5 buffer solution, mainly composed of water-soluble polyols)[10]，(2) 6 types of traditional formaldehyde fixing solutions: fixing agents: (i) 4% formaldehyde solution: 10 ml of 40% formaldehyde + 90 ml of distilled water; fixed agent (ii) neutral formaldehyde (pH 7.0): 120 ml of 40% formaldehyde solution + 880 ml of distilled water + 4 g of sodium dihydrogen phosphate + 12 g of sodium dihydrogen phosphate; fixed solution: (iii) neutral buffer formaldehyde: 40% formaldehyde 10 ml + 0.01 M pH 7.4 PBS 90 ml; fixed solution: (iv) formaldehyde physiological saline: 10 ml of 40% formaldehyde solution + 90 ml of physiological saline, pH 7.0; fixed solution: (v) 4% paraformaldehyde PB solution

pH 7.3: 40 g paraformaldehyde dissolved in 1L 0.1 M PB solution; fixed solution: (vi) 4% paraformaldehyde solution: 4 g of paraformaldehyde + 100 ml of distilled water.

Organizes materials. Fresh heart, liver, lung, and kidney tissues of mice generated from teaching experiments were selected in batches, and materials were taken and repaired, and soaked in the 7 fixed solutions. Three mice were selected from each batch, and 6 batches of experiments were repeated.

Instruments and equipment. Oven, programmed paraffin embedding device, tissue slicer; automatic staining machine, digital pathological section scanner; ultramicro spectrophotometer; and, air quality detector were used.

Methods

Fixing and embedding. Take organs from groups 1-6 of mice, with a thickness of 3 mm for the liver and lungs. After removing the capsule from the heart and kidneys, cut them in half longitudinally along the maximum plane, and half for later use. After 24 hours of fixation, the tissues were routinely dehydrated and embedded via the following steps: 70% ethanol overnight, 80% ethanol 30 min, 90% ethanol 30 min, 95% ethanol overnight, 100% ethanol I 30 min, 100% ethanol II 30 min, 100% ethanol III 30 min, the TO transparent agent I 1 h, TO transparent agent II 1 h, TO transparent agent III 1h, soft wax soaking 1.5 h, and hard wax soaking 2.5 h. After embedding, the wax blocks were stored in the refrigerator.

Slice preparation and HE staining. H&E staining was used to observe the tissue fixation effect under the section microscope.

Method: The tissue sample must be sufficiently fixed to prevent the destruction of tissue cells during handling. The thickness of the section was 4 μm; Routine HE staining, baking in oven at 65°C for 1 h, dewaxing in xylene, step-by-step hydration with gradient alcohol, rinse with distilled water; hematoxylin staining for 5 minutes, and rinse with running water. Hydrochloric acid was differentiated for 30 s, rinsed with tap water for 15 min, and stained in eosin solution. Finally dehydrated by alcohol and xylene, the tablet is sealed using a digital pathological section scanner to analyze the staining effect of the section. Then slides were submerged into graded ethanols and xylene, then covered with coverslips.

Determination of total protein content in tissues. Each group of organs was weighed 100 g and placed into an EP tube. The lysate was added at a rate of 100 g/ml, and was lysed at 4°C for 30 minutes. The supernatant was centrifuged and 100 uL was taken. The total protein content of the tissue was measured using an ultramicro spectrophotometer, and this result was used as another indicator to reflect the fixation effect of the fixative on different mice tissues and organs.

Determination of air quality in experimental areas. Comparison of formaldehyde volatilization between the new fixed solution and the traditional fixed solution: pour 300 mL of No. 1-6 fixed solution into a beaker, place them respectively in a relatively closed constant temperature space (40°C for 6 h), use an air quality detector to detect the formaldehyde content in the confined space, and use this result to reflect the volatility of formaldehyde in the fixed solution and also as an indicator of the impact of the fixed solution on the environment.

Statistical analysis

Continuous normally distributed data is expressed as the mean ± SD. All statistical calculations were carried out using the SPSS statistical software. For multiple comparisons, data were analyzed via analysis of variance (ANOVA) with the Tukey-Kramer Multiple Comparisons Test. $P < .05$ were considered significant.

RESULTS

The results were independently validated by other researchers. For different parts and textures of tissues in experimental mice, such as liver, kidney, lung, brain, cartilage, etc., there is no significant difference in the fixation effect between environment-friendly formaldehyde free fixative and traditional formaldehyde fixative in paraffin-embedded sections. Its softness and hardness are moderate, the thickness of the sections is uniform, and the continuity is good. Conventional HE staining showed complete cell morphology, clear nuclear cytoplasmic contrast, and bright and delicate staining. Microscopically, there was no significant difference in the HE staining effect (Figure 2). Statistical analysis of the fixation and staining effects of a total of 400 tissue blocks from 10 sites showed that the overall excellent rate of environment-friendly formaldehyde-free fixation solution was 92.5% (369/400), while the overall excellent rate of traditional formaldehyde fixation solution was 93% (372/400). Hence, there was no significant difference between the two.

Macroscopic Results of New Formaldehyde Free Fixation Solution and Traditional Formaldehyde Fixation Solution on Various Organs of Mice Fixed for 24 Hours

The experimental results showed that the softness and hardness of the new, lung, kidney, liver and other organs in mice fixed with formaldehyde-free fixative and traditional formaldehyde-containing fixative were moderate, with a grayish-white color. There was no significant difference in the fixation effect between different fixatives (as shown in the following Figure 1).

Comparison of results after HE staining of tissue-embedded sections fixed with seven fixative solutions

Determine from four aspects: cell morphology, the contrast between nuclear and cytoplasmic staining, color of nuclear morphology, and clarity of tissue structure. The results of fixation and dyeing effects are divided into three levels: excellent, good, and poor.

Figure 1. new, lung, kidney, liver and other organs

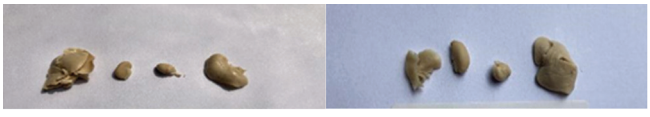

Table 1. Morphological Sub-Scores of Tissues Fixed with 7 Groups of Fixative

	fixative 1	fixative 2	fixative 3	fixative 4	fixative 5	fixative 6	fixative 7
Morphology	excellent	excellent	good	excellent	excellent	good	good
Contrast of Nuclear and cytoplasmic staining	excellent	good	excellent	good	excellent	good	good
Nuclear morphology and color	good	good	excellent	good	excellent	good	good
Organizational structure clarity	excellent	good	good	excellent	good	excellent	good

Note: Excellent: The tissue section is complete, without cracks, uniform in thickness, and without wrinkles or knife marks. The nucleus and cytoplasm have a distinct blue-red contrast, and the nucleus is not significantly swollen. Various cell components are brightly colored. Good: The structure of the tissue section is intact, with uniform thickness, a few wrinkles without knife marks, and the contrast between the nucleus and cytoplasm is distinct, with no obvious swelling of the nucleus. Poor: The tissue slices are fragmented, with incomplete structure, uneven thickness, wrinkles, and knife marks. The contrast between the nucleus and cytoplasm is poor, and some cells are even vacuolated.

Three pathologists with senior professional titles rated the slices, and the scoring results are shown in Table 1. The experimental results showed that the new aldehyde-free fixative had superior fixation effects compared to the aldehyde-containing fixative in terms of cell morphology, comparison of nuclear and cytoplasmic staining, and clarity of tissue structure. The difference in nuclear morphology and color was not significant compared to the aldehyde-containing fixative (Figure 2(a) for the lung, liver, kidney, and heart tissues shows the new aldehyde-free fixative, while Figures 2(b) to 2(g) show the aldehyde-containing fixative).

Comparison of New Formaldehyde Free Fixation Solution and Traditional Formaldehyde Fixation Solution for Protein Fixation in Mouse Tissues (Determination of Total Protein Content)

This experiment used an ultramicro spectrophotometer to compare the total protein content in the tissues before and after fixation. The detection results are shown in Table 2.

The experimental results showed that there was no significant difference in the fixation effect of the new aldehyde-free fixative and the traditional aldehyde-containing fixative on tissue proteins ($P > .05$).

Measurement and Evaluation of Air Quality in Experimental Areas of New Formaldehyde Free Fixation Solution and Traditional Formaldehyde Fixation Solution

Comparison of Formaldehyde Volatility between New Fixed Liquids and Traditional Fixed Liquids (40°C for 6 h) (mg/m^3): In this experiment, the air quality detector was used to detect the formaldehyde content before and after the stationary solution was placed in the confined space for a while, and the results reflected the volatility of formaldehyde in the stationary solution. The detection results are shown in Table 3.

The experimental results showed that there was no significant difference in the formaldehyde content in the air before and afte use of the new formaldehyde-free fixing solution, while the formaldehyde content changed significantly in the air before and after the use of the formaldehyde-containing fixing solution. The difference between the formaldehyde-free fixing solution and the formaldehyde-containing fixing solution was significant ($P < .05$). However, there was no significant difference in formaldehyde content in the air between formaldehyde-containing fixatives ($P > .05$).

Comparison of the Properties and Hazards of New Fixed Liquids and Traditional Formaldehyde Containing Fixed Liquids

In this experiment, the confidence environmental protection fixed phase is almost non-toxic and has lower volatility compared to formaldehyde formaldehyde-containing fixed phase. In terms of environmental protection, new environment-friendly fixatives can increase the safety of biological tissue preservation and fixation materials, while

Figure 2. Fixation effect of the (a) new aldehyde-free fixative and (b) six traditional aldehyde-containing fixative agents for the lung, liver, kidney, and heart tissues.

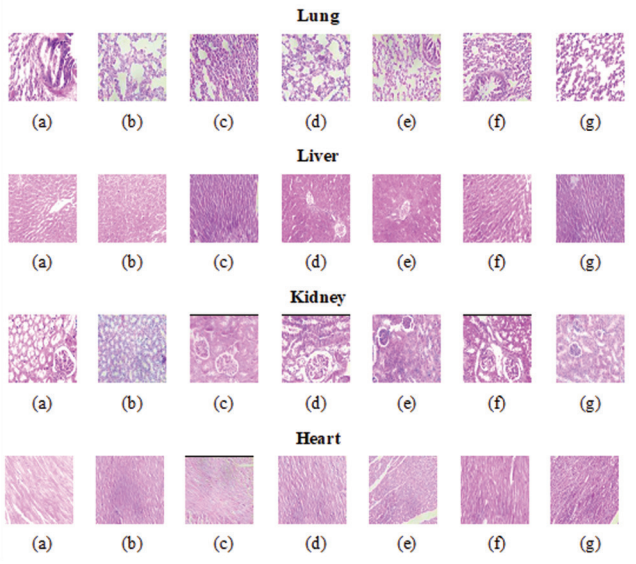

Table 2. Comparison of Total Protein Content Before and After Tissue Fixation (μg/ml)

	Heart		Liver		Lung		Kidney	
	Before fixation	After fixation	Before fixation	After fixation	Before fixation	After fixation	Before fixation	After fixation
New fixed liquid 1	0.0038	0.004	0.0047	0.005	0.0019	0.0021	0.0056	0.006
Formaldehyde containing fixing solution 2-7	0.0039	0.0042	0.0052	0.0054	0.0018	0.002	0.0051	0.0054

Table 3. Formaldehyde Content in the Air of Different Fixatives Before and After Volatilization

	fixative 1	fixative 2	fixative 3	fixative 4	fixative 5	fixative 6	fixative 7
Formaldehyde content in the air before volatilization	0.015	0.016	0.015	0.017	0.014	0.015	0.016
Formaldehyde content in the air after volatilization	0.015	0.056	0.061	0.057	0.051	0.049	0.055

Table 4. Comparison of Different Fixation Solutions

New environment-friendly fixing fluid	Formaldehyde containing fixing solution (fixing agents 1-6)
(1) Low volatility; non-toxic	(1) Volatile, chronic toxicity
(2) Environmental friendliness: increasing the safety of fixed materials for biological tissue preservation	(2) Inducing cancer and deformities
(3) Fixation effect: ensuring a good preservation and fixation effect on biological tissues	(3) Risk factors for leukemia and gene mutations
(4) No adverse reactions to the human body	(4) Reduce memory and intelligence
(5) Irritating eye conjunctiva and respiratory tract mucosa	

Table 5. Comparison of Hazards of Different Fixatives on Human Organs

	fixative 1	fixative 2	fixative 3	fixative 4	fixative 5	fixative 6	fixative 7
Irritation to cornea	None	Yes	Yes	Yes	Yes	Yes	Yes
Irritation to the respiratory tract	None	Yes	Yes	Yes	Yes	Yes	Yes
Specimen corruption and decay	None	None	None	None	None	None	None
Change in specimen color	None	None	None	None	None	None	None
Skin irritation	None	Yes	Yes	Yes	Yes	Yes	Yes
Harm to human health	None	Yes	Yes	Yes	Yes	Yes	Yes

formaldehyde fixatives can increase the possibility of cancer and deformity. At the same time, the new environment-friendly fixing solution can ensure better preservation and fixation of biological tissues with almost no adverse reactions to the human body, while formaldehyde fixing solution can cause a series of hazards such as leukemia and gene mutations (Table 4).

Comparison of the harms of new fixative and traditional formaldehyde-containing fixative on human tissues and organs

In this experiment, the harm of fixatives to human tissues and organs is mainly manifested in the stimulation of the cornea, respiratory tract skin damage, and other aspects (Table 5).

DISCUSSION

Different fixatives have different penetrating abilities to tissues, which will cause the loss of intracellular proteins, mucopolysaccharides, lipids, nucleic acids, and low molecular weight substances to varying degrees.[10-14] A good fixative can make tissues uniform, shrink slightly, and preserve the antigenicity of histiocytes. For the past decade, formaldehyde fixation has been the standard fixative used in pathology to fix tissues.[10] Formaldehyde fixative has the advantages of low price, long-term preservation of tissues, well-preserved tissue morphology, ability to perform various special staining, and reliable immunohistochemical staining results after antigen repair.[15-16] It is a proven tissue fixative. However, formaldehyde fixation solution also has drawbacks, including the ability to polymerize and form trimeric formaldehyde after being stored for too long, which affects the fixation effect.[17] Furthermore, it can form formaldehyde pigments, which can be easily confused with iron-containing hemoglobin, and thus long-term fixation can affect immunohistochemical results.[18] Long-term exposure to formaldehyde can lead to allergies, chronic respiratory diseases, and even the occurrence of tumors.

Neutral buffered formaldehyde solution is currently the most common method for the long-term preservation of tissue samples in biomedical research and pathological diagnosis, while 4% neutral formaldehyde is a routine tissue fixation reagent in pathology.[19-20] Its fixation of pathological specimens, routine HE staining, and molecular biology detection effects have been recognized by pathological experts.[21] However, formaldehyde has certain toxic side effects and poses varying degrees of harm to the environment and human health. Finding an environment-friendly fixing solution with the same effect and no toxicity has always been desirable for pathological workers.

The environment-friendly formaldehyde-free fixing solution used in this study is a new material that does not pose any harm to the environment and also to human health.[22] To understand its effectiveness as a fixing solution, we have compared the fixation effects of the new formaldehyde-free fixative with the formaldehyde fixatives on different tissues of mice to verify the value of the former.

The experimental results show that the new environment-friendly fixative has a good effect on tissue fixation compared to the aldehyde-containing fixative after 24 hours of tissue fixation, and the tissue fixation is sufficient. The experiment shows that the new environment-friendly fixative has a good effect on tissue protein fixation. Fixed tissues were observed under the H&E staining microscope through paraffin-embedded sections. The staining effect was determined with respect to four key aspects: cell morphology, the contrast between nuclear and cytoplasmic staining, color of nuclear morphology, and the clarity of tissue structure. There was no significant difference in the staining effect between the formaldehyde-free and the formaldehyde-containing fixing solutions.

Comparison of the experimental results reveals that air pollution generated from the newly developed environment-friendly fixation solution is much smaller than that of the formaldehyde-containing fixation solutions. In developed countries, substantial efforts have been made to replace formaldehyde fixative with formaldehyde-free and environment-friendly tissue fixative in pathological technology work.[23] A few publications report histological staining using glyoxal, which have verified that the immunostainings performed after glyoxal fixation were superior for the majority of the samples and targets.[24-25] In China, environment-friendly fixative solutions have not yet been widely used, and currently, the vast majority of pathologists still use formaldehyde as the fixative in their daily work.[26] The research shows that the environment-friendly formaldehyde-free fixative is as good as the formaldehyde fixative in maintaining histomorphology, preserving antigenicity and DNrVRNA, and can perform even better than the formaldehyde fixative in some aspects. Nonetheless, there are a few reports on the use of formaldehyde-free fixing solutions in China, but their composition is not clear and are yet to be tested for their application.

This study develops a new type of formaldehyde-free and environment-friendly fixative composed of natural atomic compounds and a 70% -90% pH 4.3-6.5 buffer solution. The main component is water-soluble polyols, and do not contain harmful substances such as formaldehyde, glutaraldehyde, benzene, aromatic compounds, etc. It is non-toxic, light alcohol flavored, soluble in water, and non-toxic to human health and the environment. This study conducted a large number of fixed experiments on mice tissues with different textures, and the comparative results were comprehensive and reliable. Research has shown that there is no significant difference in the staining effect between formaldehyde fixative and environment-friendly fixative for tissue fixation. The fixation and HE staining of liver, brain, and muscle tissues are even clearer. There are also a few limitations of this study. First, the numbers of the specimens were not enough. Second, there was no detection of biomarkers in the HE species, which still needs further verification.

CONCLUSIONS

The new environment-friendly formaldehyde-free fixative developed can replace formaldehyde fixative in HE staining. We will further compare and analyze the effects of special staining and immunohistochemical detection of fixed tissues between the two fixing solutions in our future research.

FUNDING

No financial support was received from any organization for the submitted work. None of the authors have any financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work.

DATA AVAILABILITY

The data can be obtained by contacting the corresponding author.

ETHICS

Consent was obtained or waived by all participants in this study.

AUTHOR DISCLOSURE STATEMENT

No conflicts of interest exist, according to the authors, with the publishing of this work.

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