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Characterization of Fixatives and their Application in Histopathology

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ABSTRACT

Background: One of the most important steps in the histopathological analysis to provide a well-qualified sample which results in excellent and reliable data. There is no universal fixative to be used in all downstream applications in the biomedical sciences and diagnostic pathology. Identifying the characteristics of each fixative and their mechanism of action can be of great help in selecting the right fixative.

Methods: A comprehensive literature searches about characterization of fixatives and their application in histopathology in English databases including Google Scholar, Science Direct, Scopus, Pub Med, and Web of Sciences, Persian SID databases to 2022.

Results: Coagulative and crosslinking agents are conventional fixatives, each providing different perspectives for maintaining tissue integrity. Investigation of the effectiveness of combined fixatives has shown that these fixatives can overcome the limitations of alcoholic and cross-linking agents and can be widely used. It is vital for pathologists and laboratory staff to have a precise knowledge of which fixative is optimal for various applications such as morphology, immunohistochemistry, and RNA/DNA analysis.

Conclusion: This review aims to discuss the classification of different types of fixatives, their mechanisms, as well as applications. It then provides practical information on the best fixative for common methods in analyzing the specimens.

Keywords: Fixatives; Histopathology; Mechanisms; Formaldehyde; Alcohol

Introduction

Fixation is an initial step in cells or tissue processing for microscopical examination in histopathology and molecular assays [1]. The outcome of fixation process is protection of the tissue from microorganisms, rapid minimization of all enzymatic and other metabolic activities, prevention from decomposition, putrefaction, and autolysis, as well as hardening and strengthening the tissue as much as possible to minimize damage [2]. During this procedure, the semi-fluid state of the cell or tissue converts to hardened specimens where their original states are preserved [3]. The studies on various aspects of fixatives have grown in the latter half of the 19th century [4]. The fixing process can be done by both physical and chemical methods. Physical fixatives include heating, microwaving, and freeze-drying [5], while chemical fixatives are classified into four groups based on their mechanisms and chemical nature, including

1. Precipitating agents: alcohols (methanol, ethanol) and ketones (acetone),
2. Cross-linking agents: aldehydes (formaldehyde, glutaraldehyde, and acrolein),
3. Oxidizing agents (osmium tetroxide and potassium permanganate), and
4. Metallic group (mercuric chloride and picric acid, tannic acid, etc.).

They can be used individually or in combination with other fixatives [6]. Fixatives are extensively used in various applications such as histological analysis for assessing the morphology as well as nuclear, cytoplasmic and membrane details in tissues; in immunohistochemistry (IHC) for investigating nuclear, membrane or cytoplasmic proteins by specific antibodies; in molecular biology to quality DNA and RNA extracted from tissue depending on the type and the condition of fixation and proteomics analysis by western blotting and mass spectrometry [7]. This review aims to discuss the general classification of fixatives, the effective parameters in the fixation process, and identifying the best fixative for common methods that are used to analyze specimens.

Methods

A comprehensive literature searches about characterization of fixatives and their application in histopathology in English databases including Google Scholar, Science Direct, Scopus, Pub Med, and Web of Sciences, Persian SID databases to 2022. The search process was performed using the following keywords and medical subject headings (MeSH) terms: «fixatives», «histopathology», « Tissue Fixation», and «Formaldehyde» alone/or in combination with each other. For study selection, first the abstracts and/or titles of the retrieved articles were reviewed. Then, the eligible full text articles were downloaded, and their data were extracted. The duplicate manuscripts, review articles, letters to the editor, congress articles, and studies with unclear data, as well as those with unavailable full text were excluded from the study.

Discussion

Precipitating Agents

The precipitating fixatives including methanol, ethanol, and acetone are the most used cellular fixatives in hematology [8]. Methanol or ethanol are commonly used for fixating peripheral blood smear before being stained with a Rowmanosky stains (e.g., wright's and giemsa) [9]. Precipitating fixatives preserve cells through a process of dehydration and precipitation of proteins [10]. Further, the use of alcohol-based fixation has a protective effect on both DNA and RNA [4]. The yield and quality of DNA and RNA extracted from tissues that are fixed with alcohol fixation are sufficient to perform several downstream molecular analyses such as PCR and cDNA library production compared to unfixed tissue [11]. Also, the DNA extracted from ethanol-fixed samples increases the number of successful PCR amplifications due to providing large fragments [12]. In addition, precipitating fixatives make the cell permeable by removing lipids from the membrane. Cell permeabilization is a process where the cell membrane becomes more permeable than normal state and allows antibodies and dyes to enter the cells [13]. Methanol and acetone can be both permeate and fix the cell at the same time. It is generally recommended to use ice-cold alcohol and to fix the cells at -20°C, as lower temperatures are thought to facilitate the fixation process and better preserve the structure [14].

The advantage of precipitating fixatives is that they properly preserve the epitopes during the fixation process, so these fixatives can be used for methods such as immunofluorescence (IFC) and IHC [15,16]. On the other hand, there have also been some limitations regarding the use of mineral fixatives, including loss of cell membrane structure as well as cytoplasmic organelles, tissue shrinkage and hardening, damage to structural elements such as microtubules and hemolysis of erythrocytes, and extraction of all soluble proteins during the fixation [13,17]. HOPE-technique (Hepes-glutamic acid buffer mediated organic solvent protection effect) is a novel method for fixation of tissues containing acetone [18]. Studies have shown that fixed tissues in HOPE were able to preserve nucleic acids without degradation. DNA and RNA can supposed to evaluation for DNA methylation by bead chip analysis, in situ hybridization (ISH), and transcription microarray analysis [19,20]. Analysis of RNA extraction and amplification by RT-PCR in fixed tissues with HOPE revealed that HOPE-fixed tissues have maximum RNA fragments (2000 bases) [21]. Also, HOPE-fixation provides good morphology and antigenic structures for IHC and Immunocytochemistry (ICC) [22].

Cross-linking Agents

Cross-linking agents consist of formaldehyde, glutaraldehyde, glyoxal, and acrolein. This group reacts with protein and lipid components of membranes, cytoskeleton, and genomic DNA [23]. They cross-link with proteins by N-terminal amino groups, side chains of cysteine, histidine, lysine and thiol, phenol, and imidazole groups of amino acids [3,24].

Formaldehyde

Formaldehyde as a fixative agent was introduced for the first time in 1893 by Ferdinand Blum [25]. Formalin contains 37-40% of formaldehyde and 60-63% of water that usually as neutral buffered formalin (NBF), which is a 10% solution of the concentrated formalin, buffered at pH 7 with phosphate salts [26]. Fixation of proteins has been done by inducing methylene bridges (CH2) between amino acids [27]. Formaldehyde is the most used

fixative that preserves tissue morphology in conditions close to live state. Thus, this fixative is widely used in histochemical stains (like hematoxylin and eosin, giemsa, propidium iodide) [28]. Formaldehyde also cross-links with nuclear proteins and nucleic acids with (A-T) rich regions and reacts with C=C and -SH bonds in unsaturated lipids [4,5].

Evaluation of lipid content and lipid droplet structure by IFC indicated that paraformaldehyde is the best fixative agent for preservation of lipid components [29]. Elsewhere, evaluation of protein localization in different organelles and cellular compartments by IFC microscopy revealed that paraformaldehyde well preserved the cell structure and detected proteins and could be used for proteome-wide studies [30]. Formaldehyde is also a selective fixative for proteomic investigation by specific applications such as amino acid sequence analysis, western blot, immunoassays, ELISA, antibody microarrays, and protein arrays [31]. Note that although formaldehyde is routinely used for IHC techniques, structure modification of many antigens by cross-linking necessitates use of antigen retrieval methods to retrieve the loss of antigenicity [32]. Other limitations of formaldehyde are slow and incomplete fixation, mislocalization, and alteration of the tertiary and quaternary conformation of target proteins, plus loss of epitopes which results in reduced efficiency of immunostaining [1,23].

Glyoxal

Glyoxal, introduced in 1943, is small dialdehyde that composed of two carbon atoms. The most important features of this fixative are safety profile, faster rate of reaction, and crosslinking under certain conditions [33]. As with formaldehyde, it is commercially available as a solution which contains hydrated forms including trimers, dimmers, and ring structures [34]. This fixative provides good cellular and molecular structure during 1-9 h depending on the specimen size [35]. Glyoxal is a reliable fixative for preservation of antigens for using in IHC without tissue antigen retrieval. Nevertheless, arginine-rich immunoreactivity of epitopes is undetectable as imidazole which is provided in response to glyoxal reaction with guanidinium group of arginine can alter the conformation of these epitopes [33]. Commercial glyoxal has a strongly acidic feature which exerts detrimental effects on tissues. The acid-free form of glyoxal could preserve the structural and macro-molecular properties of tissues, and can be used for morphological, IHC, and molecular analyses. The results of molecular analysis by RT-PCR and DNA sequencing showed that the quality of nucleic acid was well preserved by this fixative and provided less fragmented DNA (≥5.000 bp) and RNA (≥200 nt). FISH analysis by glyoxal provides a mild auto fluorescent background in response to binding to guanine and cytidine of DNA, thus complicating scoring of signals [36]. Glyoxal fixation causes lysis of RBCs and inappropriate staining of eosinophilic components [35].

Glutaraldehyde

Glutaraldehyde is a clear, colorless fixative which is soluble in water, alcohol, and organic solvents. This fixative is present as an acidic solution at concentrations from 2 to 70% [24]. Glutaraldehyde was widely used to immobilize many enzymes [24]. Enzyme immobilization is a process that improves enzyme properties such as activity, specificity, sensitivity, and stability to inhibitors [37]. Moraes, et al. applied glutaraldehyde in western blot to improve caspase-3 detection. In this regard, nitrocellulose membranes were treated with glutaraldehyde. The study demonstrated that fixation of blotted membranes with glutaraldehyde by cross-linking of protein induced reduction of protein in several washing processes and improved the sensitivity of caspase-3 immunodetection [38]. It has been reported that glutaraldehyde can also be used for inactivation of viruses [39,40]. Glutaraldehyde is the most efficient fixative for electron microscopy [41]. Also, the best fixative for preserving the structural integrity of RBCs is glutaraldehyde. This fixation maintains Hb in its natural form (oxy) and does not interfere with the hemagglutination reaction [8,41]. Rapid cross-linking of glutaraldehyde prevents disruption of RBCs and leads to no leakage of hemoglobin during fixation [42,43]. The main drawbacks of glutaraldehyde include increased background fluorescence, loss of immunogenicity through denaturation of antigens, and low penetration of antibodies to tissues [44]. The reaction of glutaraldehyde with primary amines during the cross-linking process induces O2 depletion and hypoxia in fixed tissue.

Acrolein

For the first time in 1959, acrolein was introduced as a fixative in electron microscopy. Glycerol dehydration at high temperatures results in production of acrolein [41]. Quick and deep penetration of acrolein compared to other aldehydes provides accurate localization of enzymes and other proteins [45]. Tissues fixed by acrolein can be investigated for enzyme assay [46]. Acrolein can be used in combination with other aldehydes like glutaraldehyde that provided good staining for light and electron microscopic ICC [47]. Acrolein is unstable at alkaline pH and rapidly forms insoluble polymers [34].

Oxidizing Agents

Common fixatives in this group include osmium tetroxide (OsO_4) and potassium permanganate ($KMnO_4$). A comparison of the properties of these two fixatives has shown that the rate of cell penetration of $KMnO_4$ is greater than OsO_4, but the latter is a stronger oxidant [41]. In a study, the structure of phospholipid models by electron microscope was examined by oxidizing fixatives. This fixative was mainly used to fix unsaturated lipids through reaction with double bonds [48]. Pre-and post-fixation by $KMnO_4$ and OsO_4 can prevent production of artifacts during fixation [49]. For electron microscopy, sample preparation was done with

chemical fixation using glutaraldehyde followed by post-fixation with osmium tetroxide that maintain the integrity of the samples, enhance the contrast of SEM images and provide larger intercellular spaces [41]. According to a study, degradation of carbohydrate and protein due to low rate of reaction and penetration of osmium tetroxide happened during fixation [34].

Metallic Group

Metallic group fixatives consist of mercuric chloride and picric acid. The components of this group are commonly used in combination with other fixatives, such as mercuric chloride in Zenker's solution and picric acid in Bouin's solution [3]. Fixatives that contain this group demonstrate excellent preservation of the component such as nucleus and extracellular matrix details. However, mercuric chloride and picric acid make fixed tissue stained yellow and brown [3,34].

Mixtures of Fixatives

5.9.1.Carnoy's and Methacarn Fixation: Carnoy's consists of 60% ethanol, 30% chloroform, and 10% glacial acetic acid, while methacarn is formed by replacing methanol with ethanol. Both are the best fixatives for preserving nucleic acids in tissues [50]. When comparing the two fixatives, since methacarn contains methanol, it is a better fixative than Carnoy's. Methacarn compared to carnoy's yields less hardening and little shrinkage of fixed tissues [51]. Fixation by carnoy's solution results in the good quality of DNA for analysis via PCR and Sanger sequencing [52]. Also, methacarn allows preserving the integrity of protein and nucleic acid. Gene expression can be assayed by methods such as RT-PCR and microarray analysis. Protein extraction and expression have shown that methacarn provides a good yield of protein and can be used for protein analysis, but it is not recommended for surface antigens [26,53,54].

RCL2

RCL2 is a new non-crosslinking fixative whose mechanism relies on tissue dehydration. According to the patent application (WO 2004/083369), RCL2 consists of ethanol, acetic acid, and glycerol. This fixative is reliable in terms of morphology, IHC, and molecular examinations. The quality of nucleic acid was investigated by PCR amplification and RNA integrity number. A fixed tissue with RCL2 can provide DNA and RNA amplicons larger than 800bp and 200bp [55]. Molecular assay with nucleic acid extracted from this fixative could provide good quality in fluorescent and silver in-situ hybridization (FISH and SISH), PCR, and RT-qPCR [56,57].

FineFIX

FineFIX is a formalin-free fixative which consists of ethanol, water, 1, 2-propanediol, polyvinyl alcohol, and monomeric polyhydroxy compound (US patent: 2005/0074422). By preserving the cell integrity and antigenic property of tissue, this fixative can be used for morphology, ICC, and IHC analysis. The good quality of extracted DNA/RNA from fixed tissue in FineFIX can be used in molecular investigation even for sensitive methods such as high-resolution melting method (HRM) [58]. Amplification of the extracted nucleic acid from fixed tissue by this fixative can provide DNA and RNA fragments with maximum lengths of 2400 bps and 600 bps [59].

Bouin's Solution

Bouin solution first described by Till and McCulloch in 1897, is composed of 25% of 37% formaldehyde solution, 70% picric acid, and 5% acetic acid. It causes a denaturing and cross-linking effect on proteins [3]. This fixative can be used as a staining solution though it needs to be washed in multiple steps with ethanol to remove the yellow stain in response to presence of picric acid [60]. Other studies have shown that Bouin solution can be used for evaluating the protein and nucleic acid in IHC and molecular analysis such as real-time PCR [61]. Note that the fragments less than 100 bp for RNA and 200 bp for DNA could be amplified by this fixative [62].

Davidson's Fixative

Davidson's fixative contains acetic acid, alcohol, and formalin with both cross-linking and precipitating features. This fixative can be used for histological examination of eye, bone marrow, breast, testis, and other tissues. Acid acetic makes rapid penetration and swelling of proteins with alcohol compensating this effect through shrinkage of proteins [63]. The morphology and IHC analysis in both eye and testis tissues by this fixative provides reliable preservation structural details and antigenicity [64]. Also, studies have shown that TUNEL assay which analysis DNA fragmentation can be performed by modified Davidson's fixative at lower temperatures [65]. Excessive shrinkage and poor staining are the disadvantages of this fixative, but modification of the component ratio in modified Davidson's fixative improves the morphology analysis [64].

Classification of different fixatives and details about their properties are reported in Table 1.

Table 1: Classification of different fixatives and details about their properties.

Chemical Fixative		Fixative	Mechanism of fixation	Recommend
Simple fixative	Precipitating agents	Ethanol (CH_3CH_2OH) Methanol (CH_3OH) Acetone (CH_3COCH_3)	Coagulating and dehydrating mechanism	Preserves small non- lipid molecules such as Glycogen, Nucleic Acids, preserve enzymatic activity
	Cross-linking agents	Formaldehyde (HCHO)	Cross-linking mechanism	Routine processing, Proteins
		Glutaraldehyde ($OHC-CH_2-CH_2-CHO$)		Electron microscopy, Lipids, RBC
		Acrolein ($CH_2=CH—CHO$)		phospholipids
	Oxidizing agents	Osmium tetroxide (OsO4) potassium permanganate ($KMnO_4$ etc)	unsaturated bond	Lipids, Post fixation in electron microscopy
	Metallic group	Mercuric chloride ($HgCl_2$) picric acid ($C_6H_3N_3O_7$)	forming insoluble metallic precipitates	Use in compound fixative like Zenker (Bone marrow biopsy) and Bouin
Compound fixatives	Alcohol based fixatives	Carnoy (Ethanol,chloroform,acetic acid) Methacarn (Methanol, chloroform, acetic acid)	Coagulating and dehydrating mechanism	Molecular analysis
		RCl_2 (Ethanol, acetic acid, and glycerol) Finefix (Ethanol, propanediol, polyvinyl alcohol)		Morphology, ICC, IHC and Molecular analysis
	Mix fixatives	Bouin (Formaldehyde, picric acid, acetic acid)	Both cross-linking and precipitating features	Staining, IHC, Molecular analysis
		Davidson (Acetic acid, alcohol, and formalin)		Histological examination, IHC, TUNEL assay

Glutaraldehyde-Formaldehyde

The combination of formaldehyde and glutaraldehyde can be used as an ideal fixative. Formaldehyde penetrates specimen faster than glutaraldehyde, while glutaraldehyde has a more complete and stable fixation property than formaldehyde [41]. Formaldehyde cross-linking with proteins is weaker than that of glutaraldehyde as it is a small molecule and in a more difficult state can bridge between reactive groups. Also, fixing specimens with formaldehyde is temporary and reversible [66]. Kirkeby, et al. compared the effect of glutaraldehyde, formaldehyde, and their mixture on protein fixation via SDS-electrophoresis and spectrophotometry. The result of this study showed that mixtures

of glutaraldehyde and formaldehyde offer a far stronger effect on fixation of proteins compared with their individual use [67]. Also, it has been reported that the combination of these two fixatives provides good morphology and antigenic structures especially for IHC and ICC assessments [44].

Factors Affecting Fixation Process

It is now well established that pH, penetration, tissue specimen size, osmolality, temperature, buffer, concentration, and method of application of the fixative are effective factors in the fixation process [41,68].

PH

The best result from fixation occurs at physiological pH within the range of 6-8. Proteins at their isoelectric pH show minimal solubility and viscosity, osmotic pressure, and swelling. Appropriate pH is maintained in the fixation through an environment with appropriate buffering capacity. Different buffers can be used in the fixation, but phosphate buffers which exist in living systems cells, are more physiological than any other buffers [41].

Tissue Specimen Size and Thickness

The small size of the specimen results in uniformity of fixation. Ideally, the specimen should be no larger than 0.5 mm [41]. There is a direct relationship between specimen size, fixative penetration, and fixation duration [5]. If the sample size is large, it is unfavorable for the fixative to penetrate and reach the deeper parts of the tissue, which would result in autolysis of epithelium. Among the commonly used fixatives, formaldehyde penetrates faster than either glutaraldehyde or OsO4 (41). Cross-linking fixatives are preferable for larger specimens [69].

Osmolality

The appearance of a fixed sample (size and shape) varies with the osmolarity of the fixative solution [32]. Hypertonic and hypotonic solutions lead to shrinkage and swelling, respectively [5].

Temperature

Temperature of the fixative during fixation may affect the tissue structure. By raising the temperature, the speed of chemical reactions increases between the fixative and specimen along with the rate of penetration [68]. Formaldehyde fixation is better at room temperature, while 4°C is commonly used for glutaraldehyde fixation which results in diminished shrinkage of mitochondria, granularity of cytoplasm, and volume changes [41]. Also, for electron microscopic studies or acetone fixation, 0-4°C has been reported as ideal temperature [5].

Buffer

A buffer is an aqueous solution added to maintain the optimal pH. The type and concentration of buffer are depending on the type of fixative and desired application. Phosphate, cacodylate, bicarbonate, tris, and acetate are the most used buffers. In neutral buffered formalin (NBF), pH adjusted around 7.2–7.4, in which amino groups are discharged and react with formaldehyde [70]. Moreover, fixation samples in NBF result in increased DNA quality without adverse effects on tissue morphology [71].

Concentration

The concentration of fixative depends on the type of the fixative used. Low concentrations of fixatives require longer durations of fixation while higher concentrations destroy enzyme activity and damage the structure of cells [41].

Volume Ratio

The tissue to fixative volume ratio varies widely during the fixation of specimens. The optimal fixative ratio should be 10:1, but studies showed that less volume of fixative (2:1) can provide equal diagnostic value and characteristics [69]. The fixation process is more dependent to time and temperature than the volume of fixatives [72].

Types of Tissues

Biological specimens are divided into three types including soft tissue (organs), hard tissue (bones), and dense tissue (skins). The fixation is more complicated for harder and denser tissues. For example, hard tissues such as bones require both decalcification step and a longer duration of fixation [73].

Conclusion

There are various methods for biospecimen collection, processes, and preservation that can affect the cellular, molecular, and proteomic profiles. In clinical investigation, frozen and formalin-fixed tissue is commonly used for histopathological analysis. Although snap-freezing is a good way for analyzing biomolecules, due to processing and storage limitations of this method, it is not widely used in laboratories [74]. Also, formaldehyde has properties such as easy usage, cost-effectiveness, preservation, and fast permeability to tissues. Nevertheless, its limitations such as undesirable effects on the quality of nucleic acid, reduction in detection, and immunoreactivity of proteins have led to selection of alternative fixatives in certain applications [75]. High-quality nucleic acids are essential for advanced molecular technologies to provide prognostic factors in clinical practice [16]. Molecular analysis in fixed tissues via formaldehyde causes fragmentation and alternation of sequences of nucleic acid especially at the AT-rich regions of double-stranded DNA [4,50]. It has been shown that precipitating fixatives such as acetone and methanol, as well as gentle crosslinking fixatives (paraformaldehyde, etc.) can preserve nucleic acid regarding PCR amplification [1]. Modified methacarn (8 parts of methanol and 1 part of acetic acid) is the best option for formalin substitute in regarding to nucleic acid extraction [26].

The fragmentation of nuclear DNA is one of the most characteristic events of apoptosis as evaluated by fluorescence microscopy or cytometry commonly called TUNEL assay [76]. The apoptotic cells can be identified with TUNEL staining via modified Davidson's fixative plus paraformaldehyde causing both morphologic details and minimize false positive staining in the TUNEL assay [77]. Proteomic analysis is performed by methods such as protein extraction, western blot analysis, and IHC. The comparison of different fixatives has shown that fixation of tissues with HOPE and RCL2 provides the highest yield of protein [78,79]. The fixation of tissues by NBF rendered changes in proteins and masking their epitopes, so antigen retrieval strategy is necessary for availability antibody to antigen [79]. Alcohol-based fixatives can provide desirable results for IHC analysis and there is no need to antigen retrieval for this group of fixatives. Membrane proteins such as EGFR, HER2, and interleukin receptors have shown to be better preserved in mixtures of fixatives such as FineFIX, RCL2, or HOPE over FFPE [78]. The selection of the proper fixative leads to maintaining the quality of tissues for the analytical technique. It is necessary to have comprehensive knowledge about the characteristics of each fixative which would culminate in selection of the optimal fixative for each application. Studies have suggested that a single type of fixative cannot be used for preservation of tissues to evaluate all biomarkers. Reliable information from fixed tissues heavily contributes to the proper diagnosis and treatment of diseases.

Ethical Considerations

This article has been registered and ethically approved by Mazandaran university of medical science with approval ID, IR.MAZUMS.REC.1399.255 in date 2020-05-13.

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Author Contributions

Seyedeh Farzaneh Jalali and Hadi Hassannia contributed to the conception and design of the study and Manuscript writing; Farhad Jadidi-Niaragh, Seyed Ehsan Enderami and Abdol Sattar Pagheh Collection and assembly of data; Esmaeil Akbari and Saeid Abedian Kenari Final approval of manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

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