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Review Article

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FIXATION & VARIOUS FIXATIVES USED AS AN ALTERNATIVE TO FORMALIN-A REVIEW

***Dr. Neha Vaid. Deepak Bhargava, Rajeshwar Chawla, Deepak Goyal, Ruby Bansal, Chander Udey Singh Pawar**

JCD, Vidhyapeeth, Sirsa India 125005.

***Corresponding Author: Dr. Neha Vaid**

JCD, Vidhyapeeth, Sirsa India 125005.

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ABSTRACT

Fixation is usually the first step to prepare biological specimens for microscopy. Any treatment which will preserve cell structure and its biochemical composition can be deemed to be fixation. Of course, the quality of fixation is the key for all following steps which are necessary in histological research. Hence, preservation of cells with minimal alteration of morphology and virtually no loss of molecules is essential in tissue preservation. The purpose of the present article is to provide adequate insight about the fixation and various fixatives used as an alternative to formalin.

KEYWORDS: Fixation, fixatives, biopsy.

INTRODUCTION

Biopsy of any tissue is a key to its final diagnosis as it plays a pivotal role to arrive at a final diagnosis. This biopsied tissue needs to be fixed for further procedures.[1] Fixation is aimed to preserve the tissues in a life – like state, prevent bacterial putrefaction, prevent autolysis and to increase the refractive index of the tissue.[2] Fixation is usually the first step to prepare biological specimens for microscopy.[3] Hence, it has been considered as a crucial step for preparing tissues for histopathology.[1] Fixation terminates any ongoing biochemical reactions, and may also increase the mechanical strength or stability of the treated tissues.[4] First, a fixative usually acts to disable intrinsic bio-molecules particularly proteolytic enzymes—which otherwise digests or damages the sample. Second, a fixative typically protects a sample from extrinsic damage.[5] The choice of a fixation protocol will largely depend on the analyses to be performed. Obviously, there exists no ideal fixative for all study types.[3] Thus, a pathologist must have a fair idea of the properties of these commonly available fixatives, so that a correct choice can be made depending upon the desired results.[6] This review aims to give a brief overview of the commonly used fixative formalin and other fixatives that are used as an alternative to formalin.

Theoretical basis of fixation

Fixation may be considered "a complex series of chemical events. Cells and extracellular components contain peptides and proteins, lipids and phospholipids (membranes), carbohydrates and carbohydrate complexes, various types of RNA and DNA. Some tissue elements will chemically react with the fixative, be stabilized by cross-linking and thus preserved, others may be unaffected by the fixative but trapped within a cell or tissue by other fixed elements.[7]

Qualities of ideal fixative[8]

Prevent autolysis and putrefaction.
Preserve cells, tissue and its constituents in life like manner.
Make the cellular components insoluble to reagent used in tissue processing.
Mildly hardens tissue.
Preserve tissue volume (should be isotonic), maintain shape and prevents structure deformation.
Economical (cheap), stable, readily disposable or recyclable.
Should be useful for wide variety of tissues.

Types of fixation[9]

1. Physical:
It includes (a)Heat (b) Microwave and (c) Freeze-Drying & Freeze substitution
2. Chemical:
It includes (a)Coagulant and (b) Non-Coagulant.

Types of fixatives

Classification on the basis of types of structures fixed:[3]

a. Micro-anatomical fixatives: Fixatives which are chosen for *anatomical* studies (whole tissue structure). E.g 10% Formalin, Bouin's fluid, Zenker's Fluid.

b. Cytological fixatives: Fixatives used for cell smears, cell suspensions from body fluids or prepared from tissues. Two types:

1. Cytoplasmic Fixatives. E.g Formol Saline, Formol Calcium, Champy's Fluid.
2. Nuclear Fixatives e.g Alcohol, Chloroform, Glacial acetic acid

c. Histochemical fixatives.

Classification of fixatives according to chemical composition:[6]

a. Aldehydes- Formaldehyde, Gluteral, Acrolein
b. Oxidizing Agents- Osmium Tetroxide, Potassium Permanganate,
c. Protein Denaturing Agents: Acetic acid, methyl alcohol, ethyl alcohol
d. Other cross- linking agents: Carbodimides
e. Physical: Heat, Microwave
f. Miscellaneous: Mercuric chloride, picric acid

Classification of fixatives according to the components present:[8]

Simple fixative: contains single chemical e.g, formaldehyde, Glutraldehyde, ethyl alcohol.

Compound fixative: A mixture which contains more than one chemical fixative.

Formalin based fixatives: 10% Neutral buffered formal saline, 10% Neutral buffered formalin.

Mercurial fixatives: Zenker's solution, Helly's solution, B5 fixatives.

Dichromate fixatives: Regaud's solution, Moller's solution, Orth's solution.

Picric acid fixatives: Bouin's fluid, Genre's fluid.

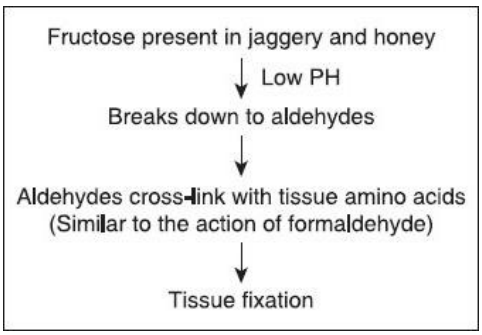
Alcohol containing fixatives: Carnoy's fluid, Acetic acid formalin.

Factors affecting fixation

1. **Time interval** from removal of tissue to fixation: fixation should be started as fast as possible. Also, avoid drying of tissue between the steps of tissue removal and fixation.[3]
2. **pH** values of different fixatives vary. In general pH is usually adjusted to the physiological range by use of a suitable buffer. After being surgically removed, pH within the tissue cells is known to decrease, due to anoxia. As many normal tertiary and quaternary structures of most proteins are stable over a limited pH range about neutrality i.e. pH of 7, many buffer systems are available to decrease this acidic environment and to preserve cellular proteins. Satisfactory fixation occurs between pH 7 and 8. The most commonly used in our laboratories is phosphate buffer.[10]
3. **Temperature of Fixation**: The diffusion of molecules (solutions) increases with increasing temperature due to increased movement and vibration of molecules. Thus, the rate of penetration of a tissue by formaldehyde is increased at higher temperatures.[11] For light microscopy initial fixation is usually carried out at room temperature and this may be followed by further fixation at temperatures up to 45°C during tissue processing.[12]
4. **Penetrability of fixative**: This depends on the diffusibility of the individual components and the size of the tissue specimens.[3] With increase in pressure, the penetration of the solution is also rapidly increased.[13] Other factors such as the porosity and the density of tissue need to be considered in deciding the appropriate size of a tissue to be fixed for a particular protocol.[14]
5. **Duration of Fixation and Size of Specimens**: The factors which govern diffusion of fixative into tissue were investigated by Medivan. He found that the depth reached by a fixative is directly proportional to the square root of duration of fixation. For most fixatives, the time of fixation is approximately equal to the (distance)- fixative must penetrate. Most fixatives such as NBF will penetrate tissue to the depth of approximately I mm in 1 hr.[15]
6. **Volume**: a high ratio of fixative to tissue will ensure good fixation process. In this sense, it is best to change the fixative solution several times during the fixation process.[3] the volume of fixative should be in excess of 20 times the volume of the tissue.[15]
7. **Concentration of Fixative**: Cost, effectiveness, and solubility determine the appropriate, concentration of fixatives. Concentrations of formalin above 10% tend to cause increased hardening and shrinkage, whereas ethanol concentration of below 70% did not remove free water from tissues efficiently.[16]
8. **Osmolality of Fixatives and Ionic Composition**: Osmolality of vehicle buffer is very important. Hypertonic and hypotonic solutions lead to shrinkage and swelling, respectively. The best results are obtained with solutions that are slightly hypertonic (1500 mOsm). Various ions (Na', K', Ca'', Mg'+) can affect cell shape and structure regardless of the osmotic effect. It is believed that the ionic composition of fluids should be as close as possible to physiological composition.[17]
9. **Vehicles and additives**: Some salts can have denaturing effects while others such as ammonium sulfate can stabilize proteins. Tannic acid can be useful because it penetrates tissues easily and precipitates polypeptides and proteins. Phenol has an accelerating effect on formaldehyde fixation. Transitional metal salts such as zinc sulfate helps in the formation of insoluble protein and polypeptide complexes, thus enhancing antigen preservation. It is possible to supplement fixatives with detergents with the aim to enhance subsequent micro-techniques. It must be noted, however, that this type of primary fixation is limited to special applications because detergents in general have deleterious effects on cellular details.[3]
10. **Tonicity**: An isotonic solution will cause neither swelling nor shrinkage of cells in the solution. Isotonic solutions give an osmotic pressure equal to that of the cell cytoplasm. Hypertonic solutions cause shrinkage, while hypotonic solutions induce

swelling. The tonicity can be adjusted by adding electrolytes (e.g., NaCl) or non-electrolytes (e.g., sucrose). Most of the fixatives are slightly hypertonic.[16]

Fixation procedures are generally of two different types: immersion by which tissue samples are immersed into the fixative solution and perfusion via bloodflow.[3]
Formaldehyde was discovered by Butlerov in 1859. It was first synthesized by Van Hoffman in 1868 who developed a practical method for its synthesis from methanol, and further established its properties. Trillat in 1889 was the first to commercially manufacture formaldehyde.[6] Formalin is traditionally a popular and widely used fixative for histopathology processing of tissues due to its ease, economic viability, fairly fast fixation, effortless processing and an array of histologic techniques that can be performed postfixation. Despite these advantages, the health and safety risks associated with formalin use is a concern.[18]



Adverse Reactions Reported After Exposure to Formaldehyde[19]

Short-Term Exposure

Eyes Blindness: Conjunctival irritation, Corneal clouding, Corneal erosion, Keratitis
Gastrointestinal System: Colitis, Dry mouth, Oral and gastric irritation, Sclerosing cholangitis Ulcers, necrosis, perforation, hemorrhage
Respiratory: Asthma, System Chest tightness and pain, Cough with or without sputum, Nose and throat irritation, Pharyngitis, Pneumonitis, Pulmonary edema, Respiratory distress, Respiratory failure, Rhinitis.

Skin Contact dermatitis Drying, crackling, scaling, and whitish discoloration of the skin Eczema, Erythema multiforme, Itching Rash, Acidosis, Anaphylactic shock, Death, Dizziness Drowsiness, Immunohemolytic anemia, Renal failure.

Long-Term Exposure

Respiratory System: Cuboidal and squamous metaplasia, Goblet cell hyperplasia, Loss of cilia formation of autoantibodies.

The International Agency for Research on Cancer branch of the World Health Organization classifies formaldehyde as carcinogenic in humans.[20] The U.S. Occupational Safety and Health Administration (OSHA) stated that employers must reduce worker exposure to formaldehyde at, or below, permissible exposure limits (PEL) and the TWA (time-weighted average) should be less than or equal to 0.75 ppm. The 15-min short term exposure limit (STEL) is 2 ppm.[21]

Substitute to formalin

1. Acid free glyoxal solution results in a histological, immunohistochemical and nucleic acid preservation not inferior to that permitted by fixation in phosphate buffered formalin (PBF).[20]
2. Honey have all the fixative properties that an ideal fixative should have and can be used as an alternative fixative to formalin.[22]
3. Jaggery has all the innovative potential to be a brilliant standby for formalin. Recently it has been reported that effectiveness of honey as fixative over jaggery shows superior results.[23]

The possible mechanism of fixation by honey and jiggery[24] (shown in Fig. 1).

Weigners fixative is a nonhazardous alternative to formalin, which provides a good morphologic preservation of most organs, a similar sensitivity for protein detection, and a superior preservation of nucleic acids. Weigners may therefore be a promising alternative to cryopreservation and may be embraced by people affected by formalin allergies.[25]

CONCLUSION

Fixation is a vital part of histotechniques. No fixative is ideal. Every fixative in some or the other way compromises the morphology, protein evaluation or histochemical staining of the tissue and therefore the fixative and fixation regime must be carefully chosen based upon the desired end-result. This is achieved by exposing the tissue to chemical compounds, call fixatives. Good fixative is most important factors in the production of satisfactory results in histopathology. Following factors are important: 1. Fresh tissue 2. Proper penetration of tissue by fixatives 3. Correct choice of fixatives. No fixative will penetrate a piece of tissue.

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