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REVIEW ARTICLE

Tissue Fixatives: A Review

Radhika Rai[1], Amit Bhardwaj[2], Shalu Verma[3]

Department of Oral Pathology, FDS,
SGT University, Gurgaon.

Department of Periodontology, FDS,
SGT University, Gurgaon.

Department of Pedodontics and Preventive Dentistry,
FDS, SGT University, Gurgaon.

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E-mail: nagpal.j@gmail.com

Abstract: Fixation is the first or the foundation step of the histotechniques done to preserve the tissues in as close a life like state as possible by preventing their autolysis and putrefaction .A number of fixatives exists, either having being in use for decades, or in the case of formaldehyde over a century. Every fixative has different properties, each indicated for a special purpose based on the type of cell component to be studied, the method of sectioning and staining employed and the type of microscopy involved. 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. This review aims to give a brief overview of the commonly available fixatives with their merits and their demerits. The fixatives discussed are: a formaldehyde containing fixative-10% Formalin, a picric acid fixative-Bouin's Fluid, an alcoholic fixative- Clarke's Fluid and a mercury chloride containing fixative- Zenker's Fluid.

Keywords: Bouin's Fluid, Clarke's Fluid, Tissue fixation, Fixative, Formalin, Zenker's Fluid.

Introduction:

DIAGNOSIS' [Greek: *'dia'*- through or by means of and *'gnosis'* - knowledge] is the process of identifying and determining the nature and cause of a disease through complete evaluation of patient & review of the lab findings. Though the attempts to establish a confirmed diagnosis began since the days of Hippocrates, establishing a confirmed diagnosis became possible only after the advent of histotechniques. Thus a proper fixation is mandatory to facilitate a correct diagnosis.

'Histotechniques' refer to the series of chemical procedures through which the tissues have to undergo before they are ready to be microscopically examined and diagnosed[1].

Fixation is the first or the foundation step of the histotechniques and is done immediately after biopsy. Fixation is a complex series of chemical events which preserves the tissues in as close a life like state as possible by preventing their autolysis and putrefaction[1]. During this process, the semi fluid state of the cell is converted into a semisolid state thus maintaining, the morphology and structural details of the tissue[2].

The clarity of all microscopic preparations depends upon the adequacy with which the tissue is fixed. Faults of fixation can not be remedied at any later stage and the finished preparation can only be as good as the primary treatment. Thus a proper fixation is mandatory to facilitate a correct diagnosis. To attain an ideal fixation it is not only essential to maintain the proper conditions but also to select an appropriate fixative[2,3]. There are many fixatives available. Though different fixatives have different features but there are certain features objectives which an ideal fixative must possess (Table I).

In this review, fixatives with different chemicals as their base constituent have been reviewed and compared. The fixatives discussed are: a formaldehyde containing fixative-10% Formalin, a picric acid fixative-Bouin's Fluid, an alcoholic fixative- Clarke's Fluid and a mercury chloride containing fixative- Zenker's Fluid.

Formalin

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 thus establishing the practical aspects of its manufacture. Trillat in 1889 was the first to commercially manufacture formaldehyde as an industrial reagent after he was issued the patent, who in turn licensed several firms in France and Germany for its manufacture[4, 5].

Ferdinand Blum in 1892 discovered that formalin could serve to be an excellent fixative when he noticed that the skin of his fingers that had come in contact with the diluted solution became hardened[5].

The molecular mechanism of tissue fixation by formaldehyde is still not well understood. However the most pos-

sible and rational explanation for the same had been given by Feldman et al in 1973. Chemical studies indicate that formaldehyde is a reactive electrophilic species that reacts readily with various functional groups of biological macromolecules in a cross-linking fashion such as with proteins, glycoproteins, nucleic acids, and polysaccharides. The most reactive sites are primary amines (for example, lysine) and thiols (cystein), and the subsequent cross-linking of these functional groups to less reactive groups, such as primary amides (glutamine, asparagine), guanidine groups (arginine), and tyrosine ring carbons is a favored process . This intra-and intermolecular cross-linking of macromolecules alters considerably the physical characteristics of tissues[3].

It was further explained by Le Botlan et al in 1983 that Formaldehyde, when dissolved in water, rapidly becomes hydrated to form a glycol called methylene glycol. When tissues are immersed in formaldehyde solutions, they are

Table I. Objectives of an Ideal Fixative [1,2]:

1.	Preserve tissue in a life-like state.
2.	Prepare tissues for subsequent processing.
3.	Prevent putrefaction & autolysis of tissues
4.	Prevent osmotic damage.
5.	Prevent shrinkage and swelling
6.	Prevent any change in volume or shape during the subsequent procedures.
7.	Preserve all cell constituents.
8.	Harden the tissues allowing easy sectioning.
9.	Convert the semi fluid consistency of cells to an irreversible semi solid consistency (sol to gel)
10.	Render tissue components resistant to extraction by water and organic solvents
11.	Optimum Optical differentiation.

There are various classifications of fixatives based on different criteria. Some of the most commonly accepted classifications are listed in Table II.

Table II. Classification of Fixatives[1]

S.No.	**Type of Fixative**	**Examples**
I.	**Classification on the Basis of Type of Structures Fixed:**	
1.	Microanatomical Fixatives	10% Formalin, Bouin's fluid , Zenker's Fluid etc.
2.	Cytological Fixatives	
a.	*Cytoplasmic Fixatives*	Formol Saline, Formol Calcium, Champy's Fluid.
b.	*Nuclear Fixatives*	Alcohol, Chloroform, Glacial acetic acid
3.	Histochemical Fixatives	Vapour Fixatives: Formaldehyde, Glutaraldehyde
II.	**Classification on the Basis of Number of Structures Fixed**	
1.	Simple Fixatives	Formaldehyde, Osmium Tetroxide, Picric acid
2.	Compound Fixatives	Formol Saline, Bouin's Fluid , Zenker's Fluid,
III.	**Classification on the Basis of Chemical Composition:**	
1.	Aldehydes	Fomaldehyde, Glutaraldehyde, Acrolein
2.	Oxidizing Agents	Osmium Tetroxide, Potassium Permanganate,
3.	Coagulants	Acetic acid, methyl alcohol, ethyl alcohol
4.	Physical Agents	Heat, Microwaves
5.	Miscellaneous	Mercuric chloride, picric acid

penetrated rapidly by methylene glycol and the fraction of formaldehyde present. Actual covalent chemical reaction of the fixative solution with tissue depends on the formaldehyde present being consumed after forming bonds with the tissue components and more formaldehyde forming from dissociation of methylene glycol.[5] Thus, equilibrium between formaldehyde as carbonyl formaldehyde and methylene glycol explains most of the mystery of why formaldehyde penetrates rapidly (as methylene glycol) and fixes slowly (as carbonyl formaldehyde) [3]. Though there are a large number of fixing solutions available but still over the last century, anatomists and pathologists have used formalin as the fixative of choice. Formalin offers a huge number of advantages: It is a stable fluid. It is easy to prepare. It has a low cost. It allows the application of most stains. Preserves morphological detail with few artefacts and it is good for frozen sections[1].. Though the use of formalin for fixation is a rule in every histopathological lab across the world but various researchers over the times have pointed out its limitations which include slow fixation[1], slow penetration[1], poor nuclear fixation and shrinkage[7]. Cross links formed by formalin with proteins hinder immunohistochemistry[8].Moreover formalin has been found to be a health hazard. It has been reported to haved toxic effects on the immune system , has acute and long standing effects on the respiratory system , lymphatic system and has been reported to be carcinogenic[9]

Bouin's Fluid

The Bouin's Fluid was introduced by Pol André Bouin a distinguished French scientist in the year 1897. Bouins fixative is a combination of picric acid, formaldehyde and acetic acid. The effects of the three chemicals in Bouin's solution balance each other. Formalin causes cytoplasm to become basophilic but this effect is balanced by the effect of the picric acid. This results in an excellent nuclear and cytoplasmic staining. The tissue hardening effect of formalin is balanced by the soft tissue fixation of picric acid. The tissue swelling effect of acetic acid is balanced by the tissue shrinking effect of picric acid[1, 2].

The fixation mechanism of Bouins is essentially because of the action of the picric acid present in it. It has both a coagulative as well as cross-linking effect on proteins.[10] It forms picrates with basic amino acids and in the process causes the proteins to precipitate. It is suggested that this is the reason that acid dye staining is so effective following picric acid fixation: that basic proteins, to which acid dyes would attach, are well preserved, but acid proteins are not adequately fixed and may be removed. Nuclear protein is also precipitated, but the DNA itself remains water soluble. This means that nuclear structure may be shown with acid dyes rather than basic dyes, and that DNA methods are unreliable.

There is no direct reaction with carbohydrates, although the protein component of carbohydrate-protein complexes may be fixed. Glycogen is unaffected directly but is either physically trapped within precipitated protein or, if it is bound to protein, is preserved along with the protein as it is fixed. This preservation of glycogen is striking enough that picric acid fixation is recommended when glycogen is of special interest, particularly if it is used in conjunction with high concentration of ethanol[1].

The major demerits of picric acid fixatives are hat they stain the tissues yellow, retain little affinity for basic dyes and cause a considerable shrinkage but still there are certain situations where Bouin's fixative is preferred over formalin. The first case is for small biopsies, because the yellow tinge imparted to the tissue facilitates visualization during embedding, without an additional step of dipping the biopsies in ink. The second is when excellent nuclear detail and glycogen preservation are desired. For example, improved preservation of nuclear detail is favourable for lymphoid lesions and testicular biopsies and prostate biopsies.[11] Bouin's fixative is also appropriate to fix tissue for measuring collagen fiber because of its color enhancement property.[12] It has also been proven to be a good fixative for IHC when vimentin was used.[7]

Clarke's Fluid

The Clarke's Fluid was introduced by JL Clarke in 1851and thus this fixative was eventually named after him. Initially the Clarke's fixative was introduced for the nervous system. It was the first histological fixative to have a published formula. The formula was 3 parts of not less than 95% or 96% Alcohol and 1 part of pure Acetic acid. The "not less than" indicates that though the original formula of Clarke used absolute alcohol but since using 100% alcohol is difficult to use for practical reasons: high price and the fact that is highly hygroscopic. 96% alcohol can be used for lab purposes [96% alcohol is 96 ml of absolute alcohol + 4 ml water for every 100ml]. This is a well known azeotrope mixture, and when alcohol is concentrated through distillation, alcohol cannot be concentrated purer than this[1].

Both Ethyl Alcohol and acetic acid play different roles and compensate for each other's disadvantages creating a unique combination in this fluid. High purity alcohol precipitates proteins and is a very fast dehydrator. Its action is equivalent to suddenly drying the cells. The result is that, by inducing water loss, alcohol shrinks cells and tissues and harden the fixed pieces. Acetic acid on the contrary, swells, expands, cells and tissues, counterbalanc-

ing alcohol action, and precipitates DNA efficiently, making it an excellent chromatin fixative, encouraging the colouration of the nucleus. Thus Clarke's appears to be, according to a more than a century-old experience, a quite balanced formula, in which each component controls the other component effects.

Prento et al [13]compared the performance of various fixatives Cellular structure was observed following routine dehydration and paraffin embedding. Histological distortion, cell shrinkage and vacuolization were prominent when the formalin or ethanol fixatives were used. In contrast, these artifacts were found occasionally and to a minor degree when Clarke's fixative were used.

Zenker's Fluid

The Zenker's Fluid was introduced by Albert von Zenker . Zenker's fixative is a rapidly acting fixative for animal tissues, containing mercuric chloride, potassium dichromate, sodium sulfate, water and acetic acid. It is a good routine fixative giving fairly rapid and even penetration.[1]
The mercuric chloride in the fixative reacts with the thiol group forming a simple dimercaptide.

$2R.SH + HgCl_2 = (RS)_2 Hg + 2H^+ + 2Cl^-$

The production of hydrogen ions makes the fixative solution more acidic. Following fixation, the ultrastructure preservation is poor but trichome methods work well. Lowman et al[14] studied the effect of different fixatives on the structure and dimensions of salivary chromosomes of drosophilla by means of phase contrast microscopy. Marked chromosomal shrinkage and structural artefacts of the chromosomes were seen when Zenker's fluid was used. Baker et al[15]studied the effect of Zenker's fluid on cytoplasmic inclusions which also did not yield good results.

Discussion

Though there are a large number of fixing solutions available but still over the last century, anatomists and pathologists have used formalin as the fixative of choice. Formalin offers a huge number of advantages: It is a stable fluid. It is easy to prepare. It has a low cost. It allows the application of most stains. Preserves morphological detail with few artefacts and it is good for frozen sections[1]. Moreover the pathologists are trained to look at sections fixed with formalin and are therefore reluctant to change the microscopic appearance of diagnostic tissues by using a different type of fixative[16]. Though the use of formalin for fixation is a rule in every histopathological lab across the world but various researchers over the times have pointed out its limitations which include slow fixation[1], slow penetration[1], poor nuclear fixation and shrinkage[7]. Cross links formed by formalin with proteins hinder immunohistochemistry. It does not assure a complete DNA and messenger RNA (mRNA) recovery, essential to many tests of molecular biology which are now under continuous development [17].Moreover formalin has been found to be health hazard affecting the skin and mucous membrane, immune system, respiratory system ,GIT, cardiovascular system, reproductive system, lymphatic system and has been even reported to be carcinogenic and cause birth defects.[9, 18]

The picric acid containing fixatives (eg. Bouin's Fluid) have a good penetration, good glycogen preservation and preserve an excellent nuclear detail[1]. So these fixatives are often recommended for areas where nuclear detail can be particularly advantageous like in lymphoid lesions and testicular biopsies[6]. But the picric acid fixatives stain the tissues yellow, retain little affinity for basic dyes and cause a considerable shrinkage[1].

The alcohol containing fixatives (eg. Clarke's fluid) quickly penetrate the tissues, so they are suitable for fixing smears and cryostat sections thus helping in a rapid diagnosis. They also offer a good nuclear fixation and preservation of the cytoplasmic elements. The disadvantage associated with alcohol fixation is tissue shrinkage.

Mercuric chloride containing fixatives (eg. Zenker's Fluid) provide excellent fixation of nucleus showing chromatin in fine detail, fix connective tissue fibres well. They enhance staining of connective tissue especially for Masson's Trichome stain. Zenker's fixative it is not stable and makes the tissues hard and brittle and leads to the brown discoloration of tissues. A major limitation of this fixative is that the mercuric chloride in it has been found to have toxic effects and can be fatal if swallowed.[14, 15]
Thus we see that every fixative has its own merits and demerits. Above all other disadvantages of any chemical being used in the lab, the one that can never be ignored its ill effects on human life. Although all fixatives discussed in the present manuscript have to be dealt carefully but unfortunately, formalin has been reported to have maximum hazardous effects. Inspite of all efforts the pathologists are still reluctant to replace this age old fixative with a safer option. The OSHA (Occupational Safety and Health Administration) regulation standard has declared Formalin as hazardous and is advocating its substitution with less dangerous chemicals. Some studies have proposed the use of natural and eco-friendly solutions like sugar, jaggery and honey as alternatives to formalin for long term tissue preservation.[18, 19] Research is being done to search for safer and technically good alternatives.

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. However, formalin remains the fixative of choice in the majority of histological laboratories. But unfortunately, formalin is a corroborated biohazard, its routine use as a fixative is a major health and safety concern and hence the quest for safer alternatives is envisaged.

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