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TISSUE FIXATIVES- AN OVERVIEW

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ABSTRACT

Fixatives play a crucial role in histopathology, as inadequate fixation can compromise the entire process, leading to inaccurate diagnoses. By categorizing fixatives into simple and compound types, such as formaldehyde and 10% formal-saline, respectively, laboratories can ensure that tissues are properly preserved, terminating biochemical reactions, enhancing tissue strength, and preventing autolysis and putrefation, ultimately facilitating accurate diagnosis. This articles is designed to produce complete review of various fixatives in histopathology.

KEYWORDS: fixation, tissue processing ,histopathology.

INTRODUCTION

Tissue processing is a critical step in histology that involves converting fragile, water-laden tissues into stable, durable specimens that can withstand the rigors of sectioning, staining, and microscopic examination. This process is essential for maintaining tissue morphology, preventing degradation, and ensuring accurate diagnosis[1].The tissue processing protocol typically involves a series of steps, including fixation, dehydration, clearing, infiltration, and embedding.Fixation is the initial and most crucial stage in histopathology, involving the use of chemicals to preserve tissue and maintain its chemical and architectural integrity after death [1]. Following death, tissues and cells undergo rapid changes, leading to their breakdown and destruction due to autolytic activity or self-destruction [1]. This process can be exacerbated by external factors, such as microorganisms that induce putrefaction and disintegration [2].To prevent or slow down these changes, fixation is essential. The tissue should never be allowed to dry, as it will shrink, and improper fixation can lead to difficulties in rectifying it at a later stage [2]. Fixation alters the physio-chemical state of tissues, affecting the stain-reactive individual cellular components [2]. The duration of fixation depends on the tissue's size, consistency, and fixative type, and a sufficient amount of fixative is necessary, with a recommended volume of approximately 50 times that of the tissue [3].The primary objective of fixation is to preserve tissues in their natural state by shielding them from harmful changes like autolysis and putrefaction . Adequate fixation is critical for tissue processing in histopathology, and it must be completed as soon as possible following tissue removal from the body after death .

DURATION OF FIXATION

Adequate fixation is the foundation of histopathology, and its importance cannot be overstated. Proper fixation is crucial for morphological interpretation, histochemical analysis, and immunohistochemical analysis, and delays or insufficient fixation can compromise diagnosis and treatment [3]. The duration of fixation depends on tissue density and fixative penetration rate, with softer tissues fixing faster than dense ones, and glutaraldehyde and Carnoy's fluid acting faster than formaldehyde . To ensure thorough fixation, laboratories employ specialized techniques, such as slicing specimens into thinner sections or injecting fixative into entire organs[4].

PRINCIPLES OF FIXATION

The principle of fixatives in histology is to preserve the tissue's morphology and prevent degradation, allowing for accurate diagnosis and research. Fixatives achieve this by:

1. Stopping Autolysis: Fixatives prevent the breakdown of tissues by enzymes, which can cause degradation and distortion of cellular structures[4].
2. Preventing Putrefaction: Fixatives inhibit the growth of microorganisms, which can cause decay and degradation of tissues.
3. Stabilizing Proteins: Fixatives coagulate or cross-link proteins, stabilizing their structure and preventing degradation.
4. Preserving Cellular Structure: Fixatives maintain the integrity of cellular membranes, organelles, and other structures, allowing for accurate visualization and diagnosis.
5. Enhancing Staining: Fixatives can enhance the staining properties of tissues, allowing for better visualization of cellular structures and components.

The mechanisms by which fixatives achieve these goals vary depending on the type of fixative used. Common fixatives include:

1. Cross-linking fixatives (e.g., formaldehyde, glutaraldehyde): These fixatives form covalent bonds between proteins, stabilizing their structure.
2. Coagulative fixatives (e.g., ethanol, acetone): These fixatives precipitate proteins, stabilizing their structure.
3. Denaturing fixatives (e.g., methanol, picric acid): These fixatives denature proteins, making them less susceptible to degradation[5].

The choice of fixative depends on the specific requirements of the tissue, the intended use of the fixed tissue, and the desired outcome. Understanding the principles of fixatives is essential for selecting the most suitable fixative and achieving optimal results in histology.

PROPERTIES OF A GOOD FIXATIVES

The ideal fixative should possess a combination of characteristics that enable it to effectively preserve tissues and cells for histopathological examination. According to [6], a perfect fixative should meet the following criteria:

1. Prevention of autolysis and putrefaction: The primary function of a fixative is to halt the breakdown of tissues by enzymes and bacteria, thereby preserving the cellular and tissue structure .
2. Maintenance of cellular interactions: Fixatives should preserve the interactions between cells and external substances, ensuring that the tissue structure remains intact [7].
3. Preservation of cellular and tissue structure: Fixatives should render cell components insoluble, maintaining the tissue structure in a lifelike condition and minimizing modifications caused by subsequent processing .
4. Enhancement of tissue component visibility: Fixatives should increase the visibility of various tissue components by emphasizing differences in refractive indices and making the tissue more permeable to subsequent reagents .
5. Stabilization of tissue: Fixatives should stabilize the tissue against the rigors of processing without causing excessive tissue shrinkage, swelling, or hardening [7].
6. Chemical inertness: Fixatives should not add to or remove from the tissue, ensuring that the chemical composition of the tissue remains unchanged .
7. Rapid and even penetration: Fixatives should penetrate the tissue and cells rapidly, evenly, and deeply, ensuring that all tissue components are properly fixed [7].
8. Prevention of distortion: Fixatives should prevent distortion caused by subsequent reagents, ensuring that the tissue structure remains intact [8].
9. Imparting suitable hardness and texture: Fixatives should impart a suitable hardness and texture to tissues, making gross cutting easier and subsequent sectioning possible [7].
10. Long-term storage: Fixatives should allow for long-term storage of specimens without significant degradation or alteration of the tissue .
11. Compatibility with subsequent staining methods: The fixative should enhance and complement subsequent immunohistochemical, molecular biology, and histological staining methods [7].

In summary, an ideal fixative should possess a combination of characteristics that enable it to effectively preserve tissues and cells for histopathological examination.

TYPES OF FIXATION

1.PHYSICAL

2. CHEMICAL

PHYSICAL METHOD

Fixation methods in histology are categorized into physical and chemical approaches[].

Chemical fixation using liquid fixatives is the most widely used method.

Chemical fixatives work through various mechanisms, including cross-linking, dehydration, and denaturation.

Cross-linking fixatives, such as formaldehyde and glutaraldehyde, stabilize tissue structure by forming covalent bonds.

Dehydrants, including ethanol and acetone, remove water from tissues, precipitating and coagulating proteins[8].

Denaturing agents, such as acetic acid and mercuric chloride, alter protein structure, making them less susceptible to degradation.

Compound fixatives combine multiple mechanisms to provide robust fixation.

HEAT FIXATION

Heat fixation is the simplest form of fixation, making each component less soluble in water.

Attaching a frozen section to a warm microscope slide partially fixes it through heat and dehydration[9].

MICROWAVE FIXATION

Microwave fixation reduces fixation times for gross specimens and histological sections to under 20 minutes.

Microwaving tissue in formalin can produce hazardous, explosive vapors, posing safety risks.

Commercial glyoxal-based fixatives are a safer, efficient alternative for microwave fixation, as they don't produce vapors when heated.

FREEZE-DRYING AND FREEZE SUBSTITUTION

Freeze-drying is a technique used to study soluble materials and small molecules .

Tissues are cut into thin blocks and immersed in liquid nitrogen to preserve their structure[9].

The water is removed in a vacuum chamber at -40°C, preventing the formation of ice crystals.

The tissue can be post-fixed with formaldehyde vapor to enhance preservation.

Freeze substitution involves immersing specimens in fixatives, such as acetone or alcohol, at -40°C .

This process slowly removes water through dissolution of ice crystals, preserving the protein structure.

The proteins are not denatured, maintaining their native conformation.

Gradually increasing the temperature to 4°C completes the fixation process[10].

APPLICATION

These methods are primarily used in research environments, where preserving the fine structure of tissues is crucial.

They are rarely used in clinical laboratory settings, where other fixation methods are more practical.

CHEMICAL METHOD

Chemical fixatives use organic or non-organic solutions to preserve tissue morphology[11].

They can be categorized into three major groups: coagulant, cross-linking, and compound fixatives.

Coagulant Fixatives

Coagulant fixatives use organic and non-organic solutions to coagulate proteins, making them insoluble.

These fixatives maintain tissue histomorphology by coagulating lipoproteins and fibrous proteins like collagen[12].

However, coagulant fixatives can result in cytoplasmic flocculation and poor preservation of mitochondria and secretory granules[12].

Dehydrant Coagulant Fixatives

Common dehydrant coagulant fixatives include alcohols (e.g., ethanol, methanol) and acetone.

Ethanol competes more strongly with methanol in interacting with hydrophobic areas of molecules[13].

Coagulant fixation begins at a concentration of 50-60% for ethanol and 80% or more for methanol.

Effects of Dehydrant Coagulant Fixatives

Removing and replacing free water from tissue with dehydrant coagulant fixatives affects proteins within the tissue[13].

Water molecules surround hydrophobic areas of proteins, stabilizing hydrophobic bonding[14].

Removing water weakens hydrophobic bonding, altering protein structure.

Molecules of water participate in hydrogen bonding in hydrophilic areas of proteins, and therefore removal of water destabilizes this hydrogen bonding. Together, these changes act to disrupt the tertiary structure of proteins[14].

Additionally, with the water removed the structure of the protein may become partially reversed, with hydrophobic groups moving to the outside surface of the protein.

Disruption of the tertiary structure of proteins (i.e. Denaturation) changes their physical properties, potentially causing insolubility and the loss of function.

Factors which influence the solubility of macromolecules include:

Temperature, pressure, and ph.

Ionic strength of the solute.

The salting-in constant, which expresses the contribution of the electrostatic interactions[6].

The salting-in and salting-out interactions.

The types of denaturing reagents

OTHER TYPES OF COAGULANT FIXATIVE

Acid coagulants, such as picric and trichloroacetic acid, alter the charges on ionizable side chains of proteins[15].

This disruption affects electrostatic and hydrogen bonding, leading to changes in protein structure.

Mechanism of Action

Acid coagulants insert a lipophilic anion into a hydrophilic region, disrupting the tertiary structure of proteins.

Trichloroacetic acid penetrates hydrophobic domains of proteins, reacting with charged amine groups and precipitating proteins.

Specific Acid Coagulants

Acetic acid coagulates nucleic acids but does not fix or precipitate proteins, making it useful as an additive to other fixatives.

Trichloroacetic acid extracts nucleic acids while precipitating proteins.

Picric acid forms salts with basic groups of proteins, causing coagulation, but may cause hydrolysis and loss of nucleic acids due to its low pH[15].

Effects on Staining and Protein Structure

Picric acid fixation produces brighter staining, but the low pH solution may compromise protein structure[15].

Neutralizing the solution may cause precipitated proteins to re-dissolve.

1.FORMALDEHYDE FIXATION

10% neutral buffered formalin (NBF) is the most commonly used fixative[16].

Formalin is an aqueous solution of formaldehyde, typically 37-40% formaldehyde.

"10% formalin" is equivalent to 4% weight-to-volume of formaldehyde.

Mechanism

Formaldehyde reacts with water to form methylene hydrate (methylene glycol), initiating the fixation process[17].

Methylene hydrate reacts with protein side chains, forming reactive hydroxymethyl side groups (-CH2-OH).

Short fixation times with 10% NBF primarily involve the formation of hydroxymethyl side chains[18].

Formaldehyde reacts with nuclear proteins and nucleic acids, stabilizing the nucleic acid-protein shell.

Formaldehyde modifies nucleotides by reacting with free amino groups.

Cross-linking reactions occur at adenine-thymidine rich regions in naked DNA[13].

Formaldehyde does not interact with carbohydrates.

The side chains of peptides or proteins have the highest affinity for formaldehyde[20].

Formaldehyde has a high affinity for lysine, cysteine, histidine, arginine, tyrosine[19].

GLUTARALDEHYDE

Glutaraldehyde is a bifunctional aldehyde that reacts with the same groups as formaldehyde.

It has an aldehyde group at both ends of the molecule, enabling extensive cross-linking.

Glutaraldehyde polymerizes and oxidizes in aqueous solutions, forming cyclic and oligomeric compounds and glutaric acid.

Glutaraldehyde requires storage at 4°C and a pH of approximately 5 for stability[17].

Reaction Mechanism

Glutaraldehyde reacts with proteins, introducing unreacted aldehyde groups that can further cross-link proteins[17].

The aldehyde groups can also react with other histochemical targets, such as antibodies, enzymes, or proteins.

Glutaraldehyde cross-linking is fastest at pH 6-7 and results in more extensive cross-linking than formaldehyde.

Cross-linking is irreversible and resistant to acids, urea, semicarbazide, and heat.

Extensive cross-linking by glutaraldehyde results in better preservation of ultrastructure[18].

Tissues fixed in glutaraldehyde must be small (0.5 mm maximum).

Glutaraldehyde does not react with carbohydrates or lipids unless they contain free amino groups.and the reactive hydroxyl groups of serine and threonine.

OSMIUM TETROXIDE FIXATION

OsO4 is a toxic solid soluble in water and non-polar solvents.

It reacts with hydrophilic and hydrophobic sites, including protein side chains, potentially causing cross-linking.

OsO4 reacts with various groups, including:

Sulfhydryl ,Disulfide, Phenolic ,Hydroxyl, Carboxyl Amide ,Heterocyclic groups

OsO4 interacts with nucleic acids, specifically:

- 2,3-glycol moiety in terminal ribose groups
- 5,6 double bonds of thymine residues

OsO4 can cause clumping of DNA, which can be prevented by:

- Pre-fixation with potassium permanganate (KMnO4)
- Post-fixation with uranyl acetate
- Adding calcium ions and tryptophan during fixation

OsO4 can cause significant loss of proteins and carbohydrates from tissues[18].

This loss may be due to the superficial penetration of OsO4.

OsO4 can be used to stain lipids in frozen sections.

Adding calcium or sodium chloride to OsO4-containing fixatives can minimize tissue swelling.

CROSS LINKING FIXATIVES FOR ELECTRON MICROSCOPY

Cell organelles must be carefully preserved for electron microscopy.

Lipids in these structures can be extracted by dehydrating fixatives, such as alcohols.

Fixatives that preserve lipids are essential for ultrastructural examination[19].

Strong cross-linking fixatives are preferred, such as:

- Glutaraldehyde
- Combination of glutaraldehyde and formaldehyde
- Carson's modified Millonig's

Post-fixation in an agent like OsO4 further stabilizes and emphasizes membranes[20.

This step enhances the preservation of lipids and other cellular structures.

MERCURIC CHLORIDE(HgCl2)

Historically, mercury-based fixatives were used to enhance staining properties, particularly with trichrome stains.

However, due to health and safety concerns and reduced reliance on special stains, they are rarely used today[21].

Disadvantages

Mercury-based fixatives form intense black precipitates of mercuric pigment in tissues, making them unsuitable for immunohistochemical and molecular studies.

These precipitates can be removed from recently fixed tissues using Lugol's iodine and sodium hypochlorite solution, but this method is ineffective for older tissues.

Toxicity and Handling

Mercury-based fixatives are toxic and should be handled with caution.

They should be dissolved in distilled water to prevent precipitation of mercury salts.

Characteristic

Mercury-based fixatives penetrate tissues slowly, requiring thin specimens.

Mercury and acid formaldehyde hematein pigments may deposit in tissues after fixation[21].

Mercury-based fixatives are currently used in some hematopoietic procedures.

Zinc sulfate is being explored as a potential replacement due to its similar fixation properties and lower toxicity.

SPECIAL FIXATIVES

DICHROMATE AND CHROMIC ACID FIXATION

Chromium trioxide is a powerful oxidizing agent.

It produces aldehydes from 1,2-diglycol residues of polysaccharides.

These aldehydes react with histochemical stains like PAS and argentaffin/argyrophil.

Chromium ions interact with carboxyl and hydroxyl side chains of proteins[21].

Chromic acid interacts with disulfide bridges and attacks lipophilic residues like tyrosine and methionine.

Fixatives containing chromate (pH 3.5-5.0) make proteins insoluble without coagulation.

Chromate makes unsaturated lipids insoluble upon prolonged (>48 hours) fixation[2].

Mitochondria are well-preserved by chromate-containing fixatives.

Chromate-containing fixatives have been used to prepare neuroendocrine tissues for staining.

Specifically, they have been used for normal adrenal medulla and related tumors (e.g., pheochromocytomas)[21].

Immunohistochemistry with neuroendocrine markers (e.g., chromagranin A and synaptophysin) has largely replaced chromate-containing fixatives.

FIXATIVES FOR DNA,RNA AND PROTEIN ANALYSIS

Z7 fixative is a unique formulation containing zinc trifluoroacetate, zinc chloride, and calcium acetate[20].

This novel fixative enables the preservation of DNA and RNA fragments.

Z7 fixative supports the detection of DNA and RNA fragments up to 2.4 kb and 361 bp, respectively.

It allows for protein analysis using 2D electrophoresis.

Nucleic acids and proteins remain stable in Z7-fixed tissues for an extended period.

The fixative demonstrates reduced toxicity compared to traditional formaldehyde-based formulations.

Z7 fixative provides a safer alternative for researchers while maintaining the integrity of biological samples.

METALLIC IONS AS A FIXATIVE SUPPLEMENT

Mercury, lead, and zinc are prominent metals used in current fixative formulations.

Zinc, in particular, is often combined with formaldehyde to create a more effective fixative.

Zinc-containing formaldehyde is considered a superior fixative for immunohistochemistry compared to formaldehyde alone.

The effectiveness of zinc formaldehyde depends on factors such as pH levels[21].

Optimizing the pH of both formaldehyde and zinc formaldehyde is crucial for achieving better fixation results.

COMPOUND FIXATIVE

Formaldehyde-based fixatives ensure consistent histomorphometric patterns.
Additional agents can be combined with formaldehyde to achieve specific effects.
Alcoholic formalin is a combination of formaldehyde and ethanol.
This mixture preserves molecules like glycogen and minimizes tissue shrinkage and hardening.
Alcoholic formalin is suitable for fixing fatty tissues, such as breast tissue, where lipid preservation is not crucial.

Applications and Benefits

Alcoholic formalin aids in identifying lymph nodes in fatty tissues.
Certain combined fixatives, including alcoholic formalin, excel at preserving antigen immunorecognition.
These fixatives are valuable for specific tissue types and application.

FACTORS AFFECTING FIXATION

Fixation is a critical step in histological processing, and several factors can influence its effectiveness. Understanding these factors is essential to achieve optimal fixation and obtain high-quality histological sections.

Length of Fixation (Time)

The ideal fixation time varies depending on the fixative used. The fixative must diffuse into the specimen's center, and sufficient time must be allowed for the fixation reactions to occur. Inadequate fixation can lead to tissue distortion, poor staining, and low-quality sections [22]. On the other hand, prolonged fixation can cause over-cross-linking, making samples fragile.

2. Temperature

Temperature plays a significant role in fixation, as it affects the rate of diffusion and chemical reactions. Increasing the temperature can accelerate fixation, but excessive heat can cause tissue degeneration [22]. Microwave fixation can involve higher temperatures (up to 65°C) for shorter periods. For electron microscopic studies, a temperature range of 0°C to 4°C is considered ideal.

3. Concentration

The concentration of the fixative agent is crucial, as low concentrations may require prolonged fixation times, while high concentrations can damage cellular structures and obliterate enzyme activities [23]. The ideal concentration varies depending on the fixative used; for example, 10% formalin is commonly used for oral soft tissue fixation .

4. Size

Tissue thickness is an essential factor in fixation. Large samples can hinder fixative penetration, leading to autolysis of epithelium. Ideally, 3 mm to 5 mm thick specimens are best suited for complete penetration by fixatives .

5. Osmolality

The osmolality of the fixative solution can affect cellular structure. Hypertonic solutions can cause cell shrinkage, while hypotonic solutions can lead to cell swelling and bursting. Using a normal phosphate-buffered saline (PBS)-based fixative is recommended [23]. Slightly hypertonic solutions often produce the best result[23].

6. Penetration Rate

The penetration rate of a fixing agent depends on its diffusion characteristics, which vary from agent to agent. Formalin and alcohol penetrate well, while glutaraldehyde penetrates poorly [23]. Mercurials and other fixatives have intermediate penetration rates.

7. Volume Ratio

The fixative-to-tissue volume ratio is critical. A minimum ratio of 20:1 is recommended, but a target ratio of 50:1 is ideal. Agitation can enhance fixation, and using small volumes of fixation fluids for larger specimens is a common cause of poor tissue preservation.

GENERAL PRECAUTIONS IN HANDLING FIXATION OF SPECIMENS

Proper handling and preparation of tissue samples are crucial for obtaining high-quality histological sections. The following guidelines should be followed:

1. Proper Labeling and Identification: Each tissue sample must be correctly labeled and identified to ensure accurate diagnosis and prevent mix-ups .
2. Refrigeration and Avoidance of Freezing: If fixation cannot be performed immediately, tissues should be refrigerated but not frozen. Slow freezing can cause ice crystal artifacts, while repeated freezing and thawing can damage cellular organelles and release enzymes [23].
3. Infection Control: Fresh tissue may be infectious, and any fresh or incompletely fixed tissue should be considered potentially infectious. Laboratory workers should take necessary precautions to prevent exposure .
4. Prevention of Drying: Tissues should not be allowed to dry, as this can cause shrinkage, distortion, and loss of cellular detail. Small tissue biopsies can be placed in a petri dish with moistened filter paper to prevent drying .

1. Tissue Thickness: Tissues should be no more than 5 mm thick, except in cases of lung edema, where thicker slices may be necessary. Thin sections allow for complete penetration by the fixative in a short time [23].
2. Fixation of Hollow Organs: Cotton soaked in fixative should be placed inside hollow organs, such as the stomach and intestines, to ensure proper fixation .

3. Fixation of Eyes: Eyes should not be dissected before fixation, as this can cause tissue collapse and wrinkling. Formal-alcohol should be injected into the eye before immersing it in fixative [23].
4. Fixation of Hard Tissues: Hard tissues, such as bone and skin, may require special fixation techniques, such as Lendrum's method, which involves soaking the tissue in a phenol solution to soften it .
5. Removal of Mucus: Excess mucus can prevent proper fixation, and tissues containing mucus should be washed with saline solution before fixation .
6. Fixation of Fatty Tissues: Fatty tissues should be cut into thin sections and fixed for a longer period to ensure proper fixation [23].
7. Fixation of Blood-Rich Tissues: Tissues containing large amounts of blood, such as blood vessels and spleen, should be flushed with saline before fixation .
1. Fixative Penetration: The fixative should penetrate the tissue from all sides, and specimens should be placed in containers that already contain fixative to prevent adhesion[23].
2. Opening of Hollow Organs: Hollow organs or specimens with natural cavities should be opened to allow immediate access to the fixative .
3. Agitation and Volume of Fixative: Gentle agitation of the specimen during its first few minutes in fixative can facilitate penetration. An adequate volume of fixative (at least 20:1) is vital to ensure proper fixation [23].
4. Reuse of Fixatives: Fixatives should not be reused, as specimens can shed cells and tissue fragments into the fixative solution, contaminating subsequent specimens[24] .

CONCLUSION

Fixatives play a crucial role in histopathology by preventing autolysis and putrefaction in tissue samples. Each fixative has its unique benefits and drawbacks. The most commonly used fixative in histopathology is 10% buffered formal saline, which is considered the gold standard due to its stable pH.[24] Adequate fixation is essential for all subsequent histopathological techniques, and various factors such as fixation duration, temperature, concentration, sample size, osmolality, penetration rate, and volume ratio can influence the fixation process.

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