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Histological Fixation Process and Fixatives

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ABSTRACT

Tissue monitoring generally includes the stages of fixation, dehydration, clearing, hardening (infiltration), paraffin blocking/paraffin(emmeding), sectioning, water removal, routine staining, and mounting. Fixation is the basic and first step in the microscopic examination of tissues. The histotechnical process, which includes components such as detection, tissue tracking and staining, basically aims to capture and visualize the state of the relationships between tissue parts inside and outside the cell and various cells at a certain time as close as possible to the living state. Maintaining the natural structure of the tissue is important for the follow-up phase. The main feature of a good fixative should protect the sample and make the macromolecules insoluble without changing the chemistry of the sample studied and allowing it to be examined as closely as possible to its living state. In histological tissue analysis including light microscope and electron microscope techniques, an appropriate fixation method is selected for each study. Detection solutions are classified in terms of content. The most commonly used fixative in light microscopic follow-up procedures is 10% formaldehyde. For the electron microscope, the gluteraldehyde-osmium tetraoxide binary is widely used for fixation purposes. Gluteraldehyde acts more slowly and is more expensive than formaldehyde. Formalin is obtained by dissolving formaldehyde in water. In addition, the fixed samples can be stored in the solution for months. With a successful fixation process, the structural properties of the tissue are preserved and thus it is possible to examine the tissue as closely as possible. Thus, better quality sections are obtained from the tissue samples taken. For this reason, it will be more efficient to interpret well-fixed samples by photographing them. In this review, which was created by using various sources, the elements to be considered for an ideal fixation were determined and it was aimed to provide an overview of successful fixation for light microscope and electron microscope.

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Introduction

In the process of histological tissue preparation, it is aimed to visualize a certain memory of cell and intercellular relationships with microscopes. The first and most important stage of the microscopic preparation process of the tissue is the fixation of the tissue (Papuçcuoğlu, 2014). Fixation aims to protect tissues, cells and cell components against autolysis (Howard and Wilson, 2014). With the detection, it is ensured that the cells and tissues are preserved as close as possible to the living state, autolysis and heterolysis are prevented, the loss of easily diffused substances (lysosomes, etc.) is prevented, and it is facilitated to stain the tissues with dyes and other reagents (Papuçcuoğlu, 2014). Detection of tissues has led to the emergence of various fixatives in the last century in order to better understand biological functions and structures. Fixation of tissues is carried out in two different ways using physical or chemical methods. Fixation should be performed immediately after death to prevent autolysis as soon as possible after removal of tissues in surgical procedures (Eltoum et al., 2001). The purpose of fixation is to ensure that the biological sample studied is preserved as closely as possible to its living state (Hassan and Mushtaq, 2014). Tissue or cell samples are prepared and stained with sensitive techniques before being examined under a microscope. Fixation also affects the quality of the other steps to be applied to the sample by fixing the samples to the environment they are in before painting. Successful fixation of a fixative therefore depends on hydrogen ion concentration, osmolarity, penetration into tissue or cell, fixation time and ambient temperature (Stranz and Kastango, 2002).

What is Fixation?

The term fixation is defined as fixation in a fixed or liquid medium (Howard and Wilson, 2014). Fixation is a process that allows the tissue to be detected with all its components, including cells. It stabilizes the in-vivo micro-anatomical image of the tissue (Grizzle, 2009). The goal of fixation is to protect the tissue, minimally alter the morphology of the cells, and help preserve the tissues so that they can withstand subsequent routine tissue follow-up stages (Fox et al., 1985). It is extremely important to use the selected and appropriate fixative for fixation. Various types of fixatives are used to understand the change in morphology and function of biological samples. If a suitable fixative for the tissue is not used, many procedures become ineffective and unusable. Therefore, the main feature of a good fixative is to protect the sample and render macromolecules insoluble, allowing the sample to be examined as close as possible to its nature without changing its chemistry. At the same time, while being observed under the microscope, the integrity between intracellular and extracellular components in the stained areas of the tissue or cell must be preserved (Bancroft and Suvarna, 2012). During the fixation process, a complex series of chemical events take place to prevent tissue deterioration, and semi-liquid cells become semi-solid, allowing cellular and extracellular structures to be examined under a microscope. In all microscopic preparations, the fixation of the tissue in the most appropriate way is mandatory, otherwise the errors that occur cannot be corrected in the following stages and cannot be correctly diagnosed (Rai et al., 2016). Bad and/or poor fixation results in changes in the shape of the cells. A non-ideal fixative flattens the cells, causing them to fade. It makes it difficult and/or impedes the identification of cells. Poor fixation causes cellular losses (Koivurinne and Shield, 2003).

Fixation Methods

Detection of tissues and organs is carried out by physical or chemical methods (Grizzle et al., 2008).

Physical Detection

Physical detection methods are not frequently used in routine histology practice. It is preferred because it minimizes the formation of artifacts in the cell content for rapid detection of small-sized tissues (Huang and Yeung, 2015). Physical fixation is the fixation made by freezing, coagulating or precipitating the components with the help of heat, organic substances and acid. Examples of fixatives used with the physical fixation method are Clark (a mixture of ethanol and acetic acid), Carnoy (a mixture of ethanol, chloroform and acetic acid) (Yamashita, 2007). Physical fixation includes independent processes such as heating and freeze-drying (Eltoum et al., 2001). Heat detection is the simplest form of detection. Heat coagulates proteins, dissolves lipids. It can be used to detect large parts, but it is not preferred because it disrupts the structure of cell components (Grizzle et al.,2008). Freeze-drying detection is a method based on freezing, rapid freezing of tissue samples and taking sections through special devices called ice cream microtomes or cryotomes (Wisse et al., 2014).

Chemical Detection

Chemical detection is the detection of tissues using chemicals. The most commonly used type of detection in routine is chemical detection. It is preferred for the detection of larger sized tissues compared to physical fixation (Huang and Yeung, 2015).

Chemical detection is divided into two according to its application:

In the first method, which is defined as immersion fixation, the tissue is divided into small pieces and taken into fixative and waited until the tissue is fixed. In the other method, it is administered into the fixative blood vessel and circulated. This method, called perfusion fixation, is preferred in studies with small laboratory animals or in tissues that can undergo rapid lysis after biopsy (Wisse et al., 2010). In these methods, fixatives are inoculated into the body of animals by diffusion. Perfusion is a slower process, requires more time, and only one fixative can be used at a time (Shostak, 2013).

Traditionally, immersion fixation method is used in light microscopic examinations and is performed at room temperature. All chemical reactions are faster at high temperatures, including during the fixation process. It is used as a heat source in microwave ovens as well as classical methods (Kok and Boon, 2010).

Classification of Fixatives

Detection of tissues has led to the emergence of various fixatives in the last century in order to better understand biological functions and structures (Eltoum et al., 2001). Therefore, to date, there is no fixative that qualifies as universal or ideal (Grizzle et al., 2001). The fixative should be selected depending on the analyzes to be performed. It may be necessary to apply different fixation protocols for different building elements within a tissue. An ideal fixative prevents tissue fragmentation and autolysis (Grizzle et al., 2001; Hewitt et al., 2008). Since fixation solutions affect the antigenicity in the tissue to some extent, the appropriate fixation solution for the tissue can be determined by trial and error (Hewitson et al., 2010).

Other important features of an ideal fixative: It should penetrate the tissues quickly, shelf life should be at least one year, it should be compatible with modern automated tissue procedures, it should facilitate the sectioning of paraffin blocks, it should ensure the storage of tissues in the long term, it should be used for various tissues including fatty, lymphoid and nerve tissues, it should protect small and large tissue samples, it should support histochemical, immunohistochemical and other special procedures, it should be cost-effective (Dapson, 1993).

Different fixatives are used for various purposes in the detection process. Fixative selection may vary depending on the nature of the tissue (Firidin, 2004). Fixative solutions are classified as acidic and basic solid and liquid (Firidin, 2004; Huang and Yeung, 2015). Chemicals can be used as a single agent for detection purposes, and solutions prepared as mixtures in different proportions can be preferred because they can meet different purposes at the same time (Özfiliz et al., 2018). Chemical substance with fixative power (usually solid) is required for chemical fixation, and carrier liquid is required for chemical substance. Chemical fixatives can be classified as follows: Aldehyde group agents: Formaldehyde, glyoxal,

paraformaldehyde, glutaraldehyde. Oxidizing agents: Potassium permanganate, potassium dichromate, osmium tetraoxide. Protein denaturing agents: Acetic acid, ethanol, methanol. Other cross-linking agents: Carbodiimides. Other agents: Mercuric chlorite, picric acid (Hassan and Mushtaq, 2015). Fixatives are divided into coagulant and non-coagulant fixatives (Hassan and Mushtaq, 2015). Coagulant fixation creates an effect by coagulating proteins, that is, making them insoluble in water. Cellular structure is mainly maintained by coagulation of lipoproteins and fibrous proteins such as collagen. They are preferred in studies where tissue histomorphology will be examined at the light microscopic level. Since they cause lumps in the cytoplasm and cannot protect the mitochondria and secretory granules well, they cannot be used in studies where thin structure will be observed. Among dehydrated and coagulating fixatives, the most commonly used alcohols (ethanol, methanol) and acetone. They disrupt the tertiary structure and solubility of proteins and cause them to lose their function. Other coagulating fixatives are picric acid and trichloroacetic acid. It disrupts the structure of electrostatic bonds and hydrogen bonds by altering the charges on the ionizable side chains of proteins, which leads to the disruption of the three-dimensional structure of proteins (Grizzle et al., 2008). Picric acid is a bright yellow crystalline substance with explosive properties. Diffusion into tissue is rapid (Özfiliz et al., 2018).

Non-coagulant detection are chemicals that produce a fixative effect within or between proteins, within or between nucleic acids, and by cross-linking between proteins and nucleic acids. Buffer solution is used to maintain the desired pH value from the tissues. The fixation solution should be in the appropriate osmotic concentration compatible with cells and tissues. Thus, the tissues will not swell and shrink. The buffer solution should not be toxic and should not cause morphological changes during detection (Huang and Yeung, 2015). Formaldehyde, gluteraldehyde, osmium tetraoxide and mercury chloride are the most preferred non-coagulable detection solutions (Bancroft, 1990).

Light is the most commonly used fixative 10% formaldehyde in microscopic follow-up procedures. Formalin is obtained by dissolving formaldehyde in water (Hayat, 1989). Neutral buffered formalin (NBF) stabilizes amino acids in proteins and offers good tissue and cell structure protection (Iyiola and Avwioro, 2011). Although formaldehyde fixation changes the antigenic structure of many proteins, it maintains its title as the most commonly used detection solution because it ultimately reveals a good morphology. Therefore, many studies are carried out to combine the characteristics of determining antigenic structures with well-preserved cellular details (Tingstedt et al., 2003).

However, in addition to all these advantages, it also has disadvantages such as slow fixation feature, slow penetration to tissues, insufficient nucleus fixation and shrinkage. However, formalin remains the preferred fixative in most histology laboratories. Unfortunately, however, formalin has been confirmed to be a biological hazard, its fixative routine use is a major health and safety issue, and therefore the search for safer alternatives continues (Kiernan, 2000). Due to its carcinogenic properties, it also poses a great threat to the environment (Van Essen et al., 2010).

In electron microscopic follow-up processes, the gluteraldehyde-osmium tetraoxide pair is widely used for fixation (Altunkaynak and Altunkaynak, 2006). Gluteraldehyde acts more slowly than folmaldehyde and is more expensive. Fixed samples can be stored in solution for months. It is also used in enzyme histochemistry (Koptagel, 2018). Osmium tetraoxide is also called osmic acid. It is sold in closed tubes of 0.5 or 1 g in pale yellow crystals. Osmium tetraoxide crystals and solution are extremely irritating and dangerous. Eyes in particular should be well protected. Mercury chloride is a white crystalline substance. It is a salt commonly used in tissue detection. However, it is not used alone due to its strong astringent effect. It provides a brighter color formation in the texture and easier staining during staining. Mercury chloride is very toxic and corrosive to metals (Özfiliz et al., 2018).

Carnoy's solution can be given as an example of fixatives used with the physical fixation method (Yamashita, 2007). Glycated acetic acid consists of absolute ethanol and chloroform. It can be used in the detection of all kinds of tissues. It shows rapid penetration and therefore some laboratories are reported to prefer Carnoy's fixative for biopsies that require urgent treatment. It is a good fixative for glycogen and plasma cells. It is also recommended in cases that require the study of nucleic acids. Detection should not be made for more than 4 hours to prevent shrinkage and hardening. Chloroform can be dangerous. Also, collagen is not well preserved and acid fast bacilli are not stained. Erythrocytes undergo lysis. Acid-soluble cell granules and pigments may be lost (Pabuçcuoğlu, 2014). Tissue sections detected in Carnoy's solution yielded more successful results than tissue sections detected in formaldehyde solution. Less toxic than formaldehyde solution; may be recommended as a strong alternative (Aktan et al., 2012).

Bouin fixation solution is prepared using water-saturated picric acid, 25 ml formalin and icy glyceal acetic acid (Hewitson et al., 2010). Bouin fixative has been found to be effective in sensitive and soft tissues such as small tissues, embryo and brain tissues (Musumeci, 2014). After the tissues are left in Bouin solution for 6-24 hours, the tissues obtained are transferred to 70% alcohol (Ding, 2015). Bouin fixative is a preferred fixative because it preserves cell/tissue morphology in reproductive organs and preserves the epitopes required for immune marking (Suvarna et al., 2013). Due to the fact that it contains both picric acid, formalin and glacial acetic acid, the swelling that occurs with acetic acid in the cell is compensated by the shrinkage that occurs with picric acid. Basophils caused by formalin in cytoplasm are corrected with picric acid. Picric acid performs fixation by both coagulation and cross-linking, and the main fixation mechanism of the Bouin solution is the action of picric acid (Howard and Wilson, 2014). Bouin fixative ensures good preservation of nuclei and glycogen, but their penetration is slow and disrupts mitochondria and kidney tissues (Wisse et al., 2010).

The Hollande solution consists of picric acid, formalin, acetic acid, copper acetate. Modification of Bouin's solution. It is used in the detection of small biopsies, especially those belonging to the gastrointestinal tract. Bouine has similar disadvantages (Papuçcuoğlu, 2014).

The modified Davidson fixative is a similar fixative to the Bouin fixative, but alcohol is used instead of picric acid in this fixative. It contains formalin, acetic acid and alcohol. It has the ability to penetrate tissues quickly. Tissues are less likely to be damaged compared to other known formalin protocols (Kelder et al., 2008). It provides excellent cellular details for eye and testicular tissues and causes cells to shrink less. Even though the photoreceptor provides worse protection of the sensory endings of cells, it is still an acceptable fixation for fixed cells such as the retina and cornea (Latendresse et al., 2002).

The B5 fixative consists of mercury chloride, sodium acetate and formalin. It is used in the routine detection of tissues such as lymph nodes, spleen and all tissues suspected of lymphoproliferative disease. As well as providing excellent cytological detail, antigen protection for lymphoid markers is also high. However, there are also disadvantages. First of all, since B5 is not a stable fixative, it needs to be prepared fresh. Since excessive fixation leads to hardening and fragility in the tissue, formalin should be taken after 2-4 hours in B5. In addition, the preservation of certain antigens can be a problem (such as keratin immunoreactivity). B5 post-fixation can be applied to the tissues detected in the form (Papuçcuoğlu, 2014).

Zenker's fixative consists of potassium dichromate, mercury chloride, and glyceal acetic acid. Zenker's fixative can be used for bone marrow biopsies. Tissues are quickly detected within 8-12 hours. Histological detail is very good. After fixation, it should be washed in the stream to remove chromate deposits, and treated with iodine to remove mercury before routine staining. Not suitable for molecular analysis and immunohistochemistry. Metal instruments should not be used with mercury fixatives (Papuçcuoğlu, 2014). Cytoplasm and fibrils are well protected. It is more suitable for fresh material. They do not protect erythrocytes well (Koptagel, 2018).

Factors Affecting Fixation

It is important that fixatives protect tissue as it is removed from living things. Healthy comparison between the experimental groups depends on the preservation of the structures of the tissues. This is related to both how the tissue is removed and with which solution it is detected. While there is no fixative ideal, each fixative influences the structure, protein content, or histochemical staining of the cell/tissue in a way, and therefore the fixative and follow-up process should be carefully selected at the outset according to the purpose (Kiernan, 2008).

- Penetration of the fixative: Penetration of the fixative into the biological sample is an important effect. This process can be relatively slow. The sample should be small and thin for successful penetration. Fixation depends not only on the penetration of the fixative, but also on the rate at which it reacts with its components into the tissue (Bancroft and Suvarna, 2012).
- Concentration of the fixative: Different cellular components, the fixative and the fixative solution react differently at different concentration. Detection solution with low concentration may require a long time. Detection solution with high concentration may cause damage by destroying enzyme activities (Huang and Yeung, 2015).
- Duration of fixation: The most appropriate time for fixation varies between fixation solutions. In order for a good fixation process to take place, sufficient time should be allowed for the diffusion of the fixation solution to work to the center of the tissue and for the detection reactions to take place. Keeping the detection time short prevents the penetration of the tissue into the fixation solution and the cross-linking of macromolecules. Tissues become fragile because the long detection time causes excessive cross-linking (Huang and Yeung, 2015). In some fixatives, prolonging the time more than necessary may cause tissue hardening, shrinkage, or increased brittleness (Papuçcuoğlu, 2014).
- Temperature: Traditionally, the detection of surgical materials is carried out at room temperature. This is because autolysis is thought to develop more slowly at room temperature and diffusion occurs more optimally. However, it is a fact that detection accelerates at higher temperatures, as in various chemical reactions. However, increasing the temperature may accelerate autolysis and cause damage to some antigens (Papuçcuoğlu, 2014).
- Tissue size: Samples should be taken as small as possible in the most appropriate way for the purpose. Care should be taken to ensure that it is not larger than 1 cm^3 (Özfiliz et al., 2018).
- Osmolarity of the fixative: Phospholipid membranes can be easily damaged by excessive hypotonic and hypertonic solutions. Hypertonic solutions cause the tissues to shrink, and hypotonic solutions cause the tissues to swell. The detection solution is desired to be slightly hypertonic (Huang and Yeung, 2015).
- Hydrogen ion concentration and buffers: pH values of fixatives vary. In general, the hydrogen ion concentration is kept within physiological limits with an appropriate buffer. A satisfactory determination takes place between pH 6 and 8 (Papuçcuoğlu, 2014).

There are many variables that will affect the quality of chemical detection. The results should be compared with live cell studies to ensure that the images to be obtained are "suitable for life"(Huang and Yeung, 2015).

Conclusion

The most important step in the histological preparation process for examining the tissues under a microscope is fixation and affects the subsequent stages. An ideal fixation should be made in order to reach the correct data in histological studies. The most suitable fixative for the tissue should be selected and the necessary method should be applied to the tissue. Successful fixation preserves the structural properties of the tissue and allows the tissue to be examined as closely as possible to its living state. Thus, better quality sections are obtained from the tissue samples taken. It is possible for a well-fixed texture to come into contact with the paint and give quality results during the photography phase.

It is thought that this review will guide the studies to be conducted on the histological preparation process.

Declarations

Conflict of Interest: The authors declare no conflicts of interest regarding the publication of this paper

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