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ACTA SCIENTIAE ANATOMICA

Acta Sci Anat. 2018;1(1):21-28.

The use of fixative solutions throughout the ages: a comprehensive review

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ABSTRACT

Preservation of biological tissue is performed by using different fixative solutions. This fact had fundamental contribution on the history and scientific development of anatomy. The history of fixative solutions can be divided in three important moments: the first one is connected to the Old Egypt; the second, to the Renascence period; and the third period occurred during the American Civil War. Several solutions were tested and used throughout history and they adapted to the needs and contexts of each time. Currently, the main fixative solutions are toxic and some of them carcinogenic, including the formaldehyde, which remains as a "gold standard". The fixative solutions are well adapted for histology laboratories, where their vapors are easily removed. On the other hand, in gross anatomy labs where usually not enough exhaustion exists, the exposure to toxic components is higher. Therefore, the necessity for better, more effective and safe solutions increased the interest for studying and several researchers have been engaged to develop a better fixative solution. This works brings a review of the historic progress of tissue fixation techniques for research and didactic purposes.

Keywords: anatomy, embalming, fixative solutions, gross anatomy, history

INTRODUCTION

Despite the natural decomposition of biological tissues, humanity is always looking for new and innovative means to enable the conservation of corpses after death [1, 2].

There are many reasons for this search. Some people have performed these procedures as a result of faith because they believe in life after death. Others have been moved by the curiosity of understanding the internal arrangement of organs for treating diseases or hunting [3-6].

Fixative solutions have also been used to preserve corpses for hygienic purposes during the transportation of cadavers across long distances to avoid contamination [7]. Techniques have been developed for different reasons, but only some are suitable in the modern world [6]. Despite modern technology, we still have difficulty in preserving pieces of corpses and tissue fragments using non-toxic substances [8, 9].

Interest in disseminating knowledge about the current fixative compounds and developing new non-toxic agents to replace existing fixatives (especially formaldehyde) has been growing and is an active research área [10, 11].

The fixative process includes natural conservation by specific factors and the preservation of biological tissue [6]. The history of this technique is commonly divided into three main periods [1, 4].

REVIEW

First period (Ancient Egypt)

Embalming began in 5000 BC in Ancient Egypt as a part of religious rituals. Ancient Egyptians believed in life after death, and therefore,

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they thought they needed to preserve their bodies [1, 2].

Two different techniques were developed during this time. In the first, the body was placed in a fetal position, wrapped in cotton fabric, and stored in small desert caves [4]. Ambient heat and sand helped to remove the humidity of the body, which was important for preservation. The growth of the Egyptian population led to increased robberies at funerary caves, which inspired the development of safer resting places, known as sarcophagi [4, 6].

The sarcophagi prevented dehumidification as a result of the desert heat, and a new embalming technique, evisceration, was created, which excluded the heart because the soul was believed to lie within it. After evisceration, the entire body was wrapped with strips soaked in special solutions, such as natron and herbal components [2], after which it was held for 70 days [4].

During this period, Persians, Syrians, and Babylonians employed similar procedures using honey and wax [4]. Alexander "the Great" had his body preserved in honey after his death in Babylon in 323 AD [12].

Second period (Renaissance)

The second period of tissue fixative history was the Renaissance in the 16th century, where new techniques were improved to preserve corpses for artistic and dissection purposes [1].

During the Dark Ages (V – XV century), laws prohibited medical courses from obtaining cadavers for dissection or study. The embalming process was allowed only for royalty, clergy, and other elite members of society [7].

The Crusades (1095 – 1291) spread the Roman Empire and led the military far from Rome. The Romans invaded different regions and had the opportunity to experience different religions, cultures, and civilizations, mainly in North Africa and Asia. Many nobles and soldiers died in battle, and due to the distance, the preservation of corpses was necessary to allow the return of their bodies to Rome. Under this context, the maceration technique, in which the body was heated until soft tissues were removed, was largely employed [4].

Frederick II, a Sicilian King of the XIV century, authorized the dissection of executed criminal corpses in the medical college of Bologna, Italy [4, 13], which increased the general knowledge of anatomical science. In 1330, Pope Boniface VIII prohibited the transportation of sectioned parts of corpses from people who died in battles, which encouraged the development of new preservation techniques [6], such as the arterial injection of new solutions (e.g., hot water, wax, ink, mercury, and arsenic) and new tools for these injections (e.g., scissors and tweezers) [2, 7].

The most famous solution used for arterial injection was a mix of alcohol and wax and was created by Jan Swammerdam, who dedicated his career to the observation of insects and small animals and tested several fixation compounds. Frederick Ruysh (1655-1717) improved this technique by working with human cadavers for didactic and funerary purposes [14].

This refinement would have created the ideal fixative solution, but the exact formulation remains unknown. Speculations indicate the use of some amount of arsenic [7, 15].

Third period (American Civil War)

The third important period of specimen preservation occurred during the American Civil War, when embalmed corpses required preparation to withstand long distances from the battlefields to families waiting to claim their bodies for burial [1, 4].

In previous wars against Native Americans and Mexicans, soldiers were buried on the battlefields because the available substances (e.g., mercury and arsenic) were considered toxic. Therefore, there were no plans to return the corpses. However, President Abraham Lincoln permitted the conservation of corpses using these toxic solutions with the addition of salt and acid [1, 4].

A major improvement in the preservation of corpses occurred in 1868 with the use of formaldehyde for gross anatomy and microscopic techniques [8, 10]. Aleksandr Mikhaylovich Butlerov discovered this substance in 1859 in Russia, but its medical applications only emerged in 1891, when Ferdinand Blum discovered its antiseptic and antimicrobial properties. Another property of formaldehyde was characterized accidentally, when Blum observed that his skin became rigid upon

contact with aqueous formaldehyde solution, which was equal to or greater than the rigidity produced by alcohol [8].

Blum fixed some tissues using formaldehyde to analyse its fixative capacity. Histochemical staining after formaldehyde fixation resulted in excellent quality, which was later confirmed by the famous histologist Frankfurt Karl Weigert [8].

After the advances provided by the discovery of formaldehyde, much time passed until another technique was used to preserve tissues. However, Dr. Gunther von Hagens described a conservation technique called plastination in 1977, which consists of the replacement of body fluids and lipids with polymerisable resins, such as polyester, epoxy, and silicone [6, 16, 17].

This technique was quickly accepted in the scientific world due to the reduction of toxic exposure, which occurs during a limited part of the process and can be avoided by using careful procedures during preparation. The greatest disadvantage is related to the high cost of production. Another negative factor is the impossibility of microscopic examinations due to the resin component. This technique is very useful for exhibitions and gross anatomy studies in laboratories [6, 17, 18]. Cholecystectomies are among the most performed surgical procedures. Variations of the bile duct system can account for leakage during and after cholecystectomies if not promptly identified [5, 6, 14, 15].

Fixative solutions

Fixative solutions are composed of chemical substances that maintain tissue integrity after death. These substances work chemically to decrease proteolytic events and avoid changes within the tissue, such as intra- and extracellular destruction [19]. Fixatives should preserve elastin and collagen to stabilize the extracellular matrix. This process consists of creating crosslinking bridges to maintain collagen structure, even if there are connections in the tissue that leads to rupture [20]. Several factors, such as viscosity, temperature, volume, pH, and osmolarity, influence the creation of these connections [8, 19, 21]. Using a fixative of a similar osmolarity to the original tissue is recommended for the preparation of tissues for electron microscopy [22, 23].

Tissue thickness and the viscosity of the fixative solution are directly related to the speed of fixative penetration into the tissue. The temperature also increases the rate of penetration, especially for the most viscous fixatives. For ideal fixation, the volume of fixative should be 20 to 30 times greater than the immersed tissue [11, 19, 21].

Aqueous buffered formaldehyde solution (4%) has been used for decades for gross anatomy (Fox et al. 1985). Other substances, such as alcohol and glutaraldehyde, are used in tissues fixed for light and electron microscopy. Other less toxic fixative solutions based on alcohol, with or without acetic acid, have been proposed as alternatives [9, 11].

Conventional fixatives

Formaldehyde

Formaldehyde is the most abundant and important aldehyde in the environment. It is a colourless gas with a strong irritating smell. It is very soluble in water and shows high chemical reactivity [8-10, 24]. When dissolved in water, formaldehyde quickly becomes formaldehyde hydrate and forms methylene glycol. When tissues are immersed in aqueous solutions of formaldehyde, they are readily penetrated by the methylene glycol fraction and residual formaldehyde. The equilibrium of the aqueous solution of these two substances favors methylene glycol. Therefore, the balance between formaldehyde carbonyl and methylene glycol explains the rapid penetration achieved by methylene glycol and the slow penetration by formaldehyde carbonyl [8, 24].

Chemical studies indicate that formaldehyde is an electrophile reactive molecule that reacts readily with several groups of biological macromolecules, such as proteins, glycoproteins, nucleic acids, and polysaccharides, by crosslinking to form methylene bridges [8, 24]. The most reactive regions are the primary amines, such as lysine, arginine, tyrosine, asparagine, histidine, glutamine, and serine [9, 10]. This mechanism of intra- and intermolecular crosslinking dramatically changes the tissues' characteristics [8, 10], and adequate fixation

is obtained after several days. Under certain conditions, these bridges can be reversible and removed by the addition of alcohol to the solution [9, 24].

Formaldehyde has some disadvantages, such as the spontaneous formation of formic acid when exposed to oxygen and light [8]. Moreover, crosslinking disguises certain antigens, which hampers immunohistochemistry and often necessitates antigen retrieval (AR) steps. DNA and RNA molecules become fragmented, which is problematic for molecular techniques. Tissue fixation by formaldehyde is poor for ultrastructural analysis [25]. However, the main disadvantage of formaldehyde is its toxicity. Formaldehyde is classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer [26], and some studies have demonstrated genotoxic effects in workers exposed to this substance [26].

Merk and Speit [27] demonstrated that the high solubility of formaldehyde in water causes rapid absorption by the respiratory and gastrointestinal tracts and causes nasal tumours in rodents. Although this chemical is present in indoor and outdoor environments, it is a risk to human health [26, 28] in work areas, such as hospitals, laboratories, universities, and scientific institutions where people might be overexposed [28]. This is especially true in dissection rooms and gross anatomy labs, where overexposure results in respiratory, eye, and skin irritation [9, 28].

Several occupational health authorities worldwide have set permissible exposure limits to formaldehyde [28]. According to the World Health Organization [29] the maximum recommended indoor value is of 0.1 mg/m3.

The exposure limit adopted by Brazilian law (Brazilian Norm NR-15 ANVISA) [28] is 2.3 mg/m3 for a maximum of 48 working hours per week. This value is significantly higher than the standards adopted elsewhere in the world.

Despite its toxic characteristics, formaldehyde remains the main fixative for corpse pieces and tissue fragments for histology [8, 9, 11, 25, 30].

Alcohol

The reaction carried by formaldehyde fixation in the tissue can be inhibited at room temperature by the addition of small amounts of alcohol, which acts as a preservative and an inhibitor [8]. This solution was used for the first time to preserve cadavers at the end of the seventeenth century [30], but this compound is mainly used as a fixative for small tissue fragments (2 to 3 mm) due to its quick penetration into the tissue [19].

Fixation based on alcohol does not preserve tissue by crosslinking proteins. Therefore, the final morphology of cells differs from those treated with formaldehyde [31]. The benefits attributed to alcohol-based fixatives include quick fixation, removal of carcinogenic vapors, better preservation of glycogen, DNA, and RNA, enhanced staining, and no AR is required for immunohistochemical analysis [9, 11, 25, 32]. The disadvantages are the variability in stained tissues, tissue shrinkage, hardening, deposition of pigment (artifacts) in blood, total or partial lysis of red blood cells, and increased flammability [9, 10].

Glutaraldehyde

Glutaraldehyde is the most effective reagent for crosslinking proteins. It may be used in at least 13 different forms depending on the solution conditions, such as pH, concentration, and temperature [33]. This substance is the best choice for routine procedures to study cellular ultrastructures [22, 33].

The glutaraldehyde molecule is larger than formaldehyde, and therefore, it penetrates tissues. However, glutaraldehyde normally rearranges itself to form permanent intramolecular, intermolecular, and intrafibrillar crosslinks, which are stable and irreversible [24].

The combination of formaldehyde with glutaraldehyde as a fixative for electron microscopy has the advantage of the quick penetration of smaller molecules of formaldehyde, which initiates the structural stabilization of the tissue. Complete stabilization is caused by the oligomers of glutaraldehyde, which penetrate slowly. This mixture is known as the Karnovsky solution, referring to Morris J. Karnovsky [34]. The original

solution contained 4% of glutaraldehyde, which was more concentrated than many authors advocate as ideal. However, some authors suggest small concentration changes according to the osmolarity of each tissue [22, 24, 35]. However, this substance together with formaldehyde remain highly toxic [33].

Saturated salt solution

A new solution that has been gaining attention in recent published papers is the satured salt solution. This method was created in 1992 and uses a combination of large amounts of salts (ammonium nitrate, potassium nitrate and sodium sulphite) together with low quantities of formaldehyde, ethylenoglycol, p-chlorocresol and boric acid [36-38].

A study performed by Hayashi, Naito [36] compared this solution with formaldehyde and Thiel's solution and concluded that it possessed sufficient bactericidal effects. One of the disadvantages of the saturated salt solution is the rigidness of the cadaveric tissue.

Lombardero, Yllera [37] also studied the saturated salt solution and despite better results, this solution caused massive hair loss (a great problem when dealing with animal specimens), metal and stainless steel corrosion (thus the authors proposed that metal containers should be avoided).

Despite recent popularity, there is no consensus regarding the perfusion method and the correct amount of formaldehyde and salts to achieve the best fixative properties of the saturated salt solution [36].

Alternative fixative solutions

Honey is a natural alternative fixative that has been investigated. Honey has proven to be a successful antibacterial solution for centuries, with the potential to preserve tissue components without any toxic effects [12, 39]. Some studies have shown that honey can also be a safe alternative to conventional methods of fixation for histochemical staining and immunohistochemistry [39, 40]. However, honey is not universally available, and it is impractical to use on a large scale due to its high cost [12].

Other formaldehyde-free traditional fixatives, such as chromic acid, mercury chloride, mercuric-acetic, mercuric-formol, picrinic acid, and Zenker and Bouin's solution, either contain heavy metals (which are toxic to the environment) or are acidic (which may degrade DNA and RNA and result in tissue that is less hardened and difficult to section). Despite their special properties, these solutions are not feasible alternatives [25, 32].

Recently, glyoxal-based formulas and alcohol-based solutions have been proposed as fixatives. These fixatives appear to be less harmful to DNA and RNA and may avoid the AR step for immunohistochemistry because protein crosslinking is reduced or absent [11, 25, 32]. Some examples of these less toxic alcohol-based fixatives include F-Solv, which contains aldehyde (Adamas, Rhenen, Netherlands), FineFIX (Milestone, Bergamo, Italy), and RCL2 (Alphelys, Plaisir, France) [9, 11, 41, 42]. These fixatives do not establish crosslinks but instead cause proteins to coagulate [9]. The reported advantages of these fixatives include quick fixation, removal of carcinogenic vapors, improved preservation of glycogen, DNA, and RNA, and no requirement of AR steps for immunohistochemical analysis [9, 11].

Another component used in alternative fixatives is acetic acid, as found in RCL2 [42]. Acetic acid complements the action of alcohol, swells collagen fibres, precipitates nucleoproteins, and works as a solvent on cytoplasmic granules [9, 11, 41]. The addition of acetic acid to fixative solutions may also allow for the fixation of larger samples [11].

Microscopic analysis shows that alcohol- and zinc-based fixatives express a higher affinity for certain stains, especially eosin, compared to regular formaldehyde solutions or glyoxal-based formulas. Nuclear structures are better preserved in alcohol-based fixatives. However, shrinking artefacts are evident in alcoholic fixatives, and the shrinkage of tissue as a whole is more evident when the alcohol concentration is higher than 50%. Furthermore, zinc-based formulas show evident tissue shrinkage [32].

Macroscopic analysis of tissues fixed by alcohol-based solutions showed differences in colour and consistency, but other features, such as shrinkage and surface changes, are more subtle [32].

When tissues fixed by these alternative solutions were macroscopically analyzed, many different characteristics were observed compared to formaldehyde. Tissues fixed with F-Solv were darker in color than formalin-fixed tissues, and tissues fixed with RCL2 and FineFIX showed a lighter color [9].

Color preservation can be sufficient to provide a first impression of inadequate fixation if discoloration is assumed to be a reliable sign of proper fixation [32]. Zinc salts and ethyl alcohol do not alter respiratory enzymes and oxygen carrier proteins, such as hemoglobin and myoglobin, which means that color fading is not induced by these chemical compounds, contrasting with formalin. When an operator is not familiar with macroscopic color changes caused by fixatives, it may impair interpretations of inadequate fixation and elongate the fixation time [32]. The consistency of tissues is also different. Samples fixed with F-Solv and FineFIX are more rigid compared to RCL2-fixed tissues, which are porous and slippery, thus creating difficulty in handling [9].

In contrast, these difficulties in the handling and sectioning of samples fixed by RCL2 were not demonstrated in another report [32]. After two months of fixation, samples of lymph nodes, liver, and intestines were difficult to recognise, especially for samples fixed with F-Solv [9]. The rate of penetration was similar between tissues fixed with FineFIX and RCL2, but samples stored in F-Solv showed incomplete fixation of the inner portion. Samples fixed and stored in RCL2 showed a considerable amount of fragments floating in the solutions [9]. All tissues fixed with alcohol-based solutions can be submitted to immunohistochemical techniques with few changes in protocols [9, 32].

Nucleic acid extraction using alcohol-based fixatives and most other formulas is superior to formalin in the quality and quantity of nucleic acids, which can be removed from paraffin blocks [9, 32].

CONCLUSION

Many techniques and fixative solutions have been used throughout the history of anatomy, but each of these is suitable for certain functions, such as embalming and time. Current desirable fixatives must be non-toxic and compatible with macroscopic and microscopic analyses, histochemical and immunohistochemical staining, and the preservation of DNA and RNA, and they must also be economical.

This perfect fixative does not exist because none of the developed or discovered solutions possess all of these characteristics, especially with regard to toxicity. The vast majority of these properties is presented by formaldehyde, which preserves tissues for gross and microscopic studies, and microscopy laboratories normally contain exhaust hoods and fume hoods that enable people to work safely with these solutions. However, the biggest toxicity problem remains in gross anatomy laboratories, where the large size of the specimens and the large area does not always allow for adequate exhaustion, which exposes users to the toxicity of the fixatives.

Therefore, there is still a need for the development of new fixative solutions that match the ideal chemical and physical requirements, especially related to toxicity, in order to contribute to the safety of students, professors, and technical staff working in gross anatomy environments.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

None.

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RESUMO

O uso de soluções fixadoras ao longo das eras: uma revisão

A preservação do tecido biológico é realizada usando diferentes soluções fixadoras, o que deu contribuição fundamental na história do desenvolvimento científico, em especial, da Anatomia. A história das soluções fixadoras pode ser dividida em três momentos importantes: o primeiro está ligado ao Antigo Egito; o segundo, ao período da Renascença; e o terceiro período ocorreu durante a Guerra Civil Americana. Várias soluções foram testadas e usadas ao longo da história e estas se adaptaram às necessidades e contextos de cada período. Atualmente, as principais soluções fixadoras são tóxicas e algumas carcinogênicas, como o formaldeído, que permanece como "padrão ouro" em alguns países. As soluções fixadoras foram adaptadas para laboratórios de histologia, onde seus vapores são facilmente removidos. Por outro lado, em laboratórios de anatomia, onde geralmente não há ventilação suficiente, a exposição do aluno aos vapores tóxicos é maior. Portanto, a necessidade de soluções melhores, mais eficazes e seguras aumentou o interesse pelo estudo e vários pesquisadores se engajaram no desenvolvimento de uma melhor solução fixadora. Este trabalho traz uma revisão do progresso histórico das técnicas de fixação de tecidos para fins de pesquisa e didática.

Palavras-chave: anatomia, embalsamamento, soluções fixadoras, história