RAFAEL CISNE DE PAULA

Análise morfológica da propriedade de compostos vegetais na conservação de tecidos cadavéricos

São Paulo

2014

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Tese apresentada ao Programa de Pós-Graduação em Anatomia dos Animais Domésticos e Silvestres da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para obtenção do Título de Doutor em Ciências

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Orientador (a):
Profa. Dra. Paula de Carvalho Papa

Co-Orientador (a):
Profa. Dra. Silvana Lima Gorniak

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Parecer do Comitê de Ética

UNIVERSIDADE DE SÃO PAULO

FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

Comissão de Ética no uso de animais

CERTIFICADO

Certificamos que o Projeto intitulado "Análise morfológica da propriedade de taninos vegetais na conservação de tecidos cadavéricos", protocolado sob o nº 2851/2012, utilizando 5 (cinco) camundongos, sob a responsabilidade do(a) Profa. Dra. Paula de Carvalho Papa, está de acordo com os princípios éticos de experimentação animal da "Comissão de Ética no uso de animais" da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo e foi aprovado em reunião do dia seis de fevereiro de 2013.

São Paulo, 6 de maio de 2013.

Denise Tabacchi Fantoni
Presidente

Folha de Avaliação

Autor: DE PAULA, Rafael Cisne

Titulo: Análise morfológica da propriedade de compostos vegetais na conservação de tecidos cadavéricos.

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Dedicatória

À Deus (Grande Arquiteto do Universo), por estar sempre presente na minha vida, mesmo quando não consigo senti-Lo, e aos inúmeros companheiros espirituais que me conduzem e fortalecem minhas intuições a cada momento.

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Epígrafe

"Podemos até reconhecer que o mundo já é pequeno para a anatomia e os anatomistas. Contando desde já com a indulgência dos leitores, acrescentaremos que a "Anatomia e seus cultores são obras eminentemente divinas", e que todos, mais cedo ou mais tarde, reconhecerão que se trata de verdadeira declaração de amor, expressão de entusiasmo de um apaixonado pelo assunto que é uma razão de vida e de um estilo de vida, que enaltece e edifica os que a ele se dedicam."

Prof. Dr. Liberato J. A. Di Dio

RESUMO

DE PAULA, R. C. **Análise morfológica da propriedade de compostos vegetais na conservação de tecidos cadavéricos.** [Morphological analysis of the properties of plant compounds to preserve cadaveric tissues]. 2014. 66 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2014.

A solução fixadora a partir do formaldeído permanece ate os dias de hoje como o composto fixador "padrão ouro" para trabalho de rotina. Porém, este é classificado como cancerígeno para os seres humanos e , portanto, representa um risco para qualquer pessoa manusear. Assim, a busca por substitutos tem sido motivada, e, mais recentemente, surgiram algumas soluções alternativas com potencial para substituí-lo. A maioria destas soluções alternativas são à base de álcool , e a maior parte delas são apenas para amostras microscópicas. A partir do conhecimento de ácido tânico para estabilizar a elastina e o colágeno, diluído em glutaraldeído, para microscopia eletrônica, bem como para próteses biológicas, emerge a ideia de uma nova solução fixadora alcoólica, contendo ácido tânico como componente principal. Em análises microscópicas coração, cérebro, intestino e rins foram coletados e preservados sendo armazenados em diferentes soluções fixadoras (10 % de formalina v/v, 70 % de álcool e solução alcoólica de ácido tânico), e preparado para procedimentos histológicos de rotina. Adicionalmente, ratos wistar inteiros foram fixados com a solução alcoólica de ácido tânico ou formalina e estudantes de medicina que praticam dissecção há pelo menos 2 anos ou mais, dissecaram estes espécimes durante o curso de dissecação, como uma alternativa para adquirir habilidades básicas de cirurgia e responderam a um questionário detalhado que inferia sobre a qualidade da peça a ser dissecada. A toxicidade do composto foi analisada por ensaio "in silico" para o sistema respiratório e a cutis. A análise microscópica mostrou que solução à base de ácido tânico pode ser melhor que os outros fixadores comuns testados diversos parâmetros, e houve diferença entre os tecidos analisados. Entretanto, o resultado microscópico mais importante foi que esta nova solução possui forte capacidade de preservar e estabilizar a elastina e o colágeno, demonstrando resultados melhores em relação aos outros fixadores. Os resultados macroscópicos revelaram também uma superioridade da solução de ácido tânico em diversos parâmetros analisados, como odor, textura e flexibilidade durante dissecção. A toxicidade da solução de ácido tânico para cútis e sistema respiratório foi considerada moderada em relação à formalina que é considerada altamente tóxica. Concluímos que a solução fixadora de ácido tânico é eficaz para macro e para microscopia, é menos tóxica do que o formaldeído e inodora. Portanto, pode ser uma alternativa real para a evitar a utilização de formol, diminuindo os fatores de risco durante a manipulação de cadáveres e tecidos fixados.

Palavras-chave**:** Ácido tânico. Dissecção. “In silico”. Formaldeído. Solução fixadora.

ABSTRACT

DE PAULA, R. C. **Morphological analysis of the properties of plant compounds to preserve cadaveric tissues.** [Análise morfológica da propriedade de compostos vegetais na conservação de tecidos cadavéricos]. 2014. 66 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2014.

The fixative solution from the formaldehyde remains until nowadays as the "gold standard" for routine work fixative compounds. Nevertheless, it is classified as carcinogenic to humans and, therefore, represents a risk to anyone handling the solution. Thus, the search for formaldehyde substitutes has been motivated, and more recently, some alternative solutions with potential to replace it have appeared. Most of these alternative solutions are alcohol-based, and most of them are only for microscopic specimens. From the knowledge that tannic acid diluted in glutaraldehyde can stabilize elastin and collagen for electron microscopy as well as for bioprotheses, emerge the idea for a new alcoholic fixative solution, containing tanic acid as the main component. For microscopical analyses heart, brain, intestine and kidneys were collected and preserved in different fixative solutions (10% v/v regular formalin, 70% alcohol and tannic acid alcoholic solution), and prepared for routine histology procedures. Additionally, whole wistar rats were fixed in the tanic acid alcohol-based solution or formalin and medicine students, who practice dissection for at least 2 or more years, dissected the rats during the dissection course, as an alternative to acquire basic surgery skills and answered a detailed questionnaire about the quality of dissected specimen. The toxicity of the compound was analyzed by “in silico” tests for cutais and respiratory system. The microscopial analysis showed that tannic acid based solution could be better than others common fixatives as tested in several parameters, and there are differences among tissues. Moreover, the most important microscopic result was that this new solution showed a strong capability to preserve and stabilize elastin and collagen, shppwing better results in relation to other analysed fixatives. The macroscopial analyses also showed better results of tannic acid solution for several parameters, as odor, texture and flexibility during dissection. The toxicity of tannic acid solution for skin and respiratory system was considered moderate in relation to formaldehyde, which is considered highly toxic. We conclude that tanic acid solution is efficient to preserve tissues for macroand microscopical studies, is less toxic than formaldehyde and odorless. Therefore, it could be a real alternative to avoid the regular use of formalin, decreasing risk factors to the ones manipulating fixed corpses and tissues.

Keywords: Dissection. Fixative solution. Formaldehyde. “In silico”. Tannic acid.

SUMÁRIO

1- INTRODUÇÃO GERAL

Devido a tendência natural dos tecidos biológicos entrarem em decomposição, levando a putrefação, o homem sempre buscou ferramentas que possibilitassem a conservação de corpos após a morte (MAYOR, 2000; SAEED, RUFAI E ELSAYED, 2001). As razões por esta busca são muitas, alguns povos realizavam estes procedimentos mediados pela fé, pois acreditavam que voltariam a viver nesses corpos, sendo, portanto, necessário conservá-los (VON HAGENS E WHALLEY, 2009). Outros foram movidos pela curiosidade de entender a disposição interna dos órgãos para caça ((VON HAGENS E WHALLEY, 2009) , ou ainda tratar enfermidades (MAYOR, 2000).

Independente da razão pela qual esta busca foi motivada, diversas técnicas foram desenvolvidas, porém as mesmas se adaptam melhor ou pior às realidades atuais (VON HAGENS E WHALLEY, 2009).

A técnica de conservação de cadáveres mais antiga é a mumificação, método este utilizado por diversos povos antigos, principalmente os egípcios (SAEED, RUFAI E ELSAYED, 2001Saeed, Rufai e Elsayed, 2001). Além das múmias preparadas pelo homem, existem também as naturais, preparadas a partir de condições climáticas ou ambientais. Em algumas partes do mundo, elementos como o clima, tais como extremo calor e frio, atuam como preservadores naturais, criando corpos que se mantem íntegros por longos períodos (ITOPA, 2011). Destas podemos citar o frio das regiões polares, regiões ricas em sal e certas substâncias químicas encontradas em pântanos, principalmente no Norte da Europa (MAYOR, 2000; RODRIGUES, 2010).

Apesar de toda tecnologia disponível, até os dias atuais vivemos a dificuldade de conservar peças cadavéricas e fragmentos de tecidos em substâncias fixadoras não tóxicas (FOX et al., 1985; MOELANS et al., 2011). Desta forma, surge a necessidade pela busca de novos agentes fixadores com propriedades atóxicas capazes de substituir as atuais, principalmente o formaldeído (BUESA, 2008; ZANINI et al., 2012).

Compostos fenólicos, como taninos extraídos de extratos vegetais são comumente utilizados para curtimento de peles, a fim de produzir couro. O mecanismo utilizado por estes compostos é a estabilização de componentes da matriz extracelular e precipitação de proteínas (CHUANG et al., 2009).

A partir do conhecimento dos efeitos do ácido tânico nos componentes da matriz extracelular, este composto foi postulado neste projeto como potencial fixador de tecidos. O mesmo apresenta grande potencial antimicrobiano e antifúngico, além de capacidade de estabilizar elastina e colágeno no tecido (ISENBURG, SIMIONESCU E VYAVAHARE, 2004; 2005; ISENBURG et al., 2006).

1. THE USE OF FIXATIVE SOUTIONS THROUGHOUT THE AGES

Abstract

Preservation of biological tissues is performed by using different fixative solutions. That 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, 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 aroused the interest for studying and several researchers have been engaged to develop them. This work brings a review of the historic progress of tissue fixation techniques for research and didactic purposes and points out new perspectives on this area.

Keywords: fixative solutions; embalming; anatomy history; gross anatomy.

2.1 Introduction

Despite the natural decomposition of biological tissues, humanity is always looking for new tools that would enable the conservation of corpses after death (SAEED; RUFAI; ELSAYED, 2001; ITOPA, 2011). There are many reasons for this search. Some people performed these procedures moved by faith, since they believed in life after death. Others were moved by curiosity of understanding the internal arrangement of organs for treating diseases or even hunting (VON STADEN, 1992; MAYOR, 2000; MALOMO; IDOWU; OSUAGWU, 2006; VON HAGENS; WHALLEY, 2009). In addition, fixative solutions were also used to preserve corpses for hygienic purposes during the transportation of cadavers for long

distances, avoiding contamination (TROMPETTE; LEMONNIER, 2009). Due to the most different reasons, several techniques were developed, but only some of them are suitable to the current reality (VON HAGENS; WHALLEY, 2009). Despite all technology available, we still have difficulty in preserving pieces of corpses and tissue fragments with non-toxic substances (FOX et al., 1985; MOELANS et al., 2011). The interest by disseminating knowledge about the current fixative compounds, as well as the search for new non-toxic agents with that could replace the existing fixatives, especially formaldehyde (SOMPURAM et al., 2004; BUESA, 2008) have been constantly growing and represents nowadays a research area in morphology.

2.2 History of tissue fixative techniques

Fixative process includes natural conservation by specific factors and the preservation of biological tissue guided by man (VON HAGENS; WHALLEY, 2009). The history of this technique is commonly divided in three main periods (MAYOR, 2000; ITOPA, 2011).

2.2.1 First period (Ancient Egypt)

Embalming started at 5000 BC, in the Ancient Egypt, due to religious rituals. They believed in life after death, hence, they thought they needed their bodies preserved (SAEED; RUFAI; ELSAYED, 2001; ITOPA, 2011).

By that time, two different techniques were developed: in the first one, the body has been placed in fetal position, wrapped in cotton fabric, and stored in little caves of the desert (MAYOR, 2000). Ambient heat and sand helped to remove the humidity of the body, what was important for the preservation purpose. The growth of Egyptian population increased the robbery at funerary caves, leading to development of safer places called sarcophagi (MAYOR (MAYOR, 2000; VON HAGENS; WHALLEY, 2009). The use of sarcophagi avoided the heat and dehumidification provided by the desert, and a new embalming technique has been created: evisceration, excluding the heart because they believed in the presence of the soul

within it. After that, the whole body was wrapped with strips soaked in special solutions, such as natron and erbal components (SAEED; RUFAI; ELSAYED, 2001), and so, held by 70 days (MAYOR, 2000).

During this period Persians, Syrians and Babylonians employed similar procedures, using honey and wax (MAYOR, 2000). Alexander "the Great" had his body preserved by using honey after his death in Babylon in 323 AC (PATIL et al., 2013).

2.2.2 Second period (Renaissance)

The second main period of the tissue fixative history is the Renaissance, Sec. XVI, where new techniques were improved in order to preserve corpses for artistical and dissection purposes (ITOPA, 2011).

During the Dark Ages (Sec. V - XV), the laws have prohibited the medical courses to obtain cadavers for dissection or study. Embalming process was allowed only to royalty, clergy, and other elite members of the society (TROMPETTE; LEMONNIER, 2009).

The Crusades (1095 – 1291) spread the Roman Empire, leading the military very far from Rome. Invading different regions, they had the opportunity to know different religions and culture civilizations, mainly in North Africa and Asia. At this time, many nobles and soldiers died in battle, and due the distance, the preservation of corpses was mandatory to allow the return of their bodies to Rome. Under this context, the maceration technique (the body was heated until soft tissues were removed) was largely employed (MAYOR, 2000).

Frederick II, a Sicilian King of the XIV century, authorized the dissection of executed criminal corpses in the medical college of Bologna, Italy (OLRY, 1999; MAYOR, 2000), increasing general knowledge of anatomical science. Moreover, Pope Boniface VIII in1330 prohibited transportation of sectioned parts of corpses from people that died in battles, which encouraged the development of new techniques for preservation (VON HAGENS; WHALLEY, 2009), such as arterial injection of new solutions (hot water, wax, ink, mercury and arsenic) and new tools for these injections (scissors and tweezers) (SAEED; RUFAI; ELSAYED, 2001; TROMPETTE; LEMONNIER, 2009).

The most famous solution used for arterial injection was a mix of alcohol and wax, created by Jan Swammerdam, who dedicated his career to the observation of insects and small animals,

testing several compounds trying to fix them. This technique was improved by Frederick Ruysh (1655-1717) when working with human cadavers for didactic and funerary purposes (BENJAMIN; MICHAEL; JUDITH, 2011).

This refinement would have created the ideal fixative solution, but the exact formulation remain unknown until nowadays. Speculations indicate the use of some amount of arsenic (TROMPETTE; LEMONNIER, 2009; IJPMA; VAN GULIK, 2013).

2.2.3 Third period (American Civil War)

The third important period of specimens preservation took place during the American Civil War, where the embalmed corpses should be prepared to undergo long distances from battles fields until home town, because families started claiming the bodies to berry (MAYOR, 2000; ITOPA, 2011).

In previous wars against natives and Mexicans, the militaries were buried on the battle fields, since available substances (mercury and arsenic) were considered toxic. Therefore, there were no plans for returning the corpses, but the President Abraham Lincoln permitted the conservation by using these toxic solutions added of salt and acid (MAYOR, 2000; ITOPA, 2011).

The major improvement on the preservation of corpses occurred in 1868, with the use of formaldehyde for gross anatomy and microscopic techniques (FOX et al., 1985; SOMPURAM et al., 2004). This substance was discovered by the Russian Aleksandr Mikhaylovich Butlerov in 1859, but its medical applications have emerged only in 1891 with Ferdinand Blum, due to the discovery of its antiseptic and antimicrobial properties. Another property was characterized accidentally when Blum observed that his skin showed rigidity after contact with the aqueous formaldehyde solution, which was equal or greater than that produced by alcohol (FOX et al., 1985). Then, Blum has fixed some tissues using formaldehyde, in order to analyze its fixative capacity. Additionally, histochemical staining after formaldehyde fixation resulted in excellent quality, which was later confirmed by the famous histologist Frankfurt Karl Weigert (FOX et al., 1985).

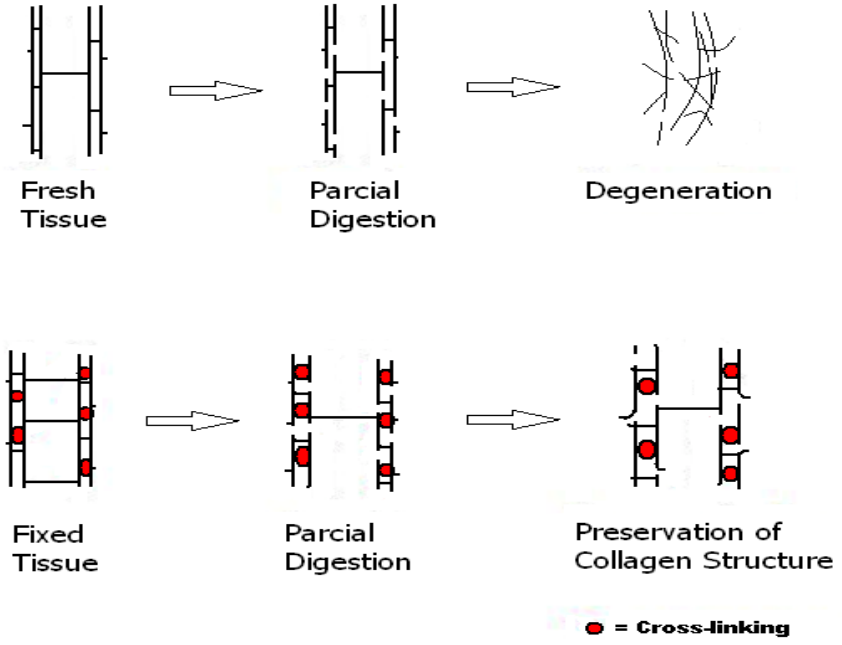
After the advance provided by the discovery of formaldehyde, it took a long time until another technique was used to preserve tissues. However, in 1977, Dr. Gunther von Hagens

described the conservation technique called plastination, which consists in the replacement of body fluids and lipids by polymerizable resins such as polyester, epoxy and silicone (GUBBINS, 1990; VON HAGENS; WHALLEY, 2009; PASHAEI, 2010). The technique was quickly accepted in the scientific world due to reduction of toxic exposure, which occurs in a limited part of the process and can be avoided by careful standard 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 resin component. This technique is very useful for exhibitions and gross anatomy studies in laboratory (REINA-DE LA TORRE; RODRÍGUEZ-BAEZA; DOMÉNECH-MATEU, 2004; VON HAGENS; WHALLEY, 2009; PASHAEI, 2010).

2.3 Fixative solutions

Fixative solutions are composed of chemicals substances that keep tissue integrity after death. These substances work chemically, decreasing proteolytic events and avoiding changes within the tissue as intra and extracellular destruction (TOLOSA et al., 2003). To make extracellular matrix stable, fixatives should achieve preservation of elastin and collagen. This process consists in creating crosslinking bridges to keep collagen structure, even if there are connections in the tissue leading to rupture (BOWES; CARTER, 1965), as shown in Figure 1. Some factors, such as viscosity, temperature, volume, pH and osmolarity of substance influence the creation of these connections (FOX et al., 1985; TOLOSA et al., 2003; ZENG et al., 2013). In the preparation of tissues for electron microscopy, the fixation in a solution with similar osmolarity to the original tissue is recommended (SPEILBERG et al., 1993; DOUGHTY; BERGMANSON; BLOCKER, 1995).

Figure 1: Schematic drawing of colagen fixation by crosslink within the tissue



Adapted from BOWES AND CARTER, 1965.

Tissue thicknesses as well as viscosity of the fixative solution are directly related to the speed of penetration into the tissue. The temperature also increases the rate of penetration, especially for the most viscous fixative. For ideal fixation, the volume of fixative should be 20 to 30 times greater than the immersed tissue (TOLOSA et al., 2003; BUESA, 2008; ZENG et al., 2013).

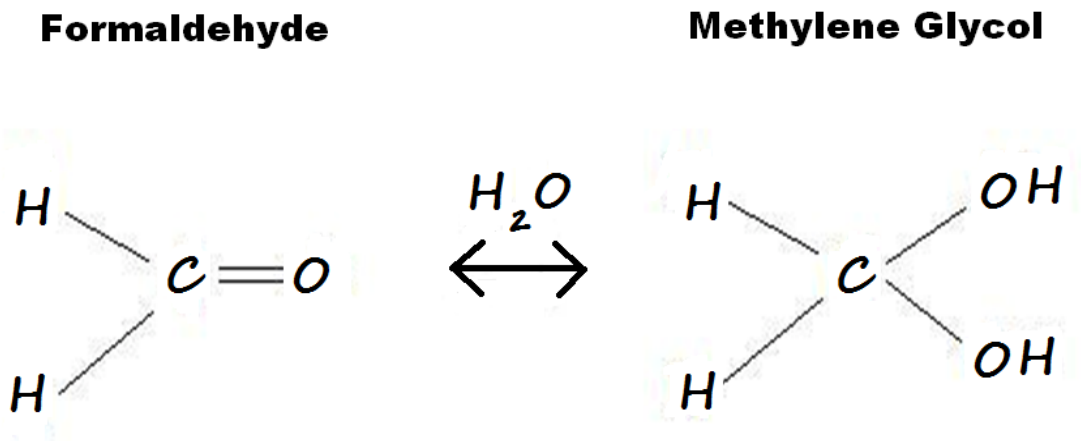
In tissues prepared for gross anatomy, the aqueous buffered solution of formaldehyde (4%) has been used for decades as a standard (FOX et al., 1985). In tissues fixed for light and electron microscopy, other substances stand out, such as alcohol and glutaraldehyde. Other less toxic fixative solutions based on alcohol, containing or not acetic acid, were proposed as alternative (BUESA, 2008; MOELANS et al., 2011).

2.4 Conventional fixatives

2.4.1 Formaldehyde

Formaldehyde is the most abundant and important aldehyde in the environment, it is a colorless gas with a strong irritating smell, it is very soluble in water and shows high chemical reactivity (FOX et al., 1985; SOMPURAM et al., 2004; MOELANS et al., 2011). When dissolved in water, it quickly becomes formaldehyde hydrate to form methylene glycol. When tissues are immersed in aqueous solutions of formaldehyde, they are readily penetrated by methylene glycol fraction and residual formaldehyde. The equilibrium on aqueous solution of these two substances must locate in favor of methylene glycol (Figure 2). Thus, the balance between formaldehyde carbonyl and methylene glycol explains most of the mystery related to the rapid penetration achieved by methylene glycol, and the slow one by formaldehyde carbonyl (FOX et al., 1985).

Figure 2: Schematic drawing showing the formation of methylene glycol from formaldehyde hydrate

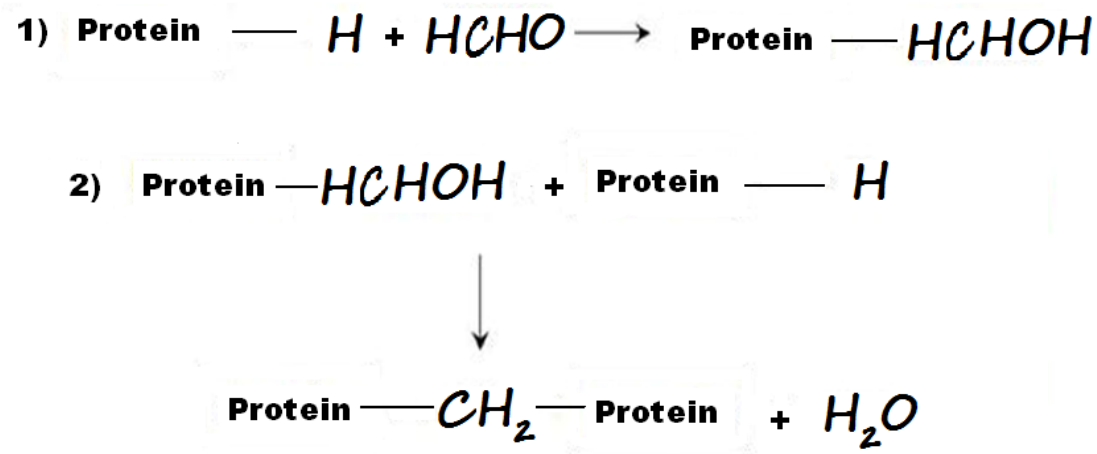


Adapted from KIERMAN, 2000.

Chemical studies indicate that formaldehyde is a electrophile reactive molecule, that reacts readily with several groups of biological macromolecules, such as proteins, glycoproteins, nucleic acids, and polysaccharides, by crosslinking forming methylene bridges (Figure 3) (FOX et al., 1985). The most reactive regions are primary amines, such as lysine, arginine, tyrosine, asparagine, histidine, glutamine and serine (SOMPURAM et al., 2004; MOELANS et al., 2011). This mechanism of intra and intermolecular crosslinking dramatically changes

the tissues characteristics (FOX et al., 1985; SOMPURAM et al., 2004), and an adequate fixation is obtained after some days. Furthermore, these bridges can be reversible under certain conditions and removed by addition of alcohol in the solution (MOELANS et al., 2011).

Figure 3: Schematic drawing of the reaction between formaldehyde and proteins to generate methylene bridges.



Adapted from KIERMAN, 2000. (1) Primary ligation between formaldehyde and protein, which occurs quickly. (2) Formaldehyde ligation to a second protein creating a crosslink of methylene.

Formaldehyde has some disadvantages, such as the spontaneous formation of formic acid when exposed to oxygen and light (FOX et al., 1985). Moreover, the crosslinking disguises certain antigens, which hampers immunohistochemistry and often needs antigen retrieval (AR) steps. The DNA and RNA molecules are fragmented, which is a problem for molecular techniques. The tissue fixation by formaldehyde for ultrastructural analysis are poor (VAN ESSEN et al., 2010). However, the main disadvantage is its toxicity. Formaldehyde is classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC, 2006), and some studies already demonstrated the genotoxic effects in workers exposed to this substance (NIELSEN; WOLKOFF, 2010). Merk and Speit (MERK; SPEIT, 1998) demonstrated that its high solubility in water causes a rapid absorption by the respiratory and gastrointestinal tract, causing nasal tumors in rodents. Although this chemicals are present in both indoor and outdoor environments, representing a risk to human health (IARC, 2006), in work areas, such as hospitals, laboratories, universities and scientific institutions, people might be overexposed (OCHS et al., 2012), especially in dissection rooms

and gross anatomy labs, causing respiratory, eye and skin irritation (MOELANS et al., 2011; OCHS et al., 2012).

Several occupational health authorities around the world have set permissible exposure limits to formaldehyde (OCHS et al., 2012). The World Health Organization recommends 0.1 mg/m^3 as maximum indoor values. The American Conference of Governmental Industry Hygiene (OCHS et al., 2012), and the European Conference proposed 0.24 mg/m^3 as threshold. The National Institute for Occupational Safety and Health of the United States has set a short-term exposure limit by 0.08 mg/m^3 and occupational exposure limit by 0.013 mg/m^3 (OCHS et al., 2012). The exposure limit adopted by Brazilian law (ANVISA) is 2.3 mg/m^3, for a maximum of 48 working hours per week. This value is significantly higher than those adopted elsewhere in the world.

Despite its toxic characteristics, the formaldehyde currently remains as the main fixative for corpse pieces and tissue fragments for histology (FOX et al., 1985; BUESA, 2008; RODRIGUES, 2010; VAN ESSEN et al., 2010; MOELANS et al., 2011).

2.4.2 Alcohol

The reaction carried by formaldehyde fixation in the tissue can be inhibited at room temperature by adding small amounts of alcohol, which acts both as a preservative and inhibitor (FOX et al., 1985). This solution was used for the first time to preserve cadavers at the end of the seventeenth century (RODRIGUES, 2010), 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 (TOLOSA et al., 2003).

The fixation based on alcohol does not preserve tissue by crosslinking of proteins, thus the final morphological aspect is not identical to that provided by the formaldehyde solution (COX et al., 2006). Benefits attributed to alcohol-based fixatives include quick fixation, removal of carcinogen vapors, better preservation of glycogen, DNA and RNA, enhanced staining and do not require antigen retrieval steps for immunohistochemistry analysis (BUESA, 2008; VAN ESSEN et al., 2010; MOELANS et al., 2011; ZANINI et al., 2012). The disadvantages are the variability in staining tissues, tissue shrinkage, hardening, deposition of pigment (artifacts) in blood, total or partial lysis of red blood cells and

increasing flammability of the environment (SOMPURAM et al., 2004; MOELANS et al., 2011).

2.4.3 Glutaraldehyde

Glutaraldehyde is the most effective reagent to perform crosslinks with proteins. It may be used in at least 13 different forms, depending on solution conditions, such as pH, concentration, temperature, etc (MIGNEAULT et al., 2004). To study cellular ultrastructure in biological tissues, this substance is considered the best choice for routine procedures (DOUGHTY; BERGMANSON; BLOCKER, 1995; 1997; MIGNEAULT et al., 2004). The glutaraldehyde molecule is greater than formaldehyde's, and therefore penetrates slower in tissue. However, glutaraldehyde normally rearranges itself to form a permanent intramolecular, intermolecular and even intrafibrilar crosslinks, which are stable and irreversible (MIGNEAULT et al., 2004).

The combination of formaldehyde with glutaraldehyde as a fixative for electron microscopy takes advantage of the quick penetration of smaller molecules of formaldehyde, which initiate the structural stabilization of the tissue. Complete stabilization is caused by the oligomers of glutaraldehyde, which penetrate slowly. This mixture is known as Karnovsky solution, referring to J. Morris Karnovsky (DOUGHTY; BERGMANSON; BLOCKER, 1995). The original solution contained 4% glutaraldehyde, which was more concentrated than many authors advocated as ideal. However, some authors suggest little concentration changes according to each tissue osmolarity (DOUGHTY; BERGMANSON; BLOCKER, 1995; 1997), although this substance remains highly toxic, as well as the formaldehyde (MIGNEAULT et al., 2004).

2.4.4 Alternative fixative solutions

A natural alternative fixative that has been tested is the honey. For centuries, honey has proven to be a successful antibacterial solution, with the potential to preserve tissue components without any toxic effect on manipulators (ÖZKAN et al., 2012; PATIL et al., 2013). Some studies have shown that honey can also be a safe alternative to conventional methods of fixation for histochemical staining and immunohistochemistry (MANDY, 2009; ÖZKAN et al., 2012). However, honey is not universally available and it is impractical to use on a large scale due to its high cost (PATIL et al., 2013).

Other formaldehyde free traditional fixatives, such as chromic acid, mercury chloride, mercuric-acetic, mercuric-formol, picrinic acid, Zenker and Bouin's either contain heavy metals (which are toxic to the environment) or are acidic (which may degrade DNA and RNA letting tissue less hardened, difficult to section). In spite of their special properties, they are not feasible alternatives (VAN ESSEN et al., 2010; ZANINI et al., 2012).

Recently, Glyoxale-based formulas and alcohol-based solutions have been proposed as fixatives. These fixatives seem to be less harmful to DNA and RNA and may avoid AR (antigen retrieval) step for immunohistochemistry, because protein crosslinking is less or absent (BUESA, 2008; VAN ESSEN et al., 2010; ZANINI et al., 2012). Some examples of these less toxic alcohol-based fixative are: the F-Solv, which contains aldehyde (Adamas, Rhenen, Netherlands) and FineFIX (Milestone, Bergamo, Italy) or RCL2 (Alphelys, Plaisir, France) (DELFOUR et al., 2006; BUESA, 2008; DENOUËL et al., 2011; MOELANS et al., 2011). These fixatives do not establish crosslinks, but protein coagulation (MOELANS et al., 2011). Advantages reported for these fixatives include quick fixation, removal of carcinogen vapors, better preservation of glycogen, DNA and RNA, and do not require antigen retrieval steps for immunohistochemistry analysis (BUESA, 2008; MOELANS et al., 2011). Another component used in alternative fixatives is the acetic acid, as found in RCL2 (DENOUËL et al., 2011). It complements the action of alcohol, swells collagen fibers, precipitates nucleoprotein and can work as a solvent on cytoplasmic granules (DELFOUR et al., 2006; BUESA, 2008; MOELANS et al., 2011). Adding acetic acid in the fixative solution may also allow fixation of larger samples (BUESA, 2008).

The miscroscopic analysis shows that alcohol-based and Zinc-based fixatives, express a higher affinity for certain stains, especially eosin, when compared to regular formaldehyde solution or Glyoxale-based formulas. Nuclear structure is better preserved in alcohol-based

fixatives. On the other hand, shrinking artifacts are evident in alcoholic fixatives, and the shrinkage of the tissue as a whole is more evident when the alcohol concentration is higher than 50%. Also, Zinc-based formulas show evident shrinkage of tissues (ZANINI et al., 2012).

The macroscopical amalysis of tissues fixed by alcohol-based solutions showed that color and consistence differences are evident at a glance, while other features, such as shrinkage and surface changes are more subtle (ZANINI et al., 2012). When tissues fixed by these alternative solutions were macroscopically analyzed, they showed many different characteristics when compared to formaldehyde and among each other. Tissues fixed with F-Solv showed up darker color than the formalin-fixed ones, while tissues fixed with RCL2 and FineFIX showed lighter color (MOELANS et al., 2011). It may be important to underscore that color preservation can be good enough to give a first impression of inadequate fixation if discoloration is assumed as a reliable sign of proper fixation (ZANINI et al., 2012). Zinc salts as well as ethyl alcohol do not alter respiratory enzymes and oxygen carrier proteins as hemoglobin and myoglobin, which means that color fading is not induced by these chemical compounds, as induced by formalin action on tissues. When the operator is not familiar with these macroscopic color changes caused by fixatives, it may induce wrong interpretation of inadequate fixation and make longer the fixation time (ZANINI et al., 2012). The consistency of the tissues is also different. Samples fixed with F-Solv and FineFIX were more rigid compared with RCL2 fixed, which is porous and slippery, causing difficulty in handling (MOELANS et al., 2011). In disagreement with previous information, these difficulties in handling and sectioning of samples fixed by RCL2 were not demonstrated by other report (ZANINI et al., 2012). After two months of fixation, samples of lymphnodes, liver and intestines were difficult to recognize, especially for samples fixed with F-Solv ((MOELANS et al., 2011). The rate of penetration was similar between tissues fixed with FineFIX and RCL2, while samples stored in F-Solv showed incomplete fixation at the inner part. Only in samples fixed by RCL2, stored tissues showed a considerable amount of fragments floating in the solutions (MOELANS et al., 2011). All tissues fixed with alcohol-based solutions showed possibility to be submitted for immunohistochemistry techniques with some few changes in protocols (MOELANS et al., 2011; ZANINI et al., 2012).

Nucleic Acid extraction by alcohol-based fixatives, as well as most of the formulas is superior to formalin, regarding to quality and quantity of nucleic acid, which could be removed from paraffin blocks (MOELANS et al., 2011; ZANINI et al., 2012).

2.5 Conclusion

Many techniques and fixative solutions were used throughout the history of anatomy, but each one is suitable for certain functions, such as embalming and time. Nowadays, a desirable fixative must be nontoxic, allowing macroscopic and microscopic analysis, histochemical and immunohistochemical staining, preservation of DNA and RNA, and must have a feasible cost (MOELANS et al., 2011). This perfect fixative does not exist, because none of the solutions developed or discovered until today is able to group all of these characteristics, especially in relation to toxicity. Although the vast majority of these properties are presented by formaldehyde, which is able to preserve tissues for gross and microscopic studies,and microscopy laboratories normally contain exhaust hoods and fume hoods, enabling people to work safely with these solutions,the biggest problem remains in gross anatomy laboratories, where the big size of specimens and the large area does not always allow adequate exhaustion, exposing their users to the toxicicity of the fixatives (ZANINI et al., 2012). Ochs et al. (OCHS et al., 2012; ZANINI et al., 2012) described several areas of a morphology department contaminated by toxic fumes coming from the formaldehyde solution at levels above those permitted by the World Health Organization.

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

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3 NEW FIXATIVE SOLUTION "FORMALDEHYDE FREE" FROM PLANT COMPOUND (NATURALFIX)

Abstract

Formaldehyde is largely used in the entire world, but it is classified as "carcinogenic to humans" (group 1) by the International Agency for Research on Cancer and, therefore, represents a risk to anyone handling the solution. Thus, the quest for formaldehyde substitutes has been motivated, and more recently, some alternative solutions with potential to replace it appeared. Most of these alternative solutions are alcohol-based solution. From the knowledge of tannic acid to stabilize elastin and collagen, diluted in glutaraldehyde, for electron microscopy as well as for bioprostheses, emerge the idea for a new alcoholic fixative solution, with it compound as main component. The experimental design followed with fixation of some tissues (heart, brain, intestine and kidney) with 10 % regular formalin solution (RFS), 70% alcohol solution (AS) and tannic acid alcohol solution (TAAS), and then, the fragments were prepared for routine histology procedures and were conducted the qualitative and quantitative analysis. The qualitative analyses demonstrate the capability of all fixative solutions to preserve the fragments, with nuclear, cytoplasmic and extracellular details. The quantitative analysis showed at first that TAAS could be better than others common fixatives tested in some parameters, with difference between tissues, mainly intestine fragments. But, the most important result was that TAAS showed a strong capability to preserve and stabilize elastin and collagen, with high superiority to RFS and AS. Therefore, TAAS seems to be a powerful and feasible alternative solution for fixing samples for microscopical analysis.

Keywords: Alcohol-based solution. Alternative solution. Fixative. Formaldehyde. Tannic acid.

3.1 Introduction

Fixatives are essential in morphological studies, as tissue needs to be kept for many years and thus prevented from degradation. As the most essential and first step of preparing specimens for histology, the fixation as well as the effects on the tissues have been widely applied and studied for more than a century (ZENG et al., 2013). With evolution of microscopic and molecular biology techniques, tissue structure needs also to be kept to allow good morphological assessment. In the same manner, it is necessary that antigens were retained for immunohistochemistry evaluations, the preservation of DNA and RNA for molecular tests, and subcellular structure must be conserved for electron microscopy (VAN ESSEN et al., 2010; MOELANS et al., 2011; ZANINI et al., 2012).

The regular formalin 10% solution (RFS) concentrated is the most famous fixative (SOMPURAM et al., 2004; MOELANS et al., 2011; ZENG et al., 2013) and used in 81% of US histology laboratories, in almost all laboratories around UK and on two thirds in the rest of the world (BUESA, 2008). However, while formaldehyde is largely used in the entire world, it is classified as “carcinogenic to humans” (group 1) by the International Agency for Research on Cancer and, therefore, represents a risk to anyone handling the solution (OCHS et al., 2012). In addition, acute inhalation exposure to formaldehyde results in irritation and burning of the mucous membranes of the nose, mouth and upper respiratory tract. In some people, exposure to formaldehyde vapors, even at very low concentrations, leads to respiratory sensitization resulting in an allergic reaction similar to asthma (COSTA; GORDON, 2013).

Furthermore, considering technical aspects, the cross-linking of formaldehyde masks antigens, which may hamper immunohistochemical analysis, and fragments nucleic acids, which impairs the extraction efficiency and quality of DNA and RNA (VAN ESSEN et al., 2010; MOELANS et al., 2011).

Thus, the quest for formaldehyde substitutes has been mainly motivated by its hazardous exposure (claiming a substitute with less toxicity) and the fact that it does not assure a complete DNA and messenger RNA (mRNA) recovery, essential to many

tests of molecular biology (MASUDA et al., 1999; BUESA, 2008; VAN ESSEN et al., 2010; MOELANS et al., 2011; ZANINI et al., 2012).

Alternatives commercially available to formalin are fairly numerous (ZANINI et al., 2012) and the main alternate fixatives include alcoholic and nonalcoholic solutions, with or without acetic acid (MOELANS et al., 2011) zinc-based solution (LYKIDIS et al., 2007) and less than 10% formalin solution (BUESA, 2008; VAN ESSEN et al., 2010).

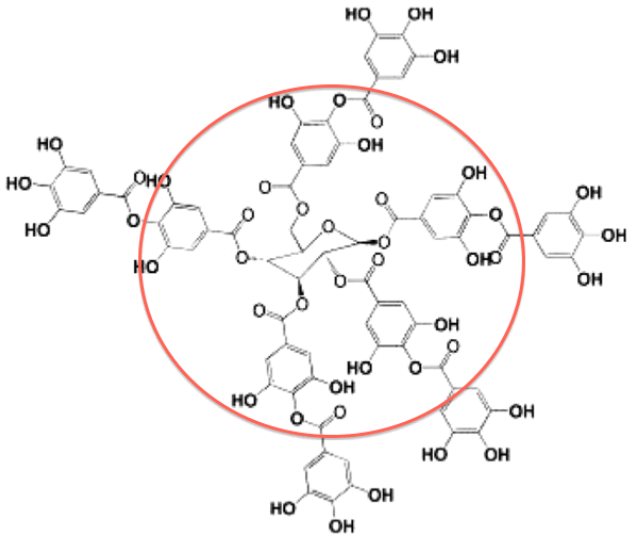
The advantages of alcoholic fixatives are: fast, act by coagulation of proteins (MOELANS et al., 2011), collapse nucleic acids that substantially reverse to their original size when rehydrated and the inclusion of methanol and acetic acid in any fixing formula will allow the fixation of larger specimens (BUESA, 2008). The tissue appearance will be different to that fixed with formalin (ZANINI et al., 2012), and because the dehydration will start simultaneously to the fixation, the processing protocols can be shorter, allowing a faster turn around time (BUESA, 2008).

Despite of this several alternatives available, in general, they are more expensive than formalin, and not easily found (BUESA, 2008). Moreover, most of these alternatives have some amounts of toxic substances.

Tannic acid (TA) is a plant polyphenol belonging to the galloylglucose family, that have a hydrophobic internal core and numerous external hydroxyl groups, with known properties to form multiple bonds with proteins, particularly those rich in proline such as elastin and collagen (ISENBURG; SIMIONESCU; VYAVAHARE, 2005; CHUANG et al., 2009). In addition, TA was proven to be an efficient antibacterial agent, reduce inflammation and antigenicity (CHUANG et al., 2009) and can be used in electron microscopy, sometimes in combination with glutaraldehyde, for ultrastructural demonstration of elastin fibers (HAIDAR et al., 1992), and more recently for bioprothetic heart valves and vascular grafts in association of glutaraldehyde (KRISHNAMOORTHY et al., 2012).

Tannic acid is composed of a central glucose molecule, called Penta-galloyl glucose (PGG), a hydrophobic portion with hydroxyl groups with one or more galloyl residues (Figure 4), which is known to be not toxic to local or systemic levels, and has the same ability TA to stabilize elastin and collagen (ISENBURG et al., 2006).

Figure 4: Schematic image of the chemical structure of polyphenol complex (TA) with the central portion of glucose (PGG - marked with red circle) and its peripheral hydroxyl compounds



Adapted from ISENBURG et al, 2006.

Thus, the purpose of the present study is to evaluate a new alcohol based solution with TA as main compound, to preserve specimens for light microscopy, as well as the use of basic and special staining techniques (Patent required).

3.2 Material and Methods

3.2.1 Materials

TA and histological staining solutions were obtained from Sigma-Aldrich Co. The 4% formaldehyde buffered solution was bought from Merck-Millipore Co., both with highest purity.

3.2.2 Tissue source and euthanasia

The study design and experimental protocols were approved by the Animal Care and Use Committee of the School of Veterinary Medicine and Animal Science of the University of Sao Paulo (protocol number 2851/2012), which based its analysis on the Guide for the Care and Use of Laboratory Animals. The experiments were performed according to guidelines of the Brazilian College for Animal Experimentation (COBEA). Fifteen Balb C mice (18-22 g) were obtained from the Animal facility of the Department of Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo. The euthanasia was accomplished by intraperitoneal injection of Xilazine (10 mg/kg) and Ketamine (80 mg/kg).

3.2.3 Experimental design

Immediately after death of each mouse, kidneys (n=5 per group), heart (n=5 per group), intestine (n=5 per group) and brain (n=5 per group) were removed and stored for 72 h at room temperature rinsed in three different solutions: a standard fixative solution using regular formalin solution (RFS), 70% alcohol solution (AS) and TA diluted (0,25 g/kg) in 70% alcohol solution (TAAS).

3.2.4 Histology preparation

Tissues underwent standard histologic procedures: alcohol dehydration (70%, 80%, 90% and 100%), followed by two baths in xylol (2x 20'). Paraffin-embedding was performed prior to sectioning the tissue in 5 μm using a microtome (MRP-09 Lupetec, São Carlos, Brazil). Sections were stained for Hematoxylin and Eosin, Gomori's trichrome and Weigert's resorcin-fuchsin after previous oxidation with oxone as described by the manufacturer (Sigma-Aldrich, St. Louis, USA).

3.2.5 Tissue Qualitative analyses

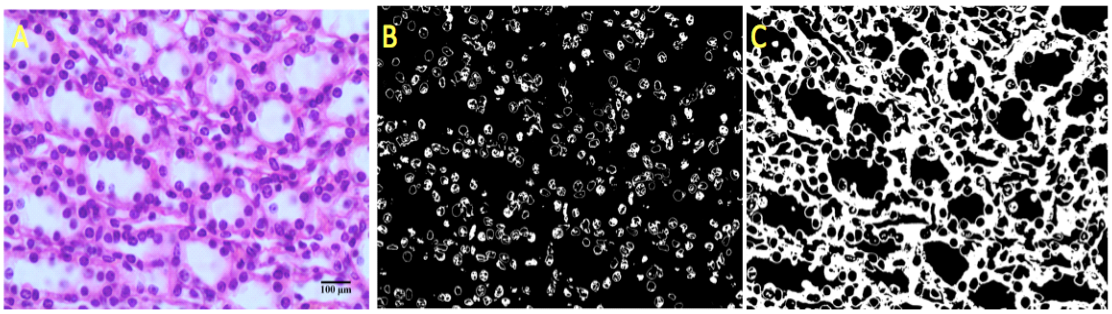
Histological aspects of the tissues stained by hematoxylin and eosin were utilized for assessment of morphological quality based on three grading systems: nuclear, cytoplasmic and extracellular matrix characteristics (as described by (COX et al., 2006).

3.2.6 Quantification method

The quantitative value of tissue integrity was analyzed by count cell method (Figure 5B) and area fraction measurement method (Figure 5 C). Both parameters are important to define the quality of fixation. The nuclei analysis has enormous potential to extract information about tissue sample, because the cell division or cell death could demonstrate the integrity and degeneration of it (FORERO; HIDALGO, 2011) .

The area fraction is another parameter, since any fixative has ability to cause shrinkage on tissues, and can provide diagnostic errors in histopathogical samples. This parameter is also utilized to determine the volume of tissue from area fraction values.

Figure 5: A= Kidney tissue prepared by H&E (100 µm – 20x). B and C= technique with segmentation by colors to convert the image in black and white (binary images 2D) for counting of nuclei (B) and measurement of area fraction (C).



From: De Paula, R. C. (2013).

The quantitative technique was performed as follows:

Cleavage and analysis of tissue sections: specimens were divided in 3 portions (upper, intermediate and lower). For each performed staining technique ten sections were obtained from upper, intermediate and lower portions. In each stained slide, ten fields (20x magnification) were randomly selected, and then, evaluated for specific characteristics.

Acquisition of images: a hundred test areas for each tissue per staining technique were analyzed. These images were digitized by a Sony CCD video camera (DXC 151-A model) coupled with a light Olympus microscope BX52 (Olympus America Inc, New York, USA).

Composition of binary images: colors from original tissue staining were used as target to convert images into Black and White (binary images 2D) using Image J software (version 1.43u - Research Services Branch, National Institute of Mental Health, Bethesda, Maryland), which took into account the parameters related to

integrity of the tissue (Figure 5). Hematoxylin staining was converted and used to count nuclei (Figure 5B), whereas Eosin staining to assess the area fraction after subtracting the area measured around the cells (Figure 5C). Gomori´s trichrome and Weigert´s resorcin-fuchsin were used to stain collagen and elastic fibers, respectively and binary images were generated after conversion of the original colors from both techniques.

Analyzing particles for quantification: All specific parameters were counted to determine the average number per slide, and the average from all slides of same tissue were used to determine mean values ± SEM. In general, the technique was carried out using an automatic cell counting and measurement of the area fraction of tissue (relative to the total area of the image) by the use of ImageJ software (Figure. 5C).

3.2.7 - Statistical Analysis

Results are expressed as means ± SEM obtained with the indicated number of animals or experiments performed and the homogeneity of the variables were analyzed with Bartlett's test. The statistical significance of differences among experimental groups was evaluated using the ANOVA (one-way). The *p* values less than 0.05 were considered statistically significant.

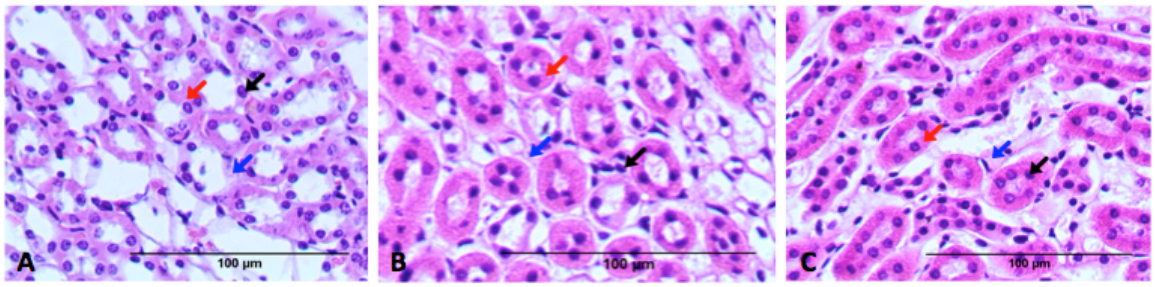
3.3 Results

3.3.1 Qualitative analysis of tissue integrity.

As expected, the samples fixed with RFS and AS showed a regular condition of tissue integrity, with preservation of cytoplasm and nucleus integrity (Fig 3). The tissue

fixed with TAAS shows similar preservation characteristics, and sometimes better than the other two fixatives (Figure 6).

Figure 6: Kidneys tissue fixed in three different fixatives solutions (A= regular formalin solution - RFS; B= alcohol solution - AS; and C= tannic acid alcoholic solution - TAAS) prepared by H&E (Bars = 100 µm – 20x). Arrows: blue for extracellular matrix, black for nucleus and red for cytoplasm.



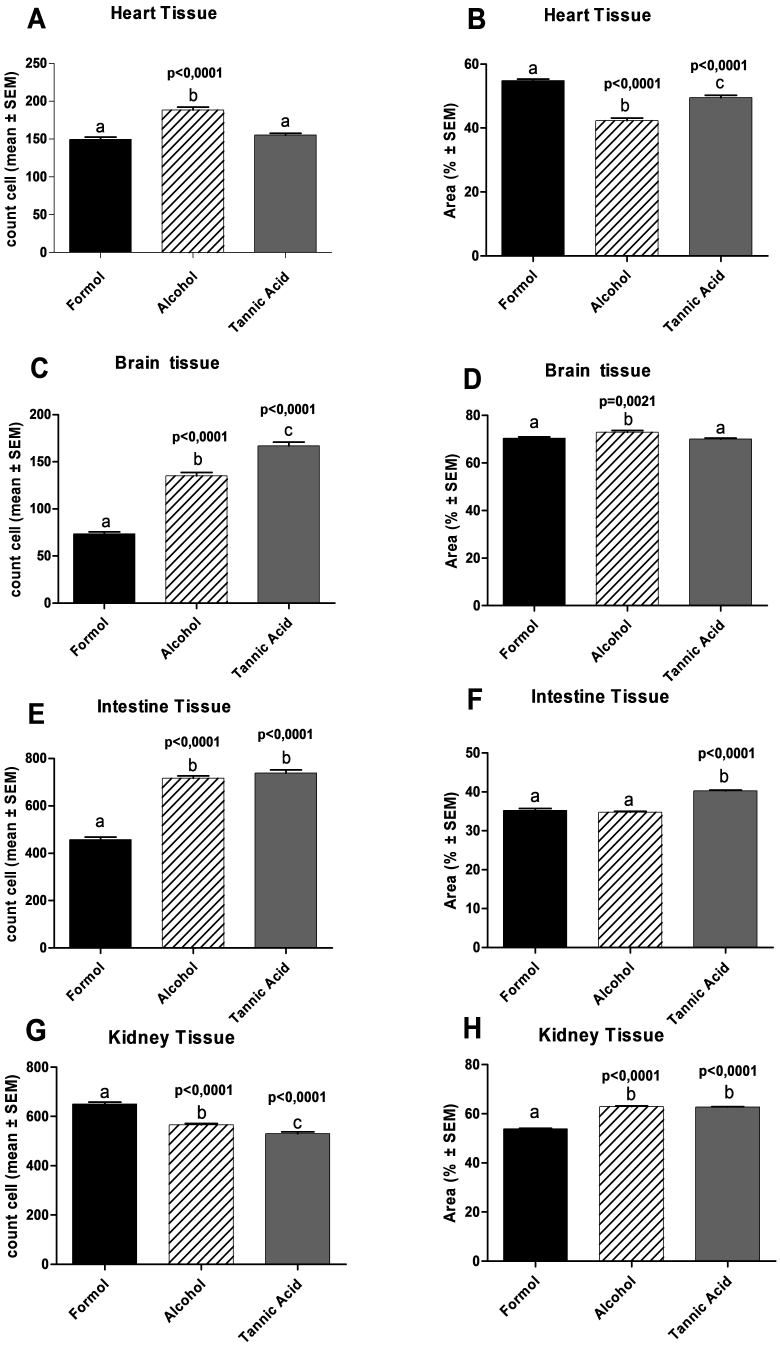
From: De Paula, R.C. (2013).

3.3.2 Quantitative analysis of tissue integrity.

The results showed that TAAS had ability to preserve the area fraction better than RFS in intestine and kidney tissue, without statistical difference in brain tissue. The TAAS was better in same parameter when compared with AS in heart and intestine, with no statistical difference in kidney specimens. Only in heart tissue the formalin appeared better than alcohol and TAAS, being TAAS better than alcohol solution (Fig 4 B, D, F and H).

The nuclei count of tissues fixed by TAAS showed better ability to preserve nuclei for brain and intestine tissues when compared with formalin solution, better than alcohol for brain tissues and with no statistical different for intestine tissues fixed by AS. The formalin was better to preserve the nuclei number just in kidney tissue, and alcohol solution only in heart (Figure 7 A, C, E and G).

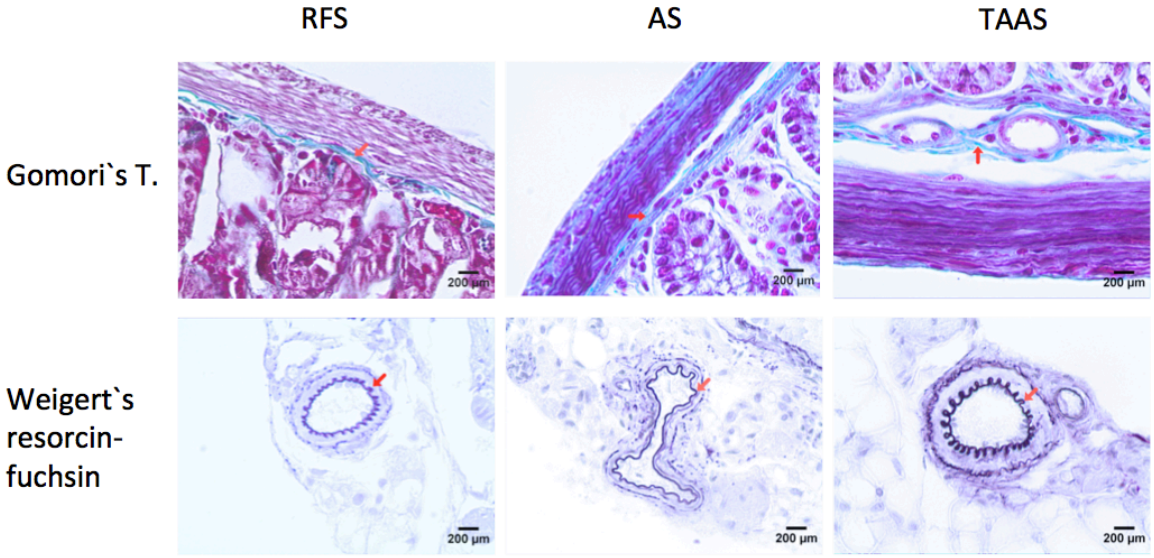
Figure 7: Quantification of nuclei count (cell) and area fraction of heart (A and B), brain (C and D), intestine (E and F) and kidney (G and H). Black column for tissues fixed with regular formalin solution (RFS), stripped column for alcohol solution (AS) and gray column for tannic acid alcoholic solution (TAAS). Different letters indicate statistical significant differences.



3.3.3 - Extracellular stability

The extracellular stability was analyzed by identification and quantification of elastin and collagen on intestine tissues. The slides were prepared for Gomori's trichrome and Weigert's resorcin-fuchsin stains after previous oxidation with oxone to analyze collagen (green color) and elastin fibers (black color) respectively (Figure 8).

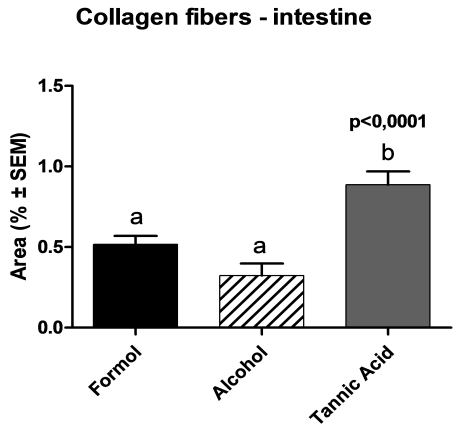
Figure 8: Representative images of intestine fixed in three different fixatives solutions (Left= Regular formalin solution - RFS; Middle= Alcohol solution - AS; and right= Tannic acid alcoholic solution - TAAS) prepared by Gomori's trichrome stain (superior line) and Weigert's resorcin-fuchsin stain (inferior line) (Bars = 100 µm). Arrows: red for collagen and elastin in Gomori's trichrome and Weigert's resorcin-fuchsin stain respectively.



From: De Paula, R. C. (2013).

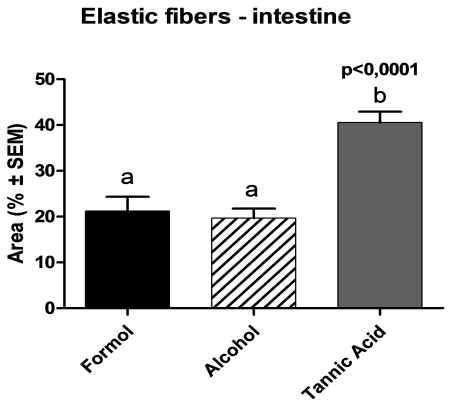
After histochemical stains analysis for collagen and elastin, the quantification using binary images was carried out. The quantification of collagen fibers on intestine showed that TAAS was better to stabilize it when compared to other fixatives (p value <0,0001), while between RFS and AS there was no statistical significant difference (Figure 9).

Figure 9: Quantification of collagen fibers in rat intestine. Black column for intestine sample fixed with regular formalin solution (RFS), stripped column for alcohol solution (AS) and gray column for Tannic acid alcoholic solution (TAAS). Different letters indicate statistical significant differences.



The quantification of elastin was also performed by binary image, as described before. The intestine showed better results for TAAS preserved specimens, while tissue fixed by RFS and AS showed no statistical difference to each other (Figure 10).

Figure 10: Quantification of elastin fibers in intestine tissues**.** Black column for tissues fixed with regular formalin solution (RFS), stripped column for alcohol solution (AS) and gray column for Tannic acid alcoholic solution (TAAS). Different letters indicate statistical significant differences.



3.4 Discussion

Even when other reagents kept appearing in more fixative formulas, formaldehyde remains as the “gold standard” fixative compound for routine work (FOX et al., 1985; BUESA, 2008; MOELANS et al., 2011). This happens due to a general consensus that formalin was the best fixative and there was therefore no need for improvement for any reason, generating what has been defined by some authors as “the formalin dogma”, a somewhat fatalistic and “addictive” approach that has severely hampered the search for alternatives to formalin in fixation procedures (ZANINI et al., 2012). Nowadays, many companies and researchers around the world have been concerned about the use of this toxic substance, as well as the large potential market involved for any substitute, leading the search to develop some new fixatives all aimed at substituting formalin (BUESA, 2008; VAN ESSEN et al., 2010; ZANINI et al., 2012).

The TA compound has been utilized to stabilize elastin (ISENBURG; SIMIONESCU; VYAVAHARE, 2004; 2005) and collagen (KRISHNAMOORTHY et al., 2012) in cardiovascular implants (ISENBURG; SIMIONESCU; VYAVAHARE, 2004) and vascular grafts (CHUANG et al., 2009) in association of glutaraldehyde. The use of this solution as fixative as proposed on this work, the TAAS, appears as an alternative less toxic than others. In fact, a study “in silico” developed in this laboratory did not show any potential toxic effect of TAAS (manuscript in preparation).

In the same manner, the results presented here shows that TAAS seems to be able to stabilize tissue degradation equal or better than RFS and AS. Schubert Werner (1990) was the first to propose the use of TA (in a hot water solution - 100°C), with or without polyethylene glycol for a rapid fixation on DE 3824936 A1 (patent deposited). Our group tested a similar solution, diluting TA in 37°C water (with high difficult) () at room temperature, but this solution was not able to preserve kidney tissues (data not shown).

Then, the TAAS was tested in a different solution. Since several authors have utilized the alcohol as main component of alternatives solutions, it has been used in the present work as well (BUESA, 2008; VAN ESSEN et al., 2010; MOELANS et al., 2011; ZANINI et al., 2012).

As other alternative fixed specimens analyzed by another authors (MOELANS et al., 2011; ZANINI et al., 2012), the TAAS as an new alternative solution showed the same capability to preserve tissues, preserving particular parameters (as nuclei detail and extracellular matrix), as well as allowing basic (H&E) and special (Gomori's trichrome and Weigert's resorcin-fuchsin) staining.

The use of pure ethanol for fixation usually causes a significative shrinkage at the edges of the specimens, together with some nuclear artifacts (TOLOSA et al., 2003), which could compromise the correct evaluation of the tissue. In fact, Buesa (2008) (BUESA, 2008) verified that when ethanol is used for small biopsies, the shrinkage of the tissue could affect the diagnosis. Due to these consequences of alcohol solution observed before in the literature, the nuclei count and area fraction have been determined to analyze the integrity of specimens. An excellent result was obtained by nuclei count, when TAAS fixed specimens of brain and intestine showed better results compared to other fixative solutions. No statistical difference has been found in heart, and kidneys were the unique tissue, in which TAAS showed no superiority than other fixative solutions. Even prepared with alcohol solution, the TAAS appears not to alter significantly the area of tissue. These results could be explained mainly by the relationship of tannic acid with extracellular matrix components, stabilizing them to keep the area of the tissue. It is an important outcome because the main problem of fixation using formaldehyde free solution is represented by the modifications on tissues that alter their morphological aspect, when compared to which is routinely considered as standard (MOELANS et al., 2011; ZANINI et al., 2012).

The modification of color generally is used to control the process of fixation (ZANINI et al., 2012). In general, alcohol-based fixatives do not change color as formalin does. The TAAS showed a high affinity as any alcohol-based solution, for dyes to the section, especially Eosin. It happens because alcohol do not alter respiratory enzymes and oxygen-carrier proteins, such as hemoglobin and myoglobin, which means that color fading, like induced by formalin action on tissues (LYKIDIS et al., 2007; ZANINI et al., 2012).

Moelans et al. (2011), compared qualitative and quantitative results for some alternative fixatives, such as: F-Solv (Adamas, Rhenen, Netherlands), FineFIX (Milestone, Bergamo, Italy) and RCL2 (Alphelys, Plaisir, France). Among the fixatives compared, RCL2 matches the acid characteristics of TAAS because it is also

an acidic solution (pH 3.10) as TAAS. The dilution of the TAAS has naturally pH 5.5 (not adjusted). Isenburg et al. (2004), assert that these stabilizing properties of the matrix components (collagen and elastin) of TA are optimized when the dilution pH is 5.5. The RCL2 utilize acetic acid as complement of a carbohydrate complex on alcohol-based solution (ZANINI et al., 2012). Moelans et al. (2011), reported some difficulties in handling and sectioning samples fixed by RCL2, and despite the similar aspects and features of tissues fixed by it solution and TAAS, this difficult was absent in TAAS fixed fragments. .

Studies are also under development to verify if it could be worth as a preservative in studies using immunohistochemistry and molecular biology techniques, to know if the DNA and RNA are also preserved.

The alternative solution proposed in the present study seems to be less toxic than all alternatives “formaldehyde free” solutions available, and to better confirm this assumption toxicological studies are still under development in our laboratory. Moreover, the results presented here show a high capability to preserve tissues with equal or superior aspects than even formalin and alcohol solution, mainly due to stabilization of elastin and collagen.

In conclusion TAAS seems to be a powerful and feasible alternative solution for fixing samples for microscopical analysis.

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4 COULD AN ALTERNATIVE SOLUTION BE BETTER THAN FORMALIN FOR MACROSCOPIC SPECIMENS?

Abstract

The most famous pedagogic tool to teach anatomy is the dissection. It trains the individual in spatial appreciation and orientation by the psychomotor activity and still is considered the best method for acquisition of medical skills and experience. The dissection occurs mainly by formalin fixed cadavers, which, as known, is a very toxic solution and irritant. Several alternative solutions appeared in the last decades, but most of them are only for microscopic specimens. The macroscopic environment remains needing a new alternative capable to replace the use of formaldehyde. Since the knowledge of tannic acid alcoholic solution (TAAS) to preserve microscopic specimens, with powerful capability to stabilize extracellular components, as elastin and collagen, it has been proposed in this study as a new fixative solution for macroscopic studies as well. Whole wistar rats were fixed in the tannic acid alcohol-based solution or formalin and medicine students, who practice dissection for at least 2 or more years, dissected the rats during the dissection course, as an alternative to acquire basic surgery skills and answered a detailed questionnaire about the quality of dissected specimen. The toxicity of the compound was analyzed by “in silico” tests for cutis and respiratory system. The results showed a higher scores for TAAS compared to 10% v/v regular formalin solution (RFS) in several parameters, as odor, texture and flexibility. The RFS appeared to be preferred only concerning the color parameter. The toxicity of TAAS for skin and respiratory system was considered moderate in relation to RFS, which is considered highly toxic. In conclusion, TAAS seems to be as good for macroscopic as for microscopy fixation. It is less toxic than formaldehyde and odorless. Therefore, it could be a real alternative for the use of RFS, decreasing risk factors to the ones manipulating fixed corpses and tissues.

Keywords: Alternative solution. Dissection. Formaldehyde. Tannic acid. Toxicity.

4.1 Introduction

Nowadays, the most commonly employed pedagogy for anatomical classes consists of didactic lectures, which discuss anatomical structure and function, as well as their clinical importance, while students navigate around the body with clinical photographs and computerized animations (SUDANG et al., 2009). Recently, several alternative pedagogical tools appeared, from ludic games to new 3D softwares (LIU et al., 2013; MULLER-STICH et al., 2013). Despite this, dissection still seems to be the best method of teaching anatomy, providing optimal examples for both students and teachers (PABST, 2002; HISLEY et al., 2008; HOLLA et al., 2009; PETERSSON et al., 2009; BOCKERS et al., 2010). Moreover, the dissection of cadavers trains the individual in spatial appreciation and orientation by the psychomotor activity, as well as cognitive development, which is essential for acquisition of medical skills and experience (SAEED; RUFAI; ELSAYED, 2001).

The dissection is made mainly in embalmed cadavers, commonly using a regular 10% v/v formalin solution - RFS (SILVA, R. M.; MATERA; RIBEIRO, 2007). This solution is based on aqueous solution of formaldehyde, which is the most famous fixative, used in more than 80% of US and UK histology laboratories, and in as much as 65% of them in other parts of the world (BUESA, 2008).

However, the formaldehyde is classified as carcinogenic (group 1) by the International Agency for Research on Cancer and, therefore, represents a risk to anyone handling the solution (OCHS et al., 2012). To minimize the exposure to formaldehyde vapors from embalmed cadavers, generally ambient air exchange is utilized, such as: downdraft airflow (directed away from the worker), or by air filtrationwith facemasks worn by workers near the cadavers. An alternative practice is to change chemicals, either perfusing bodies with a less noxious fixative that contains less (or no) formaldehyde or converting formaldehyde in the embalmed cadaver to a less noxious compound by addition of a ‘‘formaldehyde-inactivating’’ agent before dissection (WHITEHEAD; SAVOIA, 2008). Then, the quest for alternative fixative solutions become increasingly important day by day, specially for gross anatomy laboratories, in which normally the large area does not always allow adequate

exhaustion as microscopy labs do, exposing their users to the toxic fumes of the fixatives (ZANINI et al., 2012).

Several researches and companies leaded by this necessity, developed some alternative solutions, mainly including: fixatives containing alcoholic and nonalcoholic solutions, with or without acetic acid (MOELANS et al., 2011), zinc-based solution (LYKIDIS et al., 2007), glyoxale-based solution (ZANINI et al., 2012) and less than 10% formalin solution (BUESA, 2008; VAN ESSEN et al., 2010). Moreover, most of these alternatives still have some amounts of toxic substances, as well as were developed and tested to microscopic specimens. Few alternative solutions are commonly used to preserve macroscopic specimens, and these are composed principally of: glycerol, phenol, acetic acid and alcohol (RODRIGUES, 2010; MOELANS et al., 2011). There are also some kits, that may include or not some compounds described above, like Thiel`s solution (BENKHADRA et al., 2011), modified Larssen (SILVA, R. M. G.; MATERA; RIBEIRO, 2004) and Laskowski (RODRIGUES, 2010) solutions.

On our previous work, we described a new fixative kit, containing tannic acid as main compound. It is an alcohol-based solution, and showed a good capability to preserve several histological specimens for light microscopic, mainly by stabilizing elastin and collagen. Hence, the aim of this work is to evaluate the suitability of this kit solution to preserve entire body specimens (rats), as well as to allow dissection procedures, due to the shortage of fixatives with this purpose.

4.2 Material and Methods

4.2.1 Materials:

The TA of highest purity available was obtained from Sigma-Aldrich Co. The 4% formaldehyde buffered solution was bought from Merck-Millipore Co.

4.2.2 Animals:

The study design and experimental protocols were approved by the Animal Care and Use Committee of the University of Sao Paulo (protocol number 2851/2012), which is in accordance with the Guide for the Care and Use of Laboratory Animals. The experiments were performed according to guidelines of the Brazilian College for Animal Experimentation (COBEA). Sixteen Wistar rats (350-460g) were utilized as experimental model for gross dissection and obtained from laboratory animal facility of Fluminense Federal University. The euthanasia was accomplished by intraperitoneal injection of Xilazine (10 mg/kg) and Ketamine (80 mg/kg).

4.2.3 Experimental design

Immediately after death (n=8 per group), the two fixatives solutions, regular formalin (RF-10%) and 300 mL Tannic acid alcohol-based solution (TAAS-0.25%) were perfused via the left ventricle, using a peristaltic pump (60 – 80 mm/Hg). After injection, both groups were kept immersed on each fixative solution for 30 days, at room temperature, before gross dissection.

4.2.4 Analyzed parameters of dissection

A blinded evaluation of fixed specimens was carried out by fourteen medical students, who have been dissecting for 2 or more years, twice a week at Fluminense Federal University dissection program. A secret code was created to determine the specific

chemical preserved specimen, and to be sure about the blind evaluation, as follows: A= RF specimens and B=TAAS specimens. The following aspects were analyzed in each chemically preserved specimen (modified by (SILVA, R. M.; MATERA; RIBEIRO, 2007): odor, color and texture of tissues; as well as flexibility of joints and skin.

The evaluation process was based on detailed questionnaire answered by the students (Appendix 1). The quality for these aspects was graded as 0 (inadequate for dissection), 1 (reasonable quality for dissection, but adjustments in protocol are needed), or 2 (good quality for dissection).

4.2.5 *In silico* assessment

The *in silico* assessment of genotoxic effects were performed using the ACD / Labs I-Lab 2.0 server. The cutaneous and respiratory study was performed using the PredictorTM ADMET software (Simulations Plus Inc., Lancaster, CA, USA). Both programs use libraries of molecules including chemical structural information (2D or 3D) and experimental data to create statistical models that are used to predict the properties of the studied molecules as well as their toxicity.

The cutaneous toxicity: The program interprets as sensitizer compound or substance, the one, which induces allergic skin reactions in mice. A mathematical model is implemented to predict qualitatively the molecule that has the potential to induce cutaneous allergic reactions, and is based on a database of 298 molecules tested experimentally by the local lymph node murine assay (LLNA), which has been a recommended method and validated for the determination of relative potency of a chemical compound to be irritating. The end point of LLNA is the EC3, which is the estimated chemical needed to produce a 3-fold stimulation of draining lymph node cell proliferation in rats compared with controls, being used to divide concentration in classes and non-sensitizing compounds sensitizers. Compounds with EC3 lower or equal to 10 % and sensitizers are considered as sensitizer, and those above 10% as non-sensitizers. For this evaluation we used the TOX_SKIN program module.

The respiratory toxicity: The mathematical model implemented to qualitatively predict whether the molecule is toxic when inhaled was based on a library containing 314 molecules identified by inhalation studies with rats as sensitizers or not. Most molecules were obtained from AOEC (Association of Occupational and Environmental Clinics). For this evaluation was used the TOX_RESP program module.

4.2.6 Data Analysis

Results are expressed as means of percentages obtained by detailed answered questionnaires.

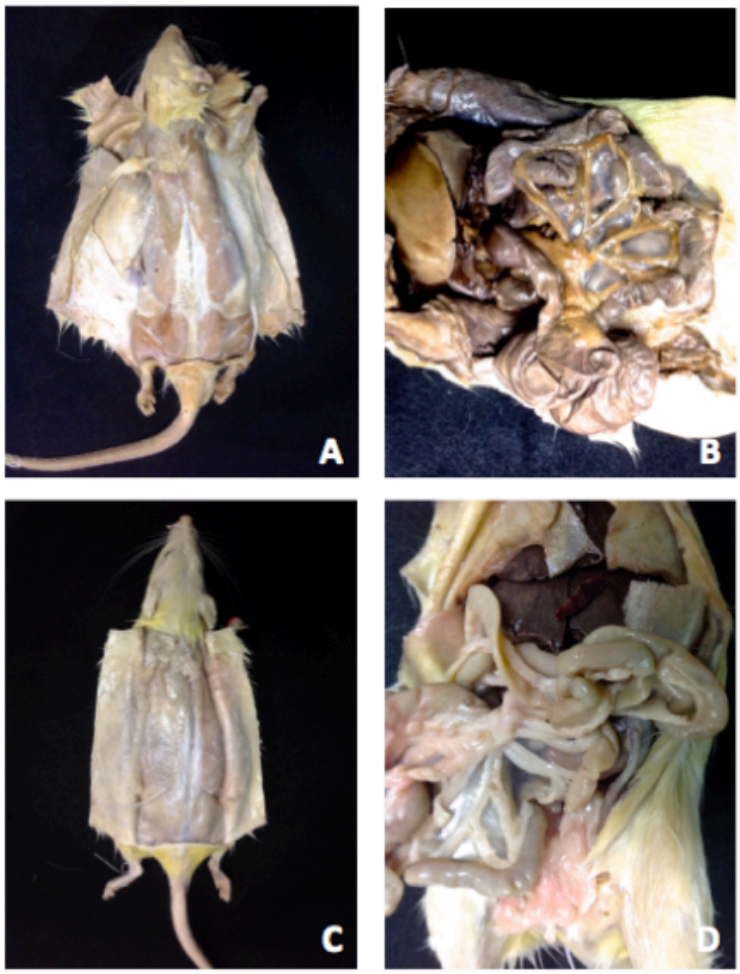
4.3 Results

4.3.1 Macroscopic analysis

The macroscopic analysis was acquired by qualitative and quantitative study, by visual observation and gross dissection respectively, being the quantitative results accessed by a blind evaluation. The parameters selected for analysis (color, texture, odor and flexibility) were unanimously considered important for dissection procedures by all respondents.

The color of chemical preserved specimens appeared different for skin, muscle and organs as expected, with TAAS darker than RFS (Figure 11). The texture of TAAS specimens seems softer and more flexible, different from RFS ones. The odor was absent in TAAS preserved specimens. All qualitative parameters described above were classified as good, based on the grade created and described before.

Figure 11: Photograph of chemical preserved specimens fixed with TAAS (A and B) and RFS (C and D).



From: De Paula, R. C. (2013).

The results exhibited a superiority of TAAS, when compared to formaldehyde, for some relevant aspects for dissection. The TAAS texture, very significant for incision and division procedures, was classified by 71,42% of responders as good for dissection, against 64,28%, who classified the texture of RFS specimens as reasonable for dissection, needing adjustments. The odor and flexibility were classified as good for dissection by 92,85% and 85,71% of respondents, whereas 42,85% and 35,71% of them thought that RFS need adjustments for these aspects (Table 1).

Table 1: Summary of quantitative results classified as, 0 (inadequate for dissection), 1 (reasonable quality for dissection, but adjustments in protocol are needed), or 2 (good quality for dissection).

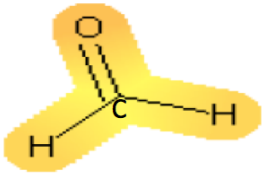
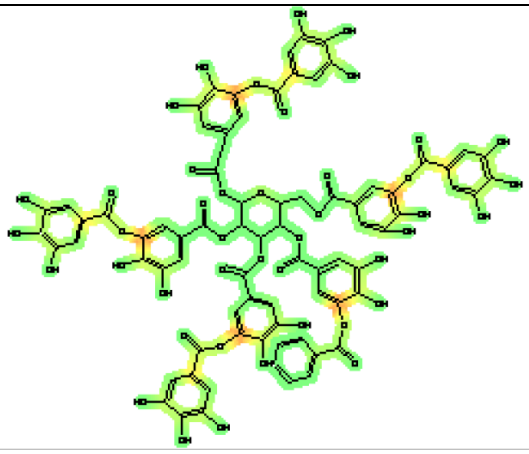
Fixative solution	**Color (%)**			**Texture (%)**			**Odor (%)**			**Flexibility (%)**		
	0	**1**	**2**	**0**	**1**	**2**	**0**	**1**	**2**	**0**	**1**	**2**
RFS	14	32	50	21	64	14	57	42		64	35	
TAAS	7	50	42		28	71		7	92		14	85

4.3.2 *In silico* assessment

a) Genotoxic effect

The mathematical model implemented to predict whether the molecule has mutagenic potential is based on a database of 8607 molecules experimentally tested by the Ames test, obtained the CCRIS (Chemical Carcinogenesis Research Information) and GENE-TOX (Genetic Toxicology Data Bank). The design of 2D structure of tannic acid and formaldehyde in the model showed that formaldehyde has a 62% probability of being mutagenic and tannic acid only 3% (Figure 12).

Table 2: Chemical structure and mutagenicity probability of formaldehyde and tannic acid. Yellow: chemical groups engaged with the mutagenic action.

Compound	Chemical structure	Mutagenicity
Formaldehyde		62%
Tannic Acid		3%

b) Cutaneous and respiratory toxicity

The molecules of formaldehyde and tannic acid were submitted to mathematical models mentioned above, with the prediction of skin and respiratory toxicity of formaldehyde 83 and 96% respectively, which indicates high respiratory toxicity as expected. The result of cutaneous toxicity of formaldehyde demonstrated to be non-sensitizer. For tannic acid, predicted cutaneous and respiratory toxicity was 42 and 36% respectively, indicating that this molecule has a moderate probability of be toxic for both cutaneous and respiratory ways (Figure 12).

4.4 Discussion

It is not news to those involved with the use of formaldehyde, as well as government associations about the danger of this substance. Nielsen (2010) (NIELSEN; WOLKOFF, 2010) (NIELSEN; WOLKOFF, 2010) reported the genotoxic effects of

formaldehyde in workers exposed to this substance. Its high solubility in water leads a rapid absorption by the respiratory and gastrointestinal tract, causing nasal tumors in rodents (MERK; SPEIT, 1998). Even with this disclosure of the hazard and toxicity of formaldehyde, as well as the emergence of alternative kits, RFS appears to remain as the main solution used for this purpose (ZANINI et al., 2012).

Principally on last decade, several alternative fixative solutions appeared (BUESA, 2008; MOELANS et al., 2011; ZANINI et al., 2012). Unfortunately, most of its alternatives have been tested only for histological specimens. However, the gross anatomy remains in need of alternative solutions, since the existing majority consists of kits containing other toxic elements (RODRIGUES, 2010).

TA has characteristics compatible for the microscopic and macroscopic fixation purposes, which lead us to test it for both. For microscopic ones, it showed a good capability to preserve tissues, mainly to preserve the extracellular components of these fragments, as elastin and collagen, and was considered even better than RFS (as showed on previous work). In the present work, the TAAS showed capability to preserve macroscopic specimens as well, being odorless and preserving even the flexibility of tissues of entire specimens, allowing and enhancing the dissection procedures.
The use of TA in association of glutaraldehyde is common for ultrastructural analyses of elastin fibers under electron microscopy (HAIDAR et al., 1992), and more recently for bioprothetic heart valves (ISENBURG; SIMIONESCU; VYAVAHARE, 2004) and vascular grafts (CHUANG et al., 2009), mainly by its properties to form multiple bonds extracellular compounds, such as elastin and collagen (ISENBURG; SIMIONESCU; VYAVAHARE, 2005; CHUANG et al., 2009; KRISHNAMOORTHY et al., 2012).

The knowledge of TA properties to preserve tissues comes from ancient Egypt, where methylgallate and inositols were identified in different parts of a 40-year old body of an unknown Egyptian mummy #90001255 (100BC) from the Guimet Natural History Museum (Lyon, France), indicating the general use of vegetable tannins for mummification (SAEED; RUFAI; ELSAYED, 2001).

Some features were analyzed to test TAAS to maintain entire specimens, and only the

color seems to be a disadvantage in comparison with formalin. The modification of color is generally used to control the process of fixation, but we should pay attention that alternative fixatives do not change color as formalin does (ZANINI et al., 2012). Then, the change in color may not be interpreted as negative, since the observed color is closer to alive specimens, which can be considered better for surgical exercises.

All other parameters appear better than formalin, including flexibility that is one of the most important parameters for dissection. The specimens showed a perfect condition of flexibility on skin and joints, keeping the “life like” appearance of them. This type of fixation that maintain the flexibility can be acquired with other fixative kits, as: Thiel`s solution (BENKHADRA et al., 2011), modified Larssen (SILVA, R. M. G.; MATERA; RIBEIRO, 2004) and Laskowski (RODRIGUES, 2010); (ISENBURG; SIMIONESCU; VYAVAHARE, 2005) solutions. Benkhadra (2011) (BENKHADRA et al., 2011) studied cadaveric fragments fixed in Thiel`s solution, but did not observe any modification of collagen in either muscle or tendon fibers that may explain the flexibility. The TAAS has the capacity to stabilize not only collagen, but elastin as well (as presented before), which may be the answer for this good flexibility and texture. Even the known modified Larssen and Laskowski have divergences on flexibility, which were shown by Silva et al (2007) (SILVA, R. M.; MATERA; RIBEIRO, 2007).

Beyond the mentioned parameters, the main important report consists in the real analysis of toxicity. Despite several alternative solutions being more famous nowadays, few or even none of them has been critically analyzed. The toxicity of skin and respiratory tract is probably absent in TAAS. This is important mainly for dissection rooms that commonly do not have enough fume hoods. Moreover, the use of just one compound diluted in alcohol should make this solution cheaper than other alternatives, which generally use a lot of secondary components.

4.5. Conclusion

Owing the number of positive characteristics of this fixative, TAAS seems to be as good for macroscopic as for microscopy. It is less toxic than formaldehyde and odorless. Therefore, could be a real alternative for the use of RFS, decreasing risk factors to the ones manipulating fixed corpses and tissues.

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APPENDIX (questionnaire):

Data assessment

Subject: Dissection Program (College of Medicine, Fluminense Federal University)

Name:

E-mail:

The quality for each aspect is: 0 (inadequate for dissection), 1 (reasonable quality for dissection, but adjustments in protocol are needed), or 2 (good quality for dissection).. Please, answer the objective following questions based on this grades.

1- On your opinion, the color of fixed tissues contributes for the dissection process?

(Yes)

(Not)

2- The color of chemically preserved specimens.

Group A= (0) (1) (2)

Group B= (0) (1) (2)

3- The texture of skin and internal disposition of organs in chemically preserved specimens:

Group A= (0) (1) (2)

Group B= (0) (1) (2)

4- Did you feel any odor of chemical compound during dissection?

(Yes)

(Not)

5- The odor in chemically preserved specimens during dissection:

Group A= (0) (1) (2)

Group B= (0) (1) (2)

6- Does the flexibility help the dissectionin your opinion? Why?

(Yes)

(Not)

7- Flexibility of joints and skin:

Group A= (0) (1) (2)

Group B= (0) (1) (2)

8- Are you in favor or not of the use of cadavers for surgical technique teaching?

(A) in favor

(B) against

9. Point out the pros and contras of both fixative methods:

10. What type of chemically preserved specimen did you preferred for dissection?

(A)

(B)

5 CONCLUSÃO GERAL

A solução alternativa proposta no presente estudo aparenta ser menos tóxica que todas as soluções alternativas “livres de formaldeído” disponíveis, além de ser inodora, diminuindo os fatores de risco para os indivíduos que manipulam tecidos biológicos fixados com esta solução.

Além disso, os resultados aqui apresentados mostram uma alta capacidade de preservar os tecidos biológicos com aspectos iguais ou superiores a formalina, assim como ao álcool, principalmente devido à estabilização de elastina e colágeno.

Em conclusão, a solução fixadora de acido tânico a base álcool parece ser uma potente e viável solução alternativa para a fixação de amostras microscópicas, assim como macroscópicas.