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Effectiveness of embalming solutions with partial replacement of formaldehyde and phenol with ethanol and sodium chloride on mice over 18 months

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Original Article

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Abstract

Animal preservation for anatomy teaching has been required to replace formaldehyde and phenol which represent a health hazard to humans. A comparative study of embalming solutions with partial replacement of formaldehyde and phenol with sodium chloride and ethanol was evaluated in mouse cadavers over an 18-month period. Twelve adult mouse carcasses were divided into six groups of two mice each. After opening the mouse's skin, the internal organs were removed and preserved with the remaining cadavers by freezing (Group I, control group) or in the respective embalming solution (Groups II to VI) and examined after 3, 6, 12 and 18 months. For the embalming solution, the formaldehyde concentration was reduced from 18% (Group II) to 0.5% (Groups III and IV), 0.1% (Group V), and 0.05% (Group VI), whereas the phenol and glycerol concentration was reduced from 2.5% and 5% (Group II) to 0.8% and 2%, (Groups III-VI), respectively. In replacement, 15% table salt/sea salt and 16% ethanol were included in Groups III and IV. The carcasses and internal organs were blind evaluated by 10 students. The evaluations were compared and analyzed between Groups and against Group 1 using ANOVA. Results show that embalmed mice in Groups III and IV retained a good color and flexibility, without decomposition or fungal growth throughout study. In contrast, mice in Groups V and VI were unsuitable at 3 months. We concluded that a suitable embalming fluid for small animal preservation was 0.5% formaldehyde, 0.8% phenol, 2% glycerol, 16% ethanol and 15% sodium chloride.

Keywords: embalming solution, ethanol, formaldehyde, mice, phenol, sodium chloride

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Introduction

Nowadays, in addition to human cadavers, various other animals of differing sizes, such as cattle, goats, sheep, pigs, chickens, dogs, horses, mice and rats, are used in gross anatomy teaching. There are several alternative methods for the preparation of these animals (Guimaraes da Silva *et al*., 2004), including the use of fresh specimens, frozen fresh specimens or preservation in embalming solution. A problem arises from the logistics of having animal samples available for anatomy classes each day and the long time requirement per animal to practise dissection skills. This is especially true for larger animal specimens that must be repeatedly returned to successive classes several times. This makes learning from a fresh specimen impractical due to its decomposition in the warm laboratory environment and the limited supply of such specimens. Freezing fresh specimens at -25 °C in-between dissection sessions is also unlikely to be practical, due to the lead time required to thaw the specimen before use and the potentially limited freezer space. Thus, the more usual approach is to keep the animals in an embalming solution.

Although traditional embalming solutions keep the specimen in a good condition, suitable for gross anatomy teaching, they suffer from the drawback of requiring time to initially preserve and maintain the animals in a satisfactory condition for the study of gross anatomy over a long period of time (Coleman and Kogan, 1998). However, more important is that the formaldehyde (a water solvated form of formalin) and phenol in traditional embalming fluids represent a health hazard. Thus, it is desirable to reduce the risk of exposure to formalin vapors, which are classified as a carcinogen (Albert *et al*., 1982), having a strong smell and causing acute irritation to the membranes, such as the eyes, nose and respiratory tract (Papst, 1987; Gardner *et al*., 1993.), as well as leading to tissues with a strong odor, discoloration and lack of flexibility (Tschernezky, 1984).

Although alcohols, such as 70-100% (v/v) ethanol, can preserve the tissues from degradation, they are unsuitable because of causing dehydration and leading to a loss of flexibility, texture and color in the tissues. Indeed, any replacement embalming solution will be required to prevent marked tissue shrinkage and distortion and inhibiting the growth of various microorganisms and their spread to other specimens and workers. It should also maintain the tissue color and texture, not to be too dry, and allow the elasticity of skin tissue and muscle texture to be as natural as possible and not too hard. There should also be an absence of putrefaction, including rotten odors.

Danvivathana *et al*., (2006) tried to reduce the proportion of formaldehyde by about 50% by introducing table salt as a substitute, and reported that the reduced formaldehyde content in the presence of salt at 0.5-0.75% (w/v) gave satisfactory results when preserving rats, in accord with that reported for preserving human cadavers (Coleman and Kogan, 1998) for gross anatomy studies.

This study comparatively evaluated the preservation of mice for up to 18 months in different embalming solutions which aimed to partially replace the formaldehyde (three reduced levels) and phenol (one reduced level) content with or without 15% (w/v) sodium choride (using table salt and sea salt) and 16% (v/v) ethanol, in terms of their suitability for gross anatomy teaching.

Materials and Methods

Animals and preservation: The carcasses of 12 healthy adult mice (*Mus musculus*) of mixed sexes and an average weight of 20-35 grams were donated by the Laboratory Animal Unit, Faculty of Veterinary Science, Chulalongkorn University. The mice were divided into six groups of two mice each. For each mouse, the skin was cut open on the thorax and abdomen and then the lungs, heart, stomach, urinary bladder, pancreas, spleen, liver and kidneys were removed and preserved together with the residual carcass by either freezing at -25 ^{o}C (Group I, control group) or in one of five embalming solutions (Groups II to VI) at 2,500 mL solution/each group of mice, as shown in Table 1, for 18 months.

Table 1 Embalming solution composition per 2.5 L for Groups II-VI

	37-40% Formaldehyde (mL)	Glycerol (mL)	Phenol (mL)	Ethanol (mL)	Tablet salt (g)	Sea salt (g)
II (Standard)	450 (18%)	125 (5 %)	62.5 (2.5%)	-	-	-
III	12.5 (0.5%)	50 (2%)	20 (0.8%)	400 (16 %)	375 (15%)	-
IV	12.5 (0.5 %)	50 (2%)	20 (0.8%)	400 (16 %)	-	375 (15%)
V	2.5 (0.1 %)	50 (2%)	20 (0.8%)	-	-	375 (15 %)
VI	1.25 (0.05 %)	50 (2%)	20 (0.8%)	-	-	375 (15 %)

Group I (reference control) mice were not embalmed but frozen.

Analysis of the ability of embalming solution to preserve the mouse cadavers and organs: The carcasses and internal organs were evaluated in a blind fashion by 10 students in a gross anatomy class after 3, 6, 12 and 18 months for their physical characteristics of smell (odor), tissue color, surface flexibility and the degree of hair loss from the skin in a skin slip assay. The evaluation scores were compared with Group I, the control group, which was frozen in-between examinations but brought to room temperature before examination.

The smell and rate of decay was determined and scored as 0-100% (More than 50 percent was decay).

With respect to the loss of hair from the skin, a skin slip test was performed by peeling off skin and hair loss from the dermis. The results were then categorized between 1 and 5, where 5, 4, 3, 2 and 1 represent 0%, less than 25%, 25-50%, 51-99%, and 100% hair loss from the skin, respectively.

The color of the organs and the structures inside the abdomen (fat, blood vessels and nerves) were scored on a three-point scale relative to that for the Group I control, where 3 was the color of the control, 2 was lighter than 3 but darker than 1, and 1 was lighter than 3 and 2.

Finally, the elasticity of the skin and contact surface of the muscles and organs were determined on a three-point scale, by pressing down on the two muscles of the leg, the lateral head and long head of the triceps brachii muscles, and the legs after the bicep femoris and semitendinosus. In addition, the surface of each organ was also scored. The three-point score was ascribed as, where is close to that of the Group I control, is less elastic but still soft and flexible, and is not flexible and solid. The results are summarized as the mean score in Table 4 for scores 3, 2, and 1, respectively.

Data analysis: The ascribed scores from each student were averaged and are presented as a mean ± 1SD. Significant differences in the average mean score between the Group I control and each treatment Group was evaluated by ANOVA, accepting significance at the $P < 0.05$ level.

Results

Odor: The smell of the rotting carcasses was recorded and analyzed as a decay rate (0-100%), with the average decay rate (%) shown in Table 2.

The frozen control (Group I) had no detectable bad (putrefying) odor and a 0% average decay at all assayed time points up to 18 months, as did the standard formaldehyde (18%) and phenol (2.5%) rich embalming fluid (Group II) as expected. However, of interest was the fact Groups III and IV, with the lower formaldehyde (0.5%) and phenol (0.8%) concentration but with ethanol (16%) and sodium chloride (15%; table or sea salt), also showed no smell or rate of decay over the 18 months. In contrast, Groups V and VI without ethanol and sodium chloride but with even lower formaldehyde (0.05-0.1%) levels exhibited a bad odor and decay at 3 months and were unsuitable (too badly decayed) at 6 months and disposed of.

Peeling of skin and hair loss from the dermis: The average hair loss score (on the five-point scale) is summarized in Table 2, and was numerically lower in all treatments compared to the Group I control but this was only significant for Groups III to VI at 3 months onwards and for Group I from 12 months onwards. Groups III and IV were significantly lower than Group II at all assayed time points, but were still less than 25% hair loss, which is typically deemed acceptable for gross anatomy (< 50% hair loss). Groups V and VI, with the putrefied samples, showed a hair loss of more than 50% at 3 months.

Table 2 Evaluation of the degree of degradation of mouse carcasses and organs and the levels of hair shed.

Group	Nature of smell (%)				Hair loss from the mouse's skin in the Skin slip test			
	3 month	6 month	12 month	18 month	3 month	6 month	12 month	18 month
I	0%^{a}	0%^{a}	0%^{a}	0%^{a}	5.0^{a}	5.0^{a}	5.0^{a}	5.0^{a}
II	0%^{a}	0%^{a}	0%^{a}	0%^{a}	4.9^{a}	4.8^{a}	4.8^{b}	4.7^{b}
III	0%^{a}	0%^{a}	0%^{a}	0%^{a}	4.2^{b}	4.2^{b}	4.3^{c}	4.0^{c}
IV	0%^{ab}	0%^{a}	0%^{a}	0%^{a}	4.3^{b}	4.2^{b}	4.1^{c}	4.4^{bc}
V	30%^{bc}	60%^{b}	NA	NA	2.6^{c}	2.4^{c}	NA	NA
VI	50%^{c}	80%^{b}	NA	NA	2.3^{c}	1.6^{e}	NA	NA

NA:not applicable

Different superscripts in a column indicate significant differences across the groups ($P < 0.05$).

Color of the organs and structures inside the abdomen: The color of the removed internal organs (lungs, heart, stomach, urinary bladder, pancreas, spleen and kidneys) and structures inside the abdomen (fat, blood vessels and nerves) were evaluated on a three-point scale, with the average values summarized in Table 3.

The results revealed that the color of all the examined excised organs and structures inside the cadavers had significantly faded in all the embalming fluids (Groups II to VI) compared to the frozen tissue control (Group I), with scores of 1.5-2.3 in Groups II, III and IV throughout the 18-month period. However, overall, the color in Groups II and IV (reduced formaldehyde and phenol level) was not significantly different than that in Group II (the standard embalming fluid) at all examined time points.

Elasticity of the skin, muscles and organs: The elasticity of the muscles in each cadaver and of the excised organs was evaluated on a three-point scale, where 3 is close to that of the Group I control, 2 is less elastic but still soft and flexible, and 1 is not flexible and solid. The results are summarized as the mean score in Table 4.

The results revealed that the elasticity of the surface of the muscles had an average score of 1.5-2.4 in Groups II, III, and IV throughout the 18-month period, which was significantly lower than that for the Group I control but was still acceptably soft and flexible. As with the color, overall, the tissue flexibility in Groups III and IV (reduced formaldehyde and phenol level) was not significantly different than that in the standard embalming fluid (Group II) at all examined time points.

Table 3 Assessment of the color of various excised organs and the internal structural contents on a three-point scale where 2 is lighter than 3 and 1 is lighter than 2 and 3.

Group	Lungs				Heart				Stomach				Urinary bladder			
	3 month	6 month	12 month	18 month	3 month	6 month	12 month	18 month	3 month	6 month	12 month	18 month	3 month	6 month	12 month	18 month
I	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}
II	1.9^{b}	2.0^{b}	1.9^{b}	1.9^{b}	2.1^{b}	1.9^{b}	2.0^{b}	2.0^{b}	2.0^{b}	2.1^{b}	2.1^{b}	1.7^{b}	1.8^{b}	1.5^{b}	2.1^{b}	1.7^{b}
III	2.4^{c}	1.7^{c}	1.8^{b}	1.9^{b}	2.4^{b}	2.0^{b}	1.9^{b}	1.9^{b}	2.1^{b}	1.7^{c}	2.0^{b}	1.6^{b}	2.2^{c}	1.6^{b}	1.5^{c}	1.6^{b}
IV	2.0^{b}	1.7^{c}	1.9^{b}	1.9^{b}	2.1^{b}	1.9^{b}	1.7^{bc}	1.9^{b}	2.1^{b}	1.7^{c}	1.9^{b}	1.6^{b}	2.3^{c}	1.5^{b}	1.6^{c}	1.5^{b}
V	2.2^{bc}	1.5^{c}	NA	NA	2.2^{b}	1.6^{c}	NA	NA	1.9^{b}	1.4^{c}	NA	NA	2.2^{c}	1.1^{c}	NA	NA
VI	1.8^{b}	1.6^{c}	NA	NA	1.9^{b}	1.7^{bc}	NA	NA	1.8^{b}	1.6^{c}	NA	NA	2.2^{c}	1.1^{c}	NA	NA

Group	Pancreas				Spleen				Kidneys			
	3 month	6 month	12 month	18 month	3 month	6 month	12 month	18 month	3 month	6 month	12 month	18 month
I	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}
II	1.8^{b}	2.0^{b}	2.0^{b}	1.7^{b}	1.7^{b}	2.1^{b}	2.0^{b}	1.9^{b}	1.9^{bd}	1.8^{b}	2.0^{b}	1.8^{b}
III	2.2^{b}	1.7^{c}	1.6^{b}	1.6^{b}	1.6^{b}	1.9^{b}	2.0^{b}	1.8^{b}	2.3^{c}	1.9^{b}	1.8^{c}	1.9^{b}
IV	2.1^{b}	1.5^{cd}	1.5^{ab}	1.5^{b}	2.2^{b}	1.9^{b}	2.1^{b}	1.7^{b}	2.2^{c}	1.9^{b}	1.8^{c}	1.7^{b}
V	1.7^{b}	1.7^{c}	NA	NA	1.9^{b}	1.4^{c}	NA	NA	1.7^{b}	2.0^{b}	NA	NA
VI	1.9^{b}	1.3^{d}	NA	NA	2.0^{b}	1.6^{c}	NA	NA	2.0^{d}	1.8^{b}	NA	NA

Group	Fat				Blood vessels				Nerves			
	3 month	6 month	12 month	18 month	3 month	6 month	12 month	18 month	3 month	6 month	12 month	18 month
I	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}
II	1.8^{b}	1.7^{b}	1.9^{b}	1.7^{b}	1.7^{b}	1.6^{b}	1.7^{b}	1.8^{b}	1.5^{b}	1.5^{b}	2.0^{b}	1.5^{b}
III	1.9^{b}	1.8^{b}	1.5^{b}	1.5^{b}	1.7^{b}	1.7^{b}	1.7^{b}	1.8^{b}	1.8^{bc}	1.5^{b}	1.9^{b}	1.6^{b}
IV	1.5^{b}	1.7^{b}	1.5^{b}	1.5^{b}	1.6^{b}	1.6^{b}	1.7^{b}	1.6^{b}	1.5^{b}	1.5^{b}	1.9^{b}	1.6^{b}
V	1.8^{bc}	1.0^{c}	NA	NA	1.7^{b}	1.2^{c}	NA	NA	2.0^{c}	1.7^{b}	NA	NA
VI	1.6^{b}	1.0^{c}	NA	NA	1.2^{c}	1.3^{c}	NA	NA	1.5^{b}	1.7^{b}	NA	NA

NA:not applicable

Different superscripts in a column indicate significant differences across the groups ($P < 0.05$).

Table 4 Evaluation of flexibility (rigidity) of the contact surface (texture) of muscles and organs (the liver as an example).

Group	Muscle				Liver			
	3 month	6 month	12 month	18 month	3 month	6 month	12 month	18 month
I	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}
II	2.2^{bc}	2.1^{bc}	2.0^{b}	2.0^{b}	1.8^{c}	1.9^{b}	1.7^{b}	2.0^{b}
III	2.4^{b}	2.4^{b}	1.9^{b}	1.9^{b}	2.3^{b}	1.7^{bc}	1.5^{b}	1.5^{b}
IV	2.4^{b}	2.3^{b}	1.9^{b}	2.1^{b}	1.7^{c}	1.6^{c}	1.6^{b}	1.7^{b}
V	2.1^{bc}	2.0^{c}	NA	NA	2.0^{bc}	1.4^{c}	NA	NA
VI	1.9^{c}	1.9^{c}	NA	NA	1.7^{c}	1.3^{c}	NA	NA

Table 4 Evaluation of flexibility (rigidity) of the contact surface (texture) of muscles and organs (the liver as an example).

Group	Muscle				Liver			
	3 month	6 month	12 month	18 month	3 month	6 month	12 month	18 month
I	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}
II	2.2^{bc}	2.1^{bc}	2.0^{b}	2.0^{b}	1.8^{c}	1.9^{b}	1.7^{b}	2.0^{b}
III	2.4^{b}	2.4^{b}	1.9^{b}	1.9^{b}	2.3^{b}	1.7^{bc}	1.5^{b}	1.5^{b}
IV	2.4^{b}	2.3^{b}	1.9^{b}	2.1^{b}	1.7^{c}	1.6^{c}	1.6^{b}	1.7^{b}
V	2.1^{bc}	2.0^{c}	NA	NA	2.0^{bc}	1.4^{c}	NA	NA
VI	1.9^{c}	1.9^{c}	NA	NA	1.7^{c}	1.3^{c}	NA	NA

NA:not applicable

Different superscripts in a column indicate significant differences across the groups ($P < 0.05$).

Discussion

Formalin is a colorless, flammable gas at room temperature but is highly soluble in water and this solution (known as formaldehyde) at concentrations above 10% can be used to kill fungi and bacteria, and prevent putrefaction. In addition, it is cheap and highly effective in preserving organisms (Seiichi and Haruto, 2004), as it reversibly reacts with the amine group of proteins to form crosslinks, as well as toxic substances, (Kierman, 2005), and so is often used as the main component in embalming fluid for the preservation of animals for teaching anatomy. However, these higher concentrations of formaldehyde evolve formalin gas which damages living tissue (especially the eyes, nasal passages and lungs) in the same manner and so is harmful to the health of teachers and students. Thus, it is of interest to develop embalming fluids with lower formaldehyde concentrations. In addition, although phenol is a good preserving agent for tissues, it again is a health hazard for skin contact and vapor inhalation, because although high concentrations of alcohols, such as ethanol, are good antimicrobial agents (Coleman and Kogan, 1998; Guimaraes da Silva *et al*., 2004; Danvivathana *et al*., 2006; Janczyk *et al*., 2011; Jaung *et al*., 2011), they dehydrate the tissues to an unacceptable level for teaching anatomy.

This study evaluated embalming solutions with decreasing levels of formaldehyde and a lower phenol concentration with or without replacement by 16% ethanol and 15% sodium chloride, either table salt or the cheaper (in Thailand) sea salt. The solutions with a reduced formaldehyde and phenol concentration (to 0.5% and 0.8%, respectively), but the addition of 16% ethanol and 15% table salt (Group III) or sea salt (Group IV) were found to be as effective as the standard higher formaldehyde and phenol containing solution (Group II) at preserving the mice for up to 18 months (longest time assayed) in a suitable condition for teaching anatomy. The mice in Groups II, III and IV all showed no marked decomposition over the 18-month period and were not significantly different from the frozen control (Group I) mice. In contrast, without the added ethanol and sodium chloride but with lower formaldehyde concentrations (Groups V and VI) there was very poor tissue preservation with marked decomposition and putrefaction after 3 months which was unacceptable by 6 months leading to the disposal of the samples. Thus, lowering the formaldehyde and phenol concentration without ethanol and sodium chloride replacement led to an unsuitable solution.

It is noteworthy that the use of 15% (w/v) table salt or sea salt with 16% (v/v) ethanol could allow the reduction in the concentration of formaldehyde from 18% to 0.5% and phenol from 2.5% to 0.8% (v/v). The action of the salt and the ethanol is to reduce the available water and also, by osmosis, to partially dry the tissue causing it to no longer be suitable for the growth of microorganisms (Coleman and Kogan, 1998), whereas ethanol also disrupts microbial membranes and the metabolism.

The degree of decay of the mouse cadavers in the different embalming solutions compared to the frozen control was mirrored in the hair loss from the skin, skin peeling and the loss of color and elasticity in the tissues. In the loss of hair from the skin of the cadavers, this was less than 25% in Groups II, III and IV throughout the 18-month period, which, although this was statistically greater than the loss from the frozen control (Group I), was within the acceptable level of less than 50% and was not significantly different between Groups II and IV from Group II. By contrast, again Groups V and VI showed more than 50% hair loss within 3 months.

In the same trend, the color of the excised organs (lung, heart, stomach, urinary bladder, pancreas, spleen and kidneys) and the structural components within the cadavers (fat, blood vessels and nerves) faded slightly (score 1.5-2.3) but significantly in Groups II, III, and IV from that in the Group I control (score 3), but did not significantly differ between the high formaldehyde and phenol containing Group II and the low formaldehyde and phenol containing groups III and IV. These results are consistent with those of Coleman and Kogan (1998) and Guimaraes da Silva *et al*., (2004), who got good results in cadaveric preservation using a low formaldehyde and high salt (Sodium chloride/table salt) composition of embalming fluid. Guimaraes da Silva *et al*., (2004) found that the use of salt as a component of embalming fluid allowed the muscles to retain an almost natural appearance. The importance of retaining the color helps in learning the correct anatomy.

The flexibility of the surface of the muscle also showed the same trend, being significantly lower (score 1.5-2.4) in Groups II, III, and IV than the Group I control (score 3), but the tissues were still soft and flexible and did not significantly differ between Group II and Groups III and IV throughout the 18-month period. These results are again consistent with the study on human cadavers by Coleman and Kogan (1998) and Guimaraes da Silva *et al*., (2004), which seems to come from the ability of the salt and ethanol to partially absorb moisture from the tissue leading to preservation of the skin, muscles and organs for a longer period of time without contamination and putrefaction by microorganisms. In addition, the lower concentration of formaldehyde at 0.5% contributed to

the muscles being more supple due to the reduced level of protein crosslinking (Kierman, 2005).

This study found that the use of 15% sodium chloride (table or sea salt) combined with 16% ethanol allowed a reduction in the concentration of toxic formaldehyde (18% to 0.5%) and phenol (2.5 to 0.8%), while still preserving the mouse cadavers in a suitable condition for anatomy teaching for up to 18 months, Although mice are small animals with a high surface to volume ratio, the study is in accord with those on humans by Coleman and Kogan (1998) and Guimaraes da Silva *et al*., (2004).

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