Comparison of DNA Quantity Extracted from Formalin and Ethanol Fixed Tissues

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Abstract

This study was performed to compare DNA quantity extracted from the cerebrum part of porcine brain tissues preserved in 10% formalin or 95% ethanol, for 8 weeks. A sample was collected and DNA extracted at least once a week using a QIAamp DNA FFPE Tissue Kit. The Qubit 4 Fluorometer was then used for DNA quantification and to study the association between preservation duration and fixatives to evaluate the effect of two factors on DNA quantity, which can support forensic officers in choosing the most effective fixative to preserve tissues for DNA testing. The results showed that the concentration of extracted DNA from the tissues preserved in 95% ethanol was significantly higher than in the 10% formalin-fixed tissues. Moreover, DNA concentration from tissues preserved in 10% formalin significantly decreased after one week of preservation, while no significant difference was observed between DNA concentration in 95% ethanol fixed tissues after preservation. In addition, the negative correlation between preservation duration and DNA quantity in both 10% formalin and 95% ethanol fixed tissues was observed. It can be described that extracted DNA concentrations from tissues preserved in 10% formalin and 95% ethanol were decreased when the preservation time increased. However, we found that DNA concentration from 10% formalin-fixed tissues **reduced than tissues preserved in 95% ethanol by 40%.** Therefore, it can be concluded that 95% ethanol has better preservation properties and can be an alternative for a suitable method to preserve DNA from brain tissues compared to the 10% formalin solution.

Keywords: DNA, ethanol, formalin, preservation

Introduction

Over the past decades, DNA fingerprint or DNA profile has played a very important role in forensic applications. It becomes reliable and weighty scientific evidence in court proceedings because the results of comparison between the DNA fingerprints from DNA samples taken from a crime scene and DNA sample from a suspect or victim can be linked and provide confirmation to convict criminals, identify victims of crimes, disaster victim identification, and human recovery in war (Primorac et al., 1996; Góes, Silva, Domingues, Sobrinho, & Carvalho, 2002; Montelius & Lindblom, 2012). DNA examination is also useful in paternity testing (Alex, 2020). Thus, preserving DNA samples in good condition until a testing process is the most important and challenging.

Formalin is one of the most widely used fixatives for biological and medical specimens due to its high reactivity, ease of preparation, low cost, and great preservation of morphologic structures with few artefacts (Werner, Chott, Fabiano, & Battifora, 2000), making it suitable for preserving tissues using in histological studies (Howat & Wilson, 2014; Miething, Hering, Hanschke, & Dressler, 2016). However, formalin is a cross-linker causing DNA degradation. Because of the gradual formation of formic acid from formaldehyde, DNA fragmentation which is inappropriate for molecular study and forensic testing will occur (Douglas & Rogers, 1998; Kumar, Maitray, Gupta, & Shukla, 2018; Usharani, Thilaga, & Mahalakshmi, 2019). For example, a controversial case of using formalin fixation occurred in Thailand in 2017, in which the organs from a cadet who died under suspicious circumstances at the academy were taken and preserved in formalin during the autopsy processes without consent from the victim's family. At a later time, the parents requested the victim's organs back. However, DNA

examination was needed to confirm that the organs belonged to the victim. The results from the Central Institute of Forensic Science, Thailand and the Faculty of Medicine, Ramathibodi Hospital indicated that DNA from the formalin-fixed tissues was extremely degraded and therefore could not be quantitatively or qualitatively analyzed. As a result, DNA profiling and confirmation could not be achieved for this case.

Ethanol, an alternative solution of fixative, has been widely used in museum and private natural history collections since the 18th century. Due to its advantages as a preservative, low price and effective ways of production in high concentrations, ethanol has been widely used as the fixative among naturalists (Marquina, Ronquist, & Lukasik, 2020). Ethanol is an alcohol-based fixative that preserves tissues through coagulation. However, its removal and replacement of free water from tissue proteins (Eltoum, Fredenburgh, Myers, & Grizzle, 2001) causes protein denaturation and tissue shrinkage, which makes it an inapplicable fixative for histological investigations (Miething et al., 2016) although some studies have shown that ethanol is an excellent fixative for DNA preserving (Gino, Varacalli, Robino, & Torre, 2004; Alqaydi & Roy, 2016), as its ability to destroy decomposing microorganisms and removal of water from the tissue, helps to slow down enzymatic processes (Marquina et al., 2020).

Thus, this study was performed to investigate the effective preserving of tissues by formalin or ethanol for DNA testing. The comparison of DNA quantity extracted from porcine brain tissues preserved in 10% formalin and 95% ethanol was studied in association with preservation duration and fixatives, using the QIAamp DNA FFPE Tissue Kit for DNA extraction and Qubit 4 Fluorometer for DNA quantification.

Methods and Materials

Sample

The fresh pig head was prepared by the butchery. The brain section from a pig head was then collected by the animal anatomist from the Department of Anatomy, Faculty of Veterinary Medicine, Kasetsart University. The cerebrum part of the brain was cut into 5-mm-thick slices (Paireder et al., 2013), the slices were then preserved using 10% formalin and 95% ethanol in sealed containers and stored at room temperature. One tissue sample was cut every week (at least once a week) for 8 weeks of each fixation, and DNA was extracted from the sample.

DNA extraction

The QIAamp DNA FFPE Tissue Kit (QIAGEN, USA), specially designed for DNA purifying from formalin-fixed paraffin-embedded tissue, was used for DNA extraction. The kit was used as recommended by the manufacturer (Paireder et al., 2013) with minor adaptation. However, the paraffin removal step was omitted because the fresh brain sample had not been embedded in paraffin.

The 80 µL of phosphate buffer solution (PBS) was added to 25 mg of tissue in a 1.5 mL microcentrifuge tube. The tissue grinder pestle was then used for homogenization, followed by centrifugation at 14,000 rpm for 10 minutes. Next, 180 µL of ATL buffer and 20 µL of proteinase K were added to the pellet and then mixed by vortexing. The mixture was incubated at 56°C for 1 hour (or until the sample had completely lysed), and then incubated at 90°C for 1 hour. After the incubation process, 200 µL of AL buffer and 200 µL of ethanol (96-100%) were added to the mixture which was then mixed thoroughly by vortexing. The mixture was carefully transferred to the QIAamp MinElute column equipped with a 2 mL-collection tube after being briefly

centrifuged. The column was then centrifuged at 8,000 rpm for 1 minute and the collection tube was discarded. AW1 buffer (500 μL) was added into the column, connected with a new collection tube, and then centrifuged at 8,000 rpm for 1 minute. The second collection tube was also discarded. This step was repeated with 500 μL of AW2 buffer, followed by centrifugation at 14,000 rpm for 3 minutes to completely dry the membrane, then the column was placed in a clean 1.5 mL microcentrifuge tube. A 50 μL volume of ATE buffer was loaded into the column and incubated for 5 minutes at room temperature. The DNA was eluted by centrifugation of the column at 14,000 rpm for 1 minute. The extracted DNA was stored at −20°C until required.

Determination of DNA concentration

The concentration of DNA extracted from porcine brain tissues preserved in 10% formalin and 95% ethanol was measured in triplicate in each sample by using Qubit 4 Fluorometer (Invitrogen, California, USA), the fluorescence-based quantitation assays, with a 485 nm excitation filter and 530 nm emission filter. A Qubit™ dsDNA BR Assay Kit was used with a Qubit 4 Fluorometer according to the manufacturer's protocol. Briefly, a 2.0 μL of DNA sample was added to 198 μL of Qubit™ working solution in a Qubit™ assay tube (acceptable tubes include thin-wall, clear 0.5 mL PCR tube). The mixture was mixed for 2-3 seconds by vortexing and incubated for 2 minutes at room temperature. The tube was then inserted in the Qubit 4 Fluorometer for reading the result.

Statistical analysis

Nonparametric statistical tests were performed using R programming language version 4.0.4 (R Core Team 2021). In all procedures, $p < 0.05$ was considered significant. The difference of the extracted DNA concentration from 10% formalin and 95% ethanol fixed porcine brain tissues were analyzed by the Mann-Whitney U test for two independent samples. The Kruskal-Wallis test was performed to test the differences of DNA concentration over the 8 weeks, then a Wilcoxon rank-sum test with Benjamini & Yekutieli (BY)'s p-value adjustment method was applied for pairwise comparison when the Kruskal-Wallis test showed a significant difference. In addition, Spearman's correlation was applied to measure the strength and direction of association between preservation duration and fixatives.

Results

Comparison of DNA quantity between 10% formalin and 95% ethanol fixed tissues.

The extracted DNA concentration of porcine brain tissues preserved in 10% formalin and 95% ethanol was measured using Qubit 4 Fluorometer every week for 8 weeks. The average DNA concentrations are shown in Table 1.

Table 1 Quantity of DNA extracted from porcine brain tissues preserved with 10% formalin and 95% ethanol

Week	10% formalin (ng/μL)		95% ethanol (ng/μL)	
	$\bar{x}$	S.D.	$\bar{x}$	S.D.
1	4.23	1.13	10.32	4.58
2	2.52	0.59	8.77	2.92
3	1.67	0.27	5.76	0.13
4	1.94	0.42	3.74	1.05
5	1.77	0.13	5.42	1.38

Table 1 (Cont.)

Week	10% formalin (ng/μL)		95% ethanol (ng/μL)	
	$\bar{x}$	S.D.	$\bar{x}$	S.D.
6	2.09	0.08	5.20	0.25
7	1.86	0.11	7.18	0.46
8	1.90	0.06	8.32	0.16

The results showed that the average concentration of extracted DNA decreased in both 10% formalin and 95% ethanol fixed tissues after one week of preservation. DNA concentration of tissues preserved in 10% formalin continued to decrease for the next three weeks, then ranged between 1.77 – 2.09 ng/μL from week 4 to week 8. The concentration of DNA from the 95% ethanol fixed tissues reduced until week 4, then increased again until week 8, as shown in Figure 1. At the week 8 of preservation, the average concentration of extracted DNA from tissue preserved in the 95% ethanol was 8.32 ± 0.16 ng/μL, whereas the extracted DNA from tissues preserved in 10% formalin was 1.90 ± 0.06 ng/μL, indicating a significant difference in DNA concentration between the 10% formalin and 95% ethanol fixed tissues (W = 96, p-value < 0.05). The extracted DNA concentration of tissues preserved in 95% ethanol (8.16 ± 3.74 ng/μL) was significantly higher than in 10% formalin (2.85 ± 1.26 ng/μL).

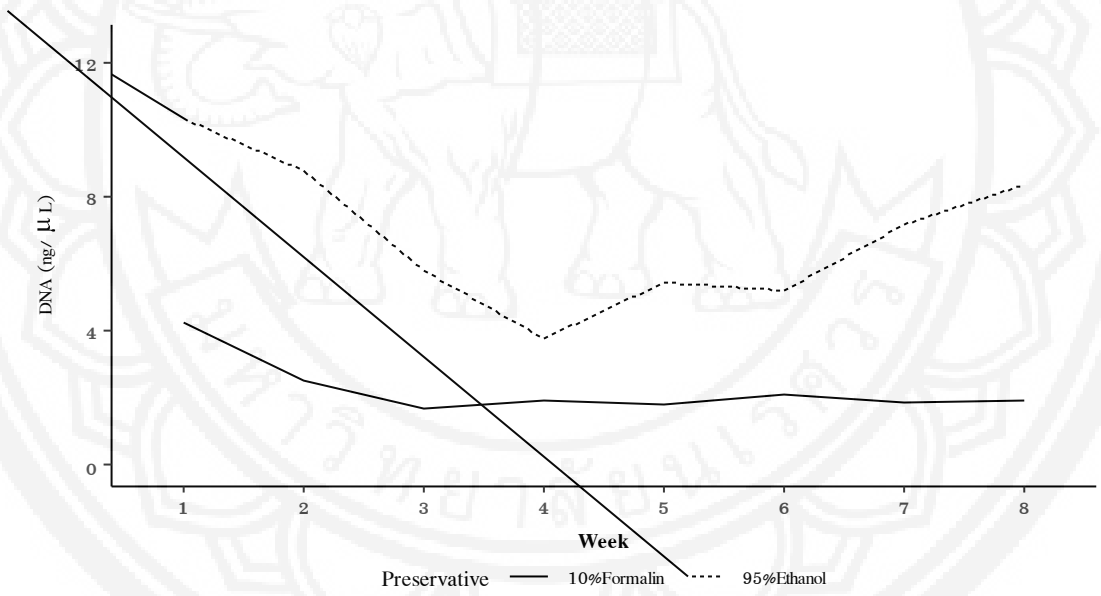

Figure 1 The mean concentration of DNA extracted from tissues preserved in 10% formalin and 95% ethanol at each preservation duration

Comparison of DNA quantity over Time

The comparison of extracted DNA concentration between the groups over the 8 weeks, using the Kruskal-Wallis test, presented a significant difference at p<0.05 in porcine brain tissues preserved in both 10% formalin and in 95% ethanol. Pairwise comparisons were performed. It was found that the concentration of DNA extracted from 10% formalin-fixed tissues in week 1 was significantly different from week 2 and subsequent weeks up to week 8 of preservation (Table 2). The DNA concentration of week 1 was significantly higher than the other weeks. In addition, the extracted DNA concentration from 10% formalin-fixed tissues in week 2 showed a

significant difference from week 5. On the contrary, the concentration of DNA extracted from 95% ethanol fixed porcine brain tissues did not show a significant difference in any paired week (Table 3).

Association between preservation duration and fixatives.

According to the study, the association between preservation duration and the fixatives can indicate the effect on the change of extracted DNA. It revealed that there was a negative correlation between preservation duration and the DNA quantity from both 10% formalin and 95% ethanol fixatives. When the preservation time increased, the extracted DNA concentration from 10% formalin and 95% ethanol fixed tissues were significantly decreased. Based on Spearman's correlation coefficients guided by Akoglu (2018), it indicated that preservation duration showed a very strong correlation ($r_S = -0.82$) with the concentration of DNA extracted from 10% formalin-fixed tissues, whereas 95% ethanol fixed tissues showed a fair correlation ($r_S = -0.42$). It implied that the DNA concentration from tissues preserved in 10% formalin reduced more than 95% ethanol fixed tissues by 40% (82%-42%=40%) with the increase in preservation time.

Table 2 Pairwise comparisons of DNA between the weeks, extracted from 10% formalin fixed tissues

Week	1	2	3	4	5	6	7	8
1	-	-	-	-	-	-	-	-
2	0.01*	-	-	-	-	-	-	-
3	0.04*	0.08	-	-	-	-	-	-
4	0.01*	0.77	1.00	-	-	-	-	-
5	0.01*	0.04*	1.00	1.00	-	-	-	-
6	0.04*	1.00	0.21	1.00	0.21	-	-	-
7	0.01*	0.21	1.00	1.00	1.00	0.21	-	-
8	0.04*	0.21	0.59	1.00	0.70	0.21	1.00	-

The numbers filled in the table were *p*-value.

* A significant difference was found between paired weeks (*p*-value < 0.05).

Table 3 Pairwise comparisons of DNA between the weeks, extracted from 95% ethanol fixed tissues

Week	1	2	3	4	5	6	7	8
1	-	-	-	-	-	-	-	-
2	1.00	-	-	-	-	-	-	-
3	0.17	0.17	-	-	-	-	-	-
4	0.17	0.17	0.17	-	-	-	-	-
5	0.27	0.17	1.00	1.00	-	-	-	-
6	0.17	0.17	0.17	0.17	1.00	-	-	-
7	1.00	1.00	0.17	0.17	0.17	0.17	-	-
8	1.00	1.00	0.17	0.17	0.17	0.17	0.17	-

The numbers filled in the table were *p*-value.

* A significant difference was found between paired weeks (*p*-value < 0.05).

Discussion

DNA fingerprinting has become one of the important pieces of forensic evidence used in the court and for the justice system, especially in the cases of personal identification or investigation of the suspect or victim of

crime. Therefore, preserving DNA samples for both quantitative and qualitative analysis is extremely vital. DNA extracted from tissues preserved in 10% formalin, a cross-linking fixative commonly used for biological and medical specimens was studied. However, 95% ethanol, an alcohol-based fixative that preserves tissues through coagulation, is alternatively applied. The most effective solution for tissue preservation and DNA testing was examined.

From our study, the DNA concentration significantly decreased in formalin-fixed tissues as preservation time increased. This is due to the gradual formation of formic acid from formaldehyde, which is the main component in formalin (Alqaydi & Roy, 2016). When the time increases, more formic acid is produced, resulting in more DNA degradation. Exposure of specimens to formaldehyde causes a direct effect on the degeneration of DNA extracted from tissues. Formaldehyde not only constructs crosslinks but can directly react with nucleotides as well (Lu et al., 2010). As a result, the nucleic acid base is added to form a hydroxymethyl (methylol) group (McGhee & Von Hippel, 1975; McGhee & Von Hippel, 1977) and generates apurinic and apyrimidinic sites through hydrolysis of the N-glycosylic bonds, leaving free pyrimidine and purine residues, and causing slow hydrolysis of the phosphodiester bonds leading to small fragments of DNA (Douglas & Rogers, 1998). These processes are not suitable for molecular biology and forensic testing (Douglas & Rogers, 1998; Kumar et al., 2018; Usharani et al., 2019). However, formalin is an excellent fixative to preserve tissues with great preservation of morphologic structures with few artefacts for histopathological study (Werner et al., 2000; Howat & Wilson, 2014; Miething et al., 2016).

Our study, however, indicated that tissues preserved in ethanol gave better results in terms of DNA quantity. Although the results demonstrated a decrease in DNA concentration, there was a higher DNA quantity in comparison with the formalin-fixed tissues. This is due to lesser chemical changes of DNA caused by ethanol (Srinivasan, Sedmak, & Jewell, 2002). Ethanol preserves tissues through coagulation while the process of removal and replacement of free water from the tissue does not interfere with nucleic acid and helps to slow down enzymatic processes (Marquina et al., 2020). Yet, ethanol exhibits several potential effects on the tissue proteins through the coagulation procedure (Eltoum et al., 2001), resulting in protein denaturation and tissue shrinkage. Therefore, ethanol is considered as being inapplicable fixative for morphological and histological studies. (Srinivasan et al., 2002; Miething et al., 2016).

Our results were consistent with the study of Alqaydi and Roy (2016), who discovered that the average DNA concentration decreased in tissues preserved in both ethanol and formalin after the first day of preservation, and continued to reduce as the preservation time increased. According to all the results of their study, they concluded that preserving tissues in formalin significantly increased DNA degradation compared with specimens preserved in ethanol. In comparison with our study, the concentration of DNA decreased in both fixatives and agreed with Alqaydi and Roy's result. However, less DNA stability was observed. Tissues preserved in formalin showed a stable concentration after week 2 (14 days) of preservation, while tissues fixed with ethanol showed an increase in DNA concentration after week 4. This may be affected by differences in tissue types (Miething et al., 2016), quantification methods (Josefiova et al., 2017), and purity of fixatives. Furthermore, Miething et al. (2016) also discovered that unbuffered formalin was one of the least suitable fixatives for DNA analysis. Loss of nuclear DNA was observed after 3 days and no longer detected from the 14 days of preservation, whereas the alcohol-based fixative was one of the most suitable substances for long-term fixation and subsequent DNA analysis. In addition,

our results were consistent with Paireder et al. (2013), in which the lowest DNA concentration from tissues preserved in unbuffered formalin compared to other fixatives consisting of less formaldehyde was found.

Conclusion and Suggestions

In summary, the results of our study showed that porcine brain tissues preserved in 10% formalin significantly decreased DNA concentration compared to 95% ethanol fixed tissue. It can be concluded that the greater ability to preserve DNA quantity from brain tissues is 95% ethanol. However, to identify the cause of death, histopathological examination is still necessary. Therefore, the authors suggest that forensic officers ought to preserve the tissues separately in both formalin and ethanol for histopathology and DNA testing. It is recommended that the DNA extracted from ethanol fixed tissue should be prioritized for investigation of the quality to ensure the process of PCR, especially for the efficacy of DNA amplification. Moreover, the different types of tissue might lead to the variation of DNA quantity (Miething et al., 2016). Thus, different tissue types are suggested and essential for the examination in forensic application. This can provide a guideline and information for the most suitable tissue under ethanol fixative for DNA testing in any specialized case.

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