Tissue preservation in extreme temperatures for rapid response to military deaths

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Highlights

- An alternative method of preservation for DNA samples transported from remote sites, including combat zones.
- The methodology involves the use of inexpensive and widely available chemicals.
- Fresh samples and those with signs of early decomposition can be preserved for at least one week up to 65°C.

Abstract

Many deployable forensic capabilities, including those used by the Australian Defense Force (ADF), employ mobile battery-operated fridge/freezers for DNA sample preservation that are not suitable for rapid response application due to their size and weight. These fridge/freezers are expensive, require regular specialised maintenance, and have a set payload. A variety of transport media are successful preservatives for DNA samples, however, there is no research specifically targeted to their suitability for operational environments where temperatures exceed 50°C.

This research examined whether sodium chloride (NaCl), ethanol, and dimethyl sulfoxide (DMSO) could preserve muscle and bone samples (fresh and early decomposition) as effectively as refrigeration, when stored at 21°C, 45°C, 55°C, and 65°C for at least one week. A total of 78 muscle and 78 bone samples were collected from an unknown deceased individual. Half of each tissue type was stored at 30°C for 48 hours to induce early decomposition. Following this, samples were stored in the transport media for one week at the above temperatures, and a control set of samples were refrigerated (-4°C) without any transport media. Preserved samples would need to provide DNA profiles comparable to the refrigerated samples for the transport media to be considered a successful replacement method.

NaCl and 70% ethanol preserved muscle samples (fresh and decomposed) up to 65°C, as well as 70% ethanol and 20% DMSO for fresh bone samples. These results were comparable with refrigeration and therefore, these preservatives could be used in rapid response operations by the military and for disaster victim identification. Conversely, under the conditions of this study, 20% DMSO and 70% ethanol failed to consistently produce full DNA profiles from decomposed bone, and NaCl performed poorly at preserving DNA from fresh and decomposed bone samples.

Keywords: disaster victim identification; tissue preservation; forensic biology, DNA profiling,

1. Introduction

The identification of human remains by many government agencies worldwide generally follow disaster victim identification (DVI) principals espoused by the International Criminal Police Organisation (INTERPOL) DVI guide (1). In a military context, a designated forensic response team and associated equipment are transported from the home country to a site often remote from the disaster site itself, where a temporary mortuary is established in order to gather and examine remains. Depending on the conditions of the remains, DNA is often a key method of identification, particularly in circumstances where fingerprints or dental identification is not possible, however, many developing countries and those subject to civil and military conflict lack the ability to type DNA. In these situations, tissue samples need to be transported to other countries for DNA analysis. It is often desirable to sample tissue near the disaster site or prior to transportation of the remains elsewhere in order to speed the identification process, particularly where local agencies retain jurisdiction of the body, there is difficulty relocating remains due to zones of conflict, or there are legal / coronial processes that result in delays in releasing bodies to a foreign entity. Several options exist for sample preservation; however, a common scenario is the utilisation of mobile battery-operated blood or tissue fridges and freezers. Samples are stored in these appliances until they can be relocated to the home country, often ahead of the remains.

There are a number of limitations associated with using fridge/freezers to preserve and transport DNA samples, which make it unsuitable for a rapid response DVI application for the military. A typical fridge/freezer used by the military weighs approximately 66kg (empty), which consumes a large proportion of the rapid response kit weight limit. They require specialised regular maintenance, the payload lacks scalability, and relies on the availability of electricity or working batteries up to 48hrs. The focus of this research is evaluating preservation of muscle and bone samples under conditions that replicate transport from a military field mortuary to another country (a period of up to one week in ambient temperatures over 50°C). Such scenarios would be encountered where agencies are required to mount a DVI response overseas, as either aid to the civilian community, or for military deaths.

This research examines three transport media to determine whether they can preserve bone and muscle samples under temperature conditions consistent with potential operational environments experienced by modern military forces (45°C, 55°C and 65°C for at least one week) (Table 1). The three chemical media chosen are, sodium chloride (NaCl), 70% ethanol, and 20% dimethyl sulfoxide (DMSO). Replacing fridge/freezers with transport media would

improve military and government capability for responding to deaths in combat and disaster scenarios by reducing the cost and weight of the response kits, and enabling a more efficient and reliable method to transport DNA samples.

Table 1: Summary of the proposed preservatives and their constituents.

Preservative	Constituents
NaCl	Solid NaCl (Analytical Reagent) (Chem-Supply)
Ethanol	100% Undenatured ethanol (Chem-supply) and sterile double distilled water (ddH ₂ O) (Invitrogen by Life Technologies) (3:1)*
DMSO	100% DMSO (Chem-Supply), 0.5M EDTA (pH 8.0) (Chem-Supply) and ddH ₂ O (2:5:3), saturated with NaCl*

* The stated concentrations are for those of the liquids before mixing of the final solution

INTERPOL guidelines state that chemical preservatives can be used to store soft tissue at room temperature but recommend against the use of formaldehyde or formalin because it degrades the DNA (1). As enzymes are active in the presence of water, the elimination of water is one of the main steps in a vast majority of preservation methods. In solid form, NaCl desiccates the sample, which inactivates nucleases and reduces the growth of pathogens (2). NaCl is a common preservative that is reported to preserve muscle tissue at room temperature for one year (3); 35°C for one month (4); and 37°C for 38 weeks (5). Michaud (6) found that liquid preservatives performed better than NaCl, most likely because they were capable of rapidly penetrating the sample to prevent decay of the DNA.

Ethanol removes water from a sample and denatures proteins and nucleases (7–9); as such, ethanol is an antimicrobial that can prevent bacterial degradation (7,10). It is the most commonly used alcohol for tissue preservation, and has been used as a successful preservative at various concentrations. For example, 100% ethanol was used to successfully preserve molluscan tissue at room temperature for 26 days (11), while 95% ethanol was used successfully to store mouse liver over a two year period, although a low DNA yield was obtained compared to DMSO and an additional procedure had to be used to protect the DNA from degradation during the extraction procedure (12). At 70%, ethanol was suitable for shorter-term storage of porcine skin and muscle tissue at room temperature (less than 6 months) (6). Michaud (6) found that 70% ethanol was as effective as DMSO in preserving DNA, and performed as well as, or better than, other concentrations (40% and 100%). For human samples, 70% ethanol and 70% ethanol containing 1mM EDTA was used to store

muscle samples at 35°C for one month (4). Both performed as successful preservatives. Based on these findings, 70% ethanol was investigated.

The mechanism of action for DMSO is only partially understood. It exerts antimicrobial activity (13) by altering the ribonucleic acid structures essential for bacterial protein synthesis (14), and is known to promote the dermal absorption of chemicals with which it is mixed (15), and thus enhances the absorption of other preservatives into the substrate (12). Compared with lysis buffer and 95% ethanol, DMSO provided the best protection from DNA degradation and provided a higher yield of DNA for mouse liver stored at room temperature for two years (12). It was used to successfully preserve avian tissue at room temperature for 24 weeks, although no PCR was conducted (7). DMSO has also been used to preserve avian blood stored at ambient temperature and 65°C, with no detectable degradation of DNA, as determined from agarose gel electrophoresis of crude DNA (16). Marine invertebrate have been successfully preserved in DMSO at ambient temperature for 26 days (11) and 28 weeks (17), while porcine skin and muscle was successfully stored 6 months (6). These studies conducted on animal samples suggests that DMSO may be a suitable preservative for human tissue, a hypothesis that was supported by Allen-Hall and McNevin (4) who successfully stored human muscle in DMSO for one month at 35°C.

A number of proprietary preservatives exist that have successfully preserved DNA samples. TypiFix™ containers are suitable for the preservation of soft human tissue when stored at room temperature for up to five months (18), while DNA Genotek Tissue Stabilising Kit, and DNAgard® have all successfully preserved fresh human muscle tissue at 35°C for up to one month (4). Sorensen et al (19) also demonstrated that DNAgard® and RNA/later® preserved fresh and decomposed human skin and muscle for up to three months at 35°C. RNA/later® was also shown to be as successful as DMSO and 70% ethanol when storing porcine tissue for DNA analysis, and performed better than NaCl (6).

Military operational environments can exceed 40°C, and after fitting military vehicles with armour kits, temperatures inside those vehicles can reach 54°C (20), while the tarmac or concrete used on airfield aprons reflects heat and further multiplies the temperature of vehicles on the ground (21). No previous literature has examined the capability of chemical media to preserve human DNA samples in the potentially high temperature ranges experienced by armed forces in modern combat zones. Therefore, it is necessary that preservatives are tested under these conditions, to determine their effect on DNA preservation.

The most important consideration when selecting a chemical preservative is that it consistently enables a DNA profile to be obtained. Other important considerations include cost, availability, and occupational health and safety during transportation and use in the mortuary and laboratory. Vapours from ethanol may form an explosive mixture with air, thus it is classified as a Dangerous Good of Class 3 (22) in accordance with International Air Transport Association Dangerous Goods Regulations. However, when packaged correctly, it is authorised for carriage aboard service aircraft (23), including both fixed and rotary wing aircraft. NaCl has minor health considerations that are addressed by correct personal protective equipment (PPE), storage and disposal. DMSO is identified as an irritant, causing both skin and eye irritation if correct storage and handling protocols are not adhered to (24). Neither NaCl nor DMSO are regulated as Dangerous Goods and are suitable for transport aboard service aircraft (24,25).

Preparation of the chemicals should be a simple task in a rapid response environment. For example, pouring solid NaCl into a vial containing a tissue sample is much easier than preparing a complex mixture of constituents which requires an accurate pH measurement. NaCl and 70% ethanol have low preparation requirements, however, DMSO requires a 24 hour (approximate) incubation period before the solution can be used to ensure the saturated NaCl has settled (26).

Scalability of an operational response is essential for DVI purposes. In addition to the large number of casualties, decomposition and fragmentation can be extensive in high impact disasters (27). One of the most important issues with the refrigerators used by the military is that they are only suitable for small DVI operations. Ideally, preservation methods should be readily capable of storing large quantities of tissue.

The shelf life of each chemical should also be considered, as it is impractical to suggest use of a chemical with a shelf-life of less than one year for inclusion in a rapid response kit. Fortunately, when stored at room temperature and protected from exposure to moisture, DMSO has a shelf life of two years (28). The shelf life of NaCl is approximately three to five years (29,30). It has been shown that the composition of an ethanol mixture changes continuously as evaporation proceeds (31), so it is recommended that pure ethanol is stored, and 70% ethanol prepared when required.

Finally, the cost per sample needs to be considered (Table 2). Cost and weight refer to the bulk chemical required (such as pure ethanol and DMSO), while cost per sample refers to the cost of the prepared chemicals for each tissue sample (assuming samples are $\sim 8\text{mm}^3$ and

are stored in a 1.5mL screw top tube with 3.5g of solid preservative or 500 μ L of liquid preservative). Compiled from: (32–35).

Table 2: Cost of common chemical preservatives.

Chemical	Weight	Cost*	Cost per sample*
NaCl	5kg	\$93.00	\$0.065
Pure Ethanol	2.5L	\$38.00	\$0.0053
Pure DMSO	2.5L	\$239.00	\$0.0096
RNAlater	100mL	\$197.00	\$0.99
DNAgard®	100mL	\$504.00	\$2.52

* Based on Australian Dollars. Prices obtained in 2017.

Given the above considerations, NaCl, DMSO, and 70% ethanol were chosen over proprietary preservatives to determine their ability to preserve muscle and bone samples (fresh and those with early decomposition) at temperatures between 21°C and 65°C, over a one week period.

2. Materials and Methods

2.1 Sample collection

Ethical approval (ETH15-0029) was obtained for the collection of muscle and bone samples from the University of Technology Sydney Body Donation Program. Seventy-eight samples of fresh muscle tissue were collected from a single donor using a muscle biopsy needle from the biceps brachii, triceps brachii and brachialis muscles. Samples were approximately 8mm³ and were stored in 1.5mL eppendorf tubes. Samples of fresh bone were also taken from the humerus using an electric bone saw and ranged from 125mm³ to 750mm³.

2.2 Sample preparation (pre-preservation)

Half of the samples from each tissue type were stored in refrigeration (fresh samples), while the remaining samples were stored in a water bath set at 30°C for 48 hours. Typically, the recovery of the bodies of military personnel is swift, however, lack of access to an incident site may result in early decomposition prior to the body reaching the mortuary. The aim of the incubation was to induce early decomposition to determine whether decomposition by-products interfered with tissue preservation.

2.3 Preservative preparation

The protocol used for preparation of the DMSO preservative solution was adapted from a protocol used on corals, released by Auburn University (26). A solution of 0.5M EDTA (pH

8.0), 100% DMSO and ddH₂O (5:2:3) was prepared. NaCl was added to saturate the solution and left to settle overnight. The solution was autoclaved before use. A stock solution of 70% ethanol was prepared by mixing absolute ethanol with ddH₂O (3:1). Solid NaCl was added directly to the samples.

2.4 Sample preservation

Fresh and decomposed muscle and bone samples were stored in each preservative at four different temperatures (room temperature (21°C), 45°C, 55°C, and 65°C) for seven days. The temperatures of the three water baths (set at 45°C, 55°C, and 65°C) were monitored during preservation using a thermometer. Three samples of fresh, and three samples of decomposed muscle and bone were stored in refrigeration with no chemical preservative as a comparison. A total of 156 samples were included in the research, comprising of three replicates for each tissue, preservative, and temperature combination. A total of 500µL of liquid preservative or 3.5g of solid preservative was used per muscle sample in a 1.5ml tube, while 10mL of liquid preservative and 7.5g of solid preservative was required for bone samples in a 50ml Falcon™ tube.

2.5 DNA extraction

All samples were washed in sterile ddH₂O to remove traces of the chemical preservatives prior to further analysis. However, it is noted that this step may be unnecessary as the DNA extraction methods should sufficiently remove traces of each preservative. Bone samples were crushed using 6970EFM Enclosed Freezer/Mill® (Spex® Sample Prep) and extracted using the protocol for DNA extraction from bodily fluids and tissues released by the FBI DNA Analysis Unit II (36), with minor changes as follows. Four microlitres of 10mg/µl proteinase K was used in step 1; samples were spun for 5 minutes at a setting of 2000 X g in step 10; 200µl of ddH₂O was used in step 12; and samples were spun in a micro-centrifuge at a setting of 10000 X g for 10 minutes in step 16. DNA was extracted from muscle samples using a QIAamp® DNA Mini Kit in accordance with the manufacturer's instructions. Negative extraction and amplification controls consisted of sterile water. The concentration of DNA (ng/µL) in each sample was estimated using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific).

2.6 STR Genotyping

DNA was amplified using the PowerPlex® 21 System (Promega®), with a final reaction volume of 25µL. Amplification was conducted using a C1000™ Thermal Cycler (Bio-Rad®) in accordance with the manufacturer's instructions. The initial bone results were unreliable (discussed in Section 4.2) so, due to kit availability, these samples were re-amplified using the

PowerPlex® S5 System (Promega®), with a final reaction volume of 25µL. Positive controls provided with each profiling kit were also tested. A master mix was prepared to deliver 0.5µL WEN500 and 15.0µL Hi-Di™ formamide (Applied Biosystems) to each sample. A 1.0µL aliquot of amplified PCR product was added to each tube. The samples were run on a 3130xl Genetic Analyser using POP 7 polymer (Applied Biosystems) and instrument injection settings of 3.0 kilovolt (kV) x 5 seconds. All other run module settings were set in accordance with the manufacturer's instructions. Electrophoresis data was generated using GeneMapper® ID software version 3.7 (Applied Biosystems). The minimum peak amplitude threshold (PAT) was designated 50 relative fluorescence units (rfu) for heterozygous alleles and 150 rfu for homozygous alleles. The total number of amplified alleles in each profile was recorded.

2.7 Data Analysis

The performance of a chemical preservative was determined by counting the number of alleles detected in each DNA profile. For each tissue type, confidence intervals (CI) were calculated for the proportion of samples that generated a full DNA profile (42 alleles for muscle samples, and 10 alleles for bone samples). Two different proportions were calculated:

1. The total number of samples (fresh and decomposed) that recorded full DNA profiles for *each preservative* pooled across the four temperatures;
2. The total number of samples that recorded full DNA profiles for each *tissue condition* within *each preservative*, pooled across the four temperatures.

CIs were calculated using the Wilson method from an online tool provided by AusVet Animal Health Services (37). The program outputs intervals using five alternative calculation methods as described in Brown et al (38).

The average allele peak height (rfu) for each loci across the five temperatures (-4°C, 21°C, 45°C, 55°C, and 65°C) was calculated to show preservation success.

3. Results

All controls performed as expected. Split peaks and pull-up were observed in a number of samples, and were likely the result of too much DNA being added during amplification.

3.1 Muscle Samples

Full DNA profiles were obtained for 76 of the 78 samples. Two partial profiles (40 and 41/42 alleles) were obtained from fresh samples stored in DMSO at 45°C (Fig 1). Fig 2 shows the average allele peak height (rfu) for each preservative at the four temperatures studied.

3.2 Bone Samples

Full DNA profiles were obtained for 72 of the 78 samples (Fig 3). Ethanol and DMSO produced full DNA profiles in all but one sample (4 and 7/10 alleles respectively), while four samples stored in NaCl returned a partial profile (4, 7, 4, and 3/10 alleles respectively). Fig 4 highlights the average allele peak height (rfu) for each preservatives across the four temperatures.

Figure 1: DNA profiling results for muscle samples

Figure 2: Average peak height for muscle samples

Figure 3: DNA profiling results for bone samples

Figure 4: Average peak height for bone samples

4. Discussion

4.1 Muscle Samples

Given the preservatives have not previously been tested above 40°C, their capacity to tolerate extreme temperatures for one week was unknown. Surprisingly, full DNA profiles were obtained for 76 out of 78 muscle samples. Partial profiles (40 and 41/42 alleles respectively) were obtained for two samples of fresh muscle stored in DMSO at 45°C. In both samples,

dropout of a single allele occurred at TPOX. Allelic dropout of a single allele also occurred at Penta E in one sample. Dropout at such loci is expected, as previous studies have shown that although different alleles at the same locus have similar probabilities of dropping out, loci with longer alleles tend to have higher dropout rates than those with shorter alleles (39–41). Decomposed muscle was preserved just as well as fresh muscle, which indicates that the preservatives were not compromised by early decomposition. DMSO lacked some ability to maintain the integrity of the muscle structure, with fragmented tissue visible in the liquid preservative. This finding was supported by Allen-Hall (42) who also found visible fragmentation following preservation of human muscle at 35°C for one month.

Given that refrigeration provided full DNA profiles for all muscle samples, any replacement method using preservatives needs to perform to this standard routinely in an operational environment. In this study ethanol and NaCl performed as well as refrigeration. In temperatures between 21°C and 65°C, it can be expected that muscle samples (fresh and decomposed) stored in NaCl or ethanol will generate a full DNA profile 86.2% to 100% of the time (95% CI). Two partial profiles were obtained for fresh samples stored at 45°C in DMSO. Based on those results, in temperatures between 21°C and 65°C, it is expected that fresh muscle samples stored in DMSO will generate a full DNA profile 55.2% to 95.3% of the time, while decomposed muscle samples will generate a full DNA profile 75.8% to 100% of the time (95% CI). These results indicate that NaCl and ethanol provided the best protection against DNA degradation.

Table 3 provides a summary of factors that would need to be considered prior to implementation into an operational environment. Of the three preservatives, NaCl is preferred for muscle as it carries minor health risks compared with the other two preservatives, is safe to transport, cost effective, is the easiest to prepare, and the sample preparation following preservation is low.

Table 3. Considerations for operational use of muscle and bone preservation methods.

	NaCl	Ethanol	DMSO
Possible Health Hazards	Low	Moderate	Moderate
Cost per sample*	Low – \$0.065	Low – \$0.0053	Low – \$0.0096
Preservative preparation	Nil	Low	High
Sample preparation^a	Muscle: Low Bone: High	Muscle: Low Bone: Moderate	Muscle: Moderate Bone: Low
Shelf life	3-5 years	3-5 years	2 years

* Australian dollars ^a Tissue sample preparation after incubation in preservative and prior to DNA extraction.

4.2 Bone Samples

The Nanodrop® can overestimate DNA quantity (43–46), and does not discriminate between amplifiable and non-amplifiable DNA. The overestimation of DNA concentration (which would have led to less DNA added to the PCR) may have caused the partial profiles observed using the PowerPlex 21® System, and no relationship was detected between the number of reportable alleles and the chemical treatment or storage temperature. Partial profiles were also obtained for all refrigerated bone samples. Due to kit availability, samples were re-amplified using the PowerPlex® S5 System. This kit enables the co-amplification and fluorescent detection of five loci (four STR loci and the Amelogenin) (47), while the PowerPlex® 21 System allows for the amplification of 20 STR loci and the Amelogenin (48); all five loci that are amplified in the PowerPlex® S5 System are also amplified in the PowerPlex® 21 System.

Reduced amplicon size multiplex PCRs are required to provide the best chance of amplifying degraded DNA (49) because the primer sites are more likely to remain intact. With the exception of locus FGA, the amplicons are larger for the PowerPlex® S5 System compared to the PowerPlex® 21 System (comparing only the loci seen in both kits), so was expected to be less sensitive.

The capacity for chemical preservatives to preserve bone samples was unknown as no previous literature could be found that investigated this topic. Following preservation for

seven days, ethanol and DMSO produced full PowerPlex S5® DNA profiles in all but one sample each. One partial profile was observed for early decomposed samples stored at 21°C in DMSO and 45°C in ethanol (4 and 8/10 alleles respectively). Four partial profiles were obtained from samples stored in NaCl (4, 7, 4 and 3/10 alleles respectively), suggesting a decline in performance compared with other preservatives. This could be because solid NaCl was unable to penetrate the bone fragments in order to prevent DNA damage during the seven-day preservation period. Michaud (6) also found that liquid preservatives were more reliable preservatives than NaCl, although their study was conducted on porcine skin and muscle tissue, and samples were stored for up to six months at room temperature. In all six samples with observed dropout, no alleles were detected for the amelogenin, and allele dropout was detected at the D18S51 locus in 5 of the 6 samples.

DMSO and ethanol performed as well as refrigeration for fresh samples, however the results were not comparable to refrigeration for decomposed samples. Based on these results, at temperatures between 21°C and 65°C, it can be expected that fresh bone samples stored in DMSO or ethanol will generate a full DNA profile 75.8% to 100% of the time, while early decomposed bone samples stored in ethanol or DMSO will generate a full DNA profile 64.6% to 98.5% of the time (95% CI). NaCl failed to produce DNA profiles equivalent to refrigerated samples, and thus it is expected that at temperatures between 21°C and 65°C, bone samples (fresh and decomposed) stored in NaCl will only generate a full DNA profile 64.1% to 93.3% of the time.

NaCl preservation caused the bone samples to become dry, and it was difficult to remove the soft tissue adhering to the bone segment prior to DNA extraction. In contrast, DMSO partially removed the soft tissue that surrounded the bone samples, simplifying the cleaning process. This should be a consideration for larger DVIs as the longer cleaning step increases turnaround times.

Figures 2 and 4 show that the average peak height obtained for each chemical preservative was well above thresholds for all loci. Some variation in peak height is present between the preservatives, though no clear trend existed and was mostly comparable to the variation obtained for refrigeration.

5. Conclusions

This research has demonstrated that 70% ethanol and NaCl are suitable for preservation of muscle samples (fresh and decomposed) for at least one week at temperatures between 21°C and 65°C, producing results that are comparable to refrigeration. Under the same conditions,

70% ethanol and 20% DMSO preserve fresh bone samples as effectively as refrigeration. These preservatives are safe, inexpensive, readily available, easy to prepare, and scalable for larger DVI operations. These media can be used to preserve DNA samples for DVI operations when no refrigeration is available, or to replace mobile fridge/freezers during transportation from the mortuary to the laboratory.

While 20% DMSO and 70% ethanol were comparable with refrigeration for preservation of fresh bone samples, they were not as effective as refrigeration for decomposed bone. DMSO did not preserve fresh muscle as well as refrigeration, and NaCl did not preserve bone samples (fresh and decomposed) as well as refrigeration.

Removing the need for refrigeration of DNA samples for rapid response field operations will enhance the military's capability to respond to deaths in combat. No previous literature could be found that has examined the effect of chemical preservatives on the storage of bone samples, and this is the first research targeted to military needs (noting however, that it can also be applied to civilian DVI). Furthermore, this is the first research that has examined the effects of preservatives at temperatures up to 65°C.

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