



FORMALIN FIXATION AT LOW TEMPERATURE PROVIDES BETTER YIELD AND INTEGRITY OF DNA

FIKSACIJA FORMALINOM NA NISKOJ TEMPERATURI OBEZBEĐUJE BOLJI PRINOS I INTEGRITET DNK

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Abstract

Introduction: Fixation with formalin, a widely used procedure to preserve tissue samples, leads to damage of DNA through crosslinking activity. The factors that could influence the fixation and integrity of DNA may be numerous: incubation time, tissue type, concentration of formalin, temperature, pH and viscosity.

Aim: The aim of this investigation was to examine the influence of incubation time and temperature of formalin fixation on the yield, purity and integrity of DNA isolated from healthy human heart myocardial tissue taken during medico-legal autopsy.

Material and methods: Heart tissue samples were fixed in phosphate-buffered formalin at +4°C in the dark, as well as at room temperature in the presence of light. The DNA was isolated after one day, then successively every day during the first week, and then on the tenth day, and after two and four-week periods using phenol-chloroform-isoamyl alcohol extraction method. The absorbances were measured at 260 nm and 280 nm, which allows calculation of yield and purity of nucleic acid in the samples. The PCR amplification of two genes, GPDH (150 bp) and β -actin (262 bp), were performed to evaluate the degree of DNA molecule fragmentation.

Results: The highest yield, purity and preserved integrity of DNA were obtained from the samples fixed in formalin at +4°C in the darkness. In these samples, GPDH and β -actin genes were amplified up to 14 days, unlike the samples that were fixed at the room temperature in which the β -actin gene was amplified up to 5 days, while the GPDH gene fragment was successfully amplified up to 10 days of fixation.

Conclusion: The temperature, presence of light and the incubation time of formalin fixation all have important influences on yield, purity and integrity of DNA during the fixation process.

Keywords:

formalin,
PCR amplification,
autopsy,
DNA degradation,
spectrophotometry



Sažetak

Uvod: Fiksacija tkiva formalinom, koja predstavlja široko korišćenu proceduru za konzervaciju tkiva, dovodi do oštećenja molekula DNK. Faktori koji mogu da utiču na proces fiksacije i integritet molekula DNK su brojni: dužina inkubacije, vrsta tkiva, koncentracija formalina, temperatura, pH, viskozitet.

Cilj: Cilj ovog istraživanja je ispitati uticaj vremena inkubacije i temperature fiksativa na prinos, čistoću i integritet DNK izolovane iz zdravog humanog srčanog tkiva, uzetog tokom sudsko-medicinske obdukcije.

Materijal i metode: Uzorci tkiva srca su fiksirani u fosfat-puferizovanom formalinu na +4°C u mraku, kao i na sobnoj temperaturi u prisustvu svetlosti. Dezoksiribonukleinska kiselina (DNK) je izolovana nakon jednog dana, potom sukcesivno svakog dana tokom prve nedelje, zatim desetog dana, i nakon dve i četiri nedelje korišćenjem metode ekstrakcije pomoću fenol-hloroform-izoamil alkohola. Apsorbance su izmerene na 260 nm i 280 nm, što je omogućilo izračunavanje prinosa i čistoće nukleinskih kiselina u uzorcima. Urađena je amplifikacija dva gena lančanom reakcijom polimeraze (PCR), glicerol-3-fosfat dehidrogenaze (GPDH) (150 bp) i β -actin-a (262 bp), kako bi se procenio stepen fragmentacije molekula DNK.

Rezultati: Najveći prinos, čistoća uzoraka, kao i očuvan integritet molekula DNK dobijeni su u uzorcima tkiva koji su fiksirani u formalinu na +4°C u mraku. U ovim uzorcima GPDH i β -actin geni su amplifikovani do 14-og dana inkubacije u formalinu, za razliku od uzoraka koji su fiksirani na sobnoj temperaturi, a u kojima je β -actin gen amplifikovan do petog dana, a GPDH do desetog dana fiksacije.

Zaključak: Temperatura formalina i dužina fiksacije u formalinu imaju značajan uticaj na prinos, čistoću i integritet molekula DNK.

Ključne reči:

formalin,
PCR amplifikacija,
obdukcija,
degradacija DNK,
spektrofotometrija

Introduction

Forensic genetics is a young interdisciplinary science which found a wide application in medico-legal practice (1). The purpose of deoxyribonucleic acid (DNA) analysis has a practical basis since the DNA molecules are unique. Among more than 7 billion people inhabiting our planet today and about 5 billion people who have settled in the past 200,000 years, there are no two persons (with the exception of monozygous twins) who had or have identical DNA molecules. Forensic analysis of DNA molecule is important in identifying a person, clarification of cases of criminal activity, determination of paternity, identifying victims of disasters, etc. Biological samples from which it is possible to isolate genomic DNA molecules are cells with a nucleus. An example of such samples are blood and bloodstains, saliva, urine, semen and seminal stains, hairs, teeth, bones, fingernails and other body fluids, tissues and organs. During the autopsy, tissue samples are taken, fixed in formalin and then embedded in paraffin. Tissue fixation is an essential step in the processing since it stops the process of decomposing and preserves the tissue morphology. It's known that prolonged tissue fixation time in formalin leads to degradation of DNA molecules, limiting the usage of these fragments in forensic genetics (2,3). Formalin leads to the formation of the cross-links between DNA and proteins (4). Therefore, it was of great importance to test how long the tissue can be fixed in formalin. The usual practice after a forensic autopsy is that the tissue fixation process is prolonged for more than a few days, and during that period the samples are not stored on +4°C. Besides the type of fixative, also the

duration of fixation, temperature, tissue type, amount of tissue and degree of autolysis impact on DNA yield and integrity (5). Due to great significance of tissue compartments, as carriers of DNA molecules, they can be used as material evidence in court processes. The main goal of this research was to investigate the yield, purity and degree of fragmentation of DNA molecules isolated from human healthy heart tissue samples were taken during a medico-legal autopsy in the Department for Forensic Medicine and Toxicology, Clinical Center of Kragujevac, Serbia. The influence of the length of fixation of heart tissue in phosphate-buffered formalin at +4°C in the dark or at room temperature in the presence of light on the integrity of DNA, isolated with phenol-chloroform-isoamyl alcohol, was studied.

Material and methods

Ethics statement

The Ethics Committee of the University of Kragujevac, Faculty of Medical Sciences, the Ethics Committee of Clinical Center of Kragujevac, Appeal Public Prosecutor's Office from Kragujevac and Higher Court in Kragujevac approved these investigations.

Tissue collection

In this study, the heart muscle tissue samples were used taken from two healthy persons who suddenly died due to violent death. The bodies of the deceased were kept

at +4°C, 24h after death and after that, the autopsy was performed. One part of the examined tissue samples were fixed in phosphate-buffered formalin (Alfapanon 10%) at +4°C in the dark, while the remaining was fixed in phosphate-buffered formalin at room temperature in the presence of light. The samples, with weight $0,091 \pm 0,009$ g, were placed in formalin in a ratio of at least 20 parts of fixative to one part of the tissue (v/v). Duration of formalin fixation was as follows: one day, then successively isolated every day during the first week, on the tenth day, and later after two and four weeks. The control samples were parts of tissue without formalin fixation, from which DNA was isolated immediately after autopsy.

DNA isolation and agarose gel electrophoresis

DNA was isolated from two samples from each heart, for each time point. For phenol-chloroform-isoamyl alcohol (PCI) extraction, 300 µL of digestion TNS buffer (TE buffer, 10% SDS, 3 M NaCl, ampoules-deionized water) and 50 µL of proteinase K (Thermo Scientific) were added to the macerated samples and incubated overnight at 56°C. An equal volume of phenol-chloroform-isoamyl alcohol solution (25:24:1) was added to each sample and centrifuged for 5 minutes at 4000 rpm at 4°C. The supernatant was transferred to a new tube and an equal volume of chloroform-isoamyl alcohol solution (24:1) was added and centrifuged for 5 minutes at 4000 rpm at 4°C. The supernatant was transferred to a new tube. The DNA was precipitated with 2.5 volume of 100% ice-cold ethanol and 0.1 volume of 3 M NaCl, followed by overnight storage at -20°C. The precipitated DNA was centrifuged 30 minutes at 15 000 rpm at 4°C, and the supernatant removed and added into pellets 70% ice-cold ethanol followed by centrifugation 15 minutes, at 15 000 rpm at 4°C. The supernatant was removed and the pellets were dried at room temperature for a few hours. The DNA was resuspended in the 50 µL of TE buffer (10 mM Tris-HCl and 1mM EDTA) and stored at -20°C.

Spectrophotometry

Yield and purity of the DNA samples were obtained spectrophotometrically (UV-1800 Shimadzu UV spectrophotometer, Japan). The absorbances were measured at 260 nm and 280 nm. The reading at 260 nm allows calculation of the yield of nucleic acid per 1 mg per tissue. One OD unit corresponds to approximately 50 µg/ml of DNA. The OD260/OD280 ratio represents the ratio of absorbance of nucleic acids and proteins and provides an estimate of the purity of the DNA. Pure preparations of DNA have OD260/OD280 values more than 1,5.

Polymerase Chain Reaction

The fragments of GPDH (glycerol-3-phosphate dehydrogenase) and β-actin (ACTB actin beta) genes were amplified from isolated DNA using PCR. For all PCRs that were performed 4 samples were taken per each time point or each fixation method. The PCR amplification was performed in the final volume of 25 µl containing: 12.5 µl One Taq 2x Master Mix with Standard Buffer (New England Biolabs

Inc.); primers (0.5 µl of 10 µM forward primer and 0.5 µl of 10 µM reverse primer (Invitrogen by Thermo Fisher Scientific), 1µl of DNA sample (approximately 1 ng/µl of genomic DNA) and sterile bidistilled water up to 25 µl. The nucleotide sequences of primers (Invitrogen by Thermo Fisher Scientific) are shown in **Table 1**. The PCR amplification was performed in a PCR apparatus (Techne genius, PCR Thermal Cycler Model FGEN02TP, Eppendorf). The following PCR conditions were used for each PCR: initial denaturation at 94°C, 30 seconds; 30 cycles of 94°C, 30 seconds, 60°C 1 minute, and 68°C 1 minute; final extension at 68°C for 5 minutes. Negative controls, as well as positive controls, were included in each manipulation. Negative controls contained all PCR compounds except the DNA. The positive control is DNA isolated from the tissue immediately after the autopsy. The PCR products were visualized on 2% agarose gel stained with ethidium bromide (10 mg/ml). After electrophoresis, the gel was photographed under ultraviolet light.

Table 1. Primers used for PCR and amplicons length (bp)

Primer name	Primer sequence	Amplicon length (bp)
GPDH-20 F GPDH-20 R	CAG ATGCCCCAGGTGAGTGAA ACTGCCTCACTCCTTACTCCT	150
β-actin-20 F β-actin-20 R	TGCTAAAGACCGTGGGGAAC TGTGACCCCTTTC TCCTCA	262

Statistical analysis

SPSS version 20.0 was used for statistical analysis and p-value < 0,05 was considered statistically significant. For comparing the differences between two groups, samples were kept at different temperatures (formalin fixation at +4°C and at room temperature) and the non-parametric Mann Whitney test was used. The Friedman test was applied to determine the differences between the different time points.

Results

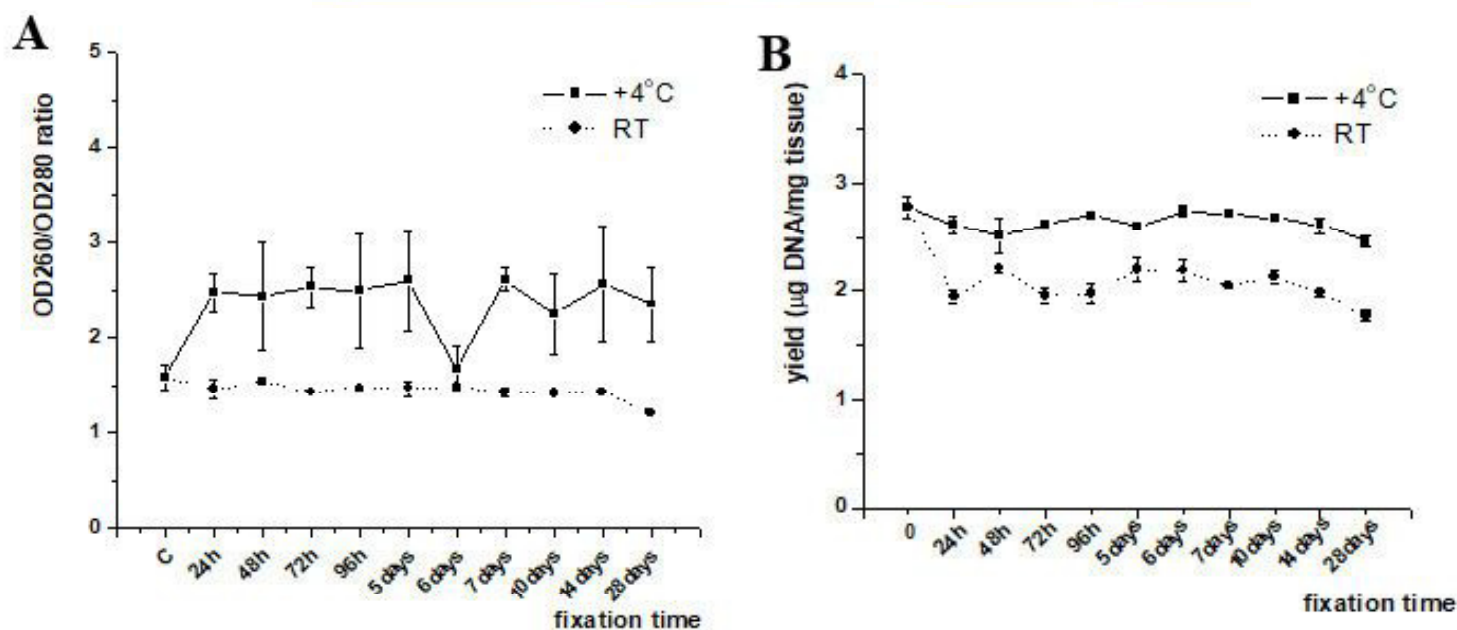
The results shown in **Table 2** represent measured values of OD260 and OD280, as well as values of yield and purity of DNA isolated from heart muscle and fixed in phosphate-buffered formalin. The yield of isolated DNA was satisfactory. Total yield and quality of DNA extracted were highly dependent on the fixation method used (**Table 2** and **Figure 1**).

Based on the spectrophotometric results, we conclude that a higher OD260/OD280 ratio ($U = 10,000$; $p = 0,000$) of DNA (**Figure 1A**), as well as higher yield ($U = 3,000$; $p = 0,000$) (**Figure 1B**), was obtained from the samples fixed at +4°C in the darkness, in comparison with samples fixed at room temperature in the presence of light and in comparison with control samples. These differences show a high statistical significance.

The method of formalin fixation has a statistically significant impact ($U = 16,000$; $p = 0,025$) on the efficiency

Table 2. The values of absorbance measured on 260 nm and 280 nm and estimated values of purity and yield of DNA (μg DNA per 1 mg of tissue) isolated from human healthy heart tissue fixed in phosphate-buffered formalin at $+4^\circ\text{C}$ in the darkness or at room temperature (RT) on the light.

College	OD260	OD280	OD260/OD280	Yield [μg DNA/mg tissue]
Cold fixation ($+4^\circ\text{C}$)				
Control	0,36 \pm 0,01	0,10 \pm 0,01	1,58 \pm 0,13	1,77 \pm 0,30
24 h	0,22 \pm 0,01	0,08 \pm 0,01	2,48 \pm 0,19	1,61 \pm 0,08
48 h	0,18 \pm 0,01	0,06 \pm 0,01	2,43 \pm 0,57	1,52 \pm 0,16
72 h	0,22 \pm 0,01	0,08 \pm 0,01	2,53 \pm 0,21	1,62 \pm 0,01
96 h	0,18 \pm 0,01	0,06 \pm 0,01	2,49 \pm 0,59	1,70 \pm 0,02
5 days	0,19 \pm 0,01	0,06 \pm 0,01	2,59 \pm 0,52	1,60 \pm 0,01
6 days	0,21 \pm 0,01	0,13 \pm 0,02	1,67 \pm 0,23	1,74 \pm 0,05
7 days	0,21 \pm 0,01	0,07 \pm 0,01	2,61 \pm 0,12	1,72 \pm 0,01
10days	0,21 \pm 0,01	0,07 \pm 0,01	2,25 \pm 0,42	1,68 \pm 0,01
14 days	0,19 \pm 0,01	0,06 \pm 0,01	2,56 \pm 0,61	1,61 \pm 0,07
28 days	0,17 \pm 0,01	0,06 \pm 0,01	2,35 \pm 0,39	1,46 \pm 0,05
Range	0,17-0,22	0,06-0,13	1,67-2,75	1,46-1,77
RT fixation				
Control	0,36 \pm 0,01	0,10 \pm 0,01	1,58 \pm 0,13	1,77 \pm 0,30
24 h	0,14 \pm 0,01	0,09 \pm 0,01	1,45 \pm 0,09	1,95 \pm 0,06
48 h	0,15 \pm 0,01	0,10 \pm 0,01	1,54 \pm 0,03	1,21 \pm 0,03
72 h	0,13 \pm 0,01	0,09 \pm 0,01	1,43 \pm 0,01	1,90 \pm 0,07
96 h	0,14 \pm 0,01	0,09 \pm 0,01	1,47 \pm 0,02	1,98 \pm 0,09
5 days	0,16 \pm 0,01	0,11 \pm 0,01	1,47 \pm 0,08	1,30 \pm 0,10
6 days	0,15 \pm 0,01	0,10 \pm 0,01	1,48 \pm 0,02	1,19 \pm 0,09
7 days	0,16 \pm 0,01	0,11 \pm 0,01	1,42 \pm 0,04	1,06 \pm 0,02
10days	0,16 \pm 0,01	0,12 \pm 0,01	1,41 \pm 0,01	1,13 \pm 0,06
14 days	0,14 \pm 0,01	0,10 \pm 0,01	1,43 \pm 0,01	1,98 \pm 0,03
28 days	0,11 \pm 0,01	0,09 \pm 0,01	1,21 \pm 0,02	1,78 \pm 0,05
Range	0,11-0,16	0,09-0,12	1,21-1,60	1,78-1,77

**Figure 1.** Differences in purity (A) and yield (B) of DNA isolated from human healthy heart tissue fixed in phosphate-buffered formalin at $+4^\circ\text{C}$ in the darkness or at room temperature (RT) on the light.

of the amplification of the selected genes (**Figure 2**) in different time points. The PCR amplicons could be detected in all DNA samples, isolated from formalin-fixed heart tissues at $+4^\circ\text{C}$ in the darkness up to 14 days of fixation (**Figure 2A**). After this time point, there are no visible amplicons on the agarose gel. The results of amplification and gel electrophoresis of DNA from heart samples fixated at room temperature to the light show a successful amplification of both genes up to 5 days of fixation (duplex PCR), while GPDH gene has been successfully amplified after 10 days of fixation (**Figure 2B**). There was no gene amplification in the tissue samples fixed after the indicated incubation period.

Discussion

Molecules of DNA are often degraded in biological materials due to the influence of environmental factors that may be of chemical (acids, bases, oxidizing agents), physical (temperature, sunlight, humidity) or biological (bacteria, fungi) origin. The most common type of degradation of DNA molecules is the fragmentation of long chains into smaller fragments, sizes up to several hundred nucleotides and less. Biological materials are often scarce or contain some inhibitory substances that can make it difficult to isolate DNA molecules from the sample. As a

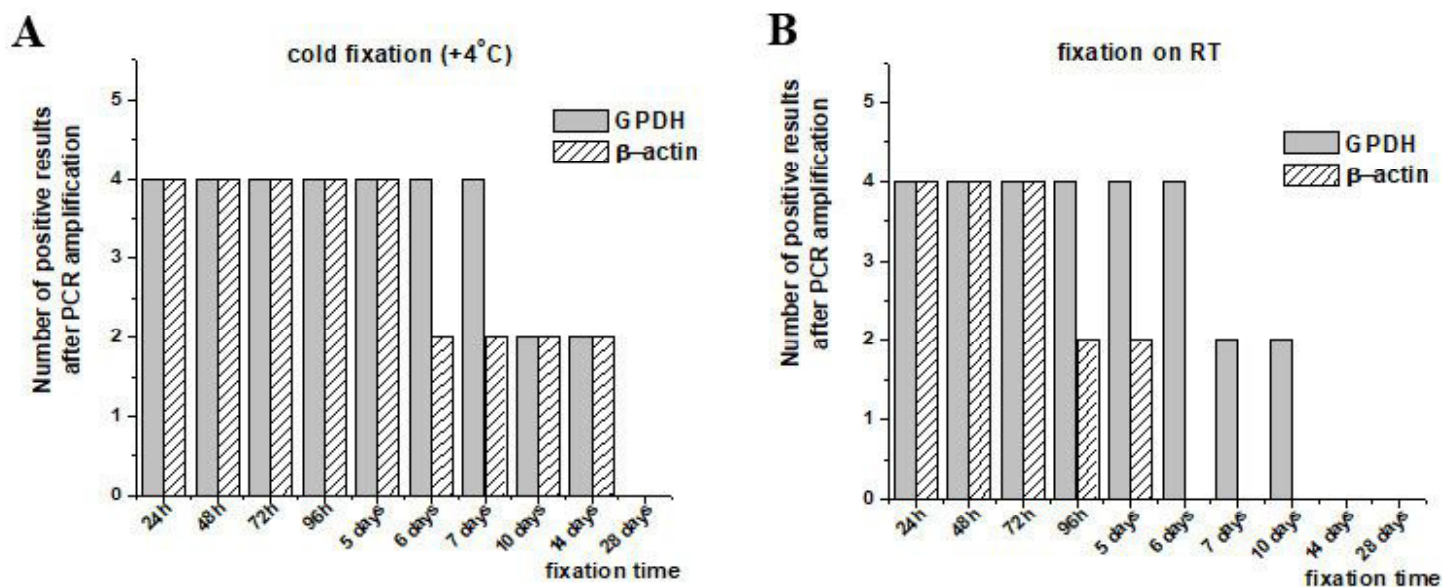


Figure 2. Comparison of successful amplification of GPDH and β -actin genes in DNA samples isolated from human healthy heart tissue fixed in phosphate-buffered formalin at + 4°C in the darkness (A) or at room temperature (RT) on the light (B).

result, a small amount of DNA from the samples is obtained. The discovery of the polymerase chain reaction (PCR), based on the replication of the DNA segments under strictly controlled conditions, resulting in an exponential increase in the number of DNA segments in the reaction mixture, allowed the application of this method in forensic genetics in the biological traces analysis with great success (6).

Archival fixed tissues from autopsy are potentially large source for forensic testing (7). In retrospective studies, preserved tissues are often the only accessible biological samples. Tissue samples are collected primarily for the histopathological analyses, but it is identified that this biological samples are suitable for the molecular analyses. In our laboratory, different tissues taken during autopsy are fixed in formalin, embedded in paraffin blocks and kept in the archives for many years. However, the usability of these samples has certain limitations. A crucial step in the procedure of preserving tissue samples in paraffin blocks is fixation with formalin. Longer duration of formalin fixation adversely affects the quality of tissue DNA, but presumably has no effect on histopathology (8). The 4% formaldehyde solution in water (formalin) has been used since XIX century with a few minor changes (9). The last decades has been in use neutral buffered formalin (NBF), buffered with several different buffers such as calcium carbonate, magnesium carbonate, citrate, Tris and most commonly phosphate buffers (5). Fixation helps to preserve morphology and structure of cells in the tissues, so that they can be studied (10). Problem with using formalin as fixative is generation of cross-linking between nucleic acids and proteins (4) by forming methylene bridges between the amino groups of purine and pyrimidine bases as well as between these nucleotide bases and histones (11-13), also cutting of the phosphodiester backbone of DNA (11) which leads to nucleic acids fragmentation. However, these chemical reactions cannot be solely responsible for fragmentation and degradation of nucleic acids. Different reaction conditions can strongly influence the nature, yield and half-life of chemical

modifications. The factors that could influence the fixation are tissue type, incubation time, concentration of formalin and other conditions (temperature, pH, viscosity etc) (14). Since there are different data in the literature on the influence of various factors on tissue fixation, it is important to define the conditions and to align the formalin fixation protocols of the tissue, so that a quality DNA from these samples can be obtained later.

The quality of DNA isolated from different organs often varies due to the variation in cellular composition of different tissues. Also, different tissues give different yield of DNA molecules. Tissue samples isolated from organs which have high cell density, such as heart, brain, liver and spleen tissue, have been use for molecular analysis because it shown better PCR amplification of DNA molecule (15). DNA isolated from chest cavity organs (heart and lungs) shows better quality than DNA that is isolated from tissue originating from the abdominal cavity, due to pancreatic enzymes and accelerated bacterial growth leading to faster tissue lysis (16). Our results are consistent with literature data. The yield and the purity of DNA isolated from heart tissue were satisfactory. However, the temperature of tissue fixation has a significant effect on the yield and on the purity of the DNA obtained. Better yield and purity DNA were obtained at a lower temperature. A low fixation temperature reduces the activity of enzymes in the cell (including enzymes that degrade DNA and proteins), resulting in higher yields and lower degradation of isolated DNA (12).

The temperature of heart tissue formalin fixation also affected amplifiability of extracted DNA. Based on PCR amplification performance, the samples that were at +4°C during formalin fixation produced better results. In our study, the GPDH gene, 150bp in length, as well as β actin gene - 262 bp, were amplified in heart samples up to 14 days at cold formalin fixation (+4°C), but only up to 5 days (β actin gene) and 10 days (GPDH gene) in samples that were fixed at room temperature. A few studies have shown that low temperatures and short fixation time decrease the

extent of DNA degradation (14). The speed of formalin fixation process depends on the rate of diffusion of formalin across the tissue and the rate of chemical reaction with cellular components (17). Formalin fixation process requires at least 1 h per 1 mm of tissue thickness. In practice, it was recommended that the duration of formalin fixation is up to 48 h (18). Our results have shown that the formalin fixation incubation time can be longer, without significant consequences on DNA amplifiability. Moreover, increase in temperature of formalin fixation will favor the disassociation of formaldehyde from the polymers. A low fixation temperature reduces the activity of enzymes in the cell (including enzymes that degrade DNA and proteins), resulting in higher yields and lower degradation of isolated DNA (12).

In conclusion, the presently proposed procedure of tissue formalin fixation results in lower degree of DNA fragmentation. Fixation of the tissue in the buffered formalin up to +4°C, in the dark, improves the degree of preservation of molecular structures in the tissue over a longer period of time than fixation at room temperature in the presence of light. Fixation of tissues longer than two weeks, in a buffered formalin up to +4°C in the dark, leads to extensive fragmentation of DNA, making the samples unusable for STR typing after this period. The tissue fixation in buffered formalin at room temperature in the presence of light longer than 5 days, leads to fragmentation of DNA, and the samples are subsequently useless in DNA analysis. Considering the usual practice that the samples are not incubate in formalin in the refrigerator, it is important to emphasize that in this case it is necessary to shorten the length of formalin incubation in order to obtain unfragmented DNA. The possibility of obtaining high quality DNA from archival tissues gives prospects for wider molecular analysis and profiling than presently feasible.

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