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FORMALIN FIXATION OF HUMAN HEALTHY AUTOPSIED TISSUES: THE INFLUENCE OF TYPE OF TISSUE, TEMPERATURE AND INCUBATION TIME ON THE QUALITY OF ISOLATED DNA

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FIKSACIJA HUMANIH ZDRAVIH AUTOPSIJSKIH TKIVA FORMALINOM: UTICAJ VRSTE TKIVA, TEMPERATURE I VREMENA INKUBACIJE NA KVALITET IZOLOVANE DNK

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ABSTRACT

Formalin fixation is a widely used method in histopathology that has certain limits. Formalin often leads to the degradation of DNA molecules in cancer tissues, which makes tissues unusable for molecular analysis. The other factors may also affect the quality of DNA isolated from fixed tissues. The aim of this study is to determine the impact of the incubation time and temperature on the quality of DNA molecules isolated from various healthy human tissues. The brain, lung and kidney tissues, excluded during the forensic autopsies of people who died of violent death, were fixed in phosphate-buffered formalin from 24h to two months. After the completion of the incubation period, the DNA was isolated using phenol-chloroform-isoamyl alcohol extraction method and the concentration and purity of the samples were determined spectrophotometrically. The degree of degradation of DNA was assessed by PCR reaction, by amplification of gene fragments which lengths were 150bp (GPD1) and 262bp (β-actin). The highest concentration, purity and preserved integrity of DNA were obtained from the brain samples. With prolonged tissue incubation times in formalin, the concentration and integrity of DNA decreased in all tissue samples, especially in the brain tissue, while the purity of DNA remained unchanged. Also, tissue fixation at +4°C contributed to a better quality of isolated DNA compared to DNA isolated from tissue fixed at room temperature. We can conclude that the type of human healthy tissue, temperature and the incubation time of formalin fixation have important influence on the concentration, purity and integrity of DNA during fixation of tissues excluded in the course of forensic autopsy.

***Keywords**: forensic autopsy, formalin, DNA isolation, PCR, spectrophotometry.*

SAŽETAK

Formalinska fiksacija je široko korišćena metoda u histopatologiji, koja ima i određena ograničenja. Formalin često dovodi do degradacije molekula DNK u tkivu tumora, što čini fiksirano tkivo neupotrebljivim za molekularnu analizu. I drugi faktori mogu uticati na kvalitet molekula DNK izolovanih iz fiksiranih tkiva. Cilj ovog rada je utvrditi uticaj dužine inkubacije tkiva u formalinu i uticaj temperature na kvalitet molekula DNK izolovanih iz različitih zdravih ljudskih tkiva. Tkiva mozga, pluća i bubrega, koja su izuzeta tokom sudsko-medicinskih obdukcija ljudi koji su umrli nasilnom smrću, fiksirana su u puferizovanom formalinu od 24h do dva meseca. Nakon isteka određenog inkubacionog perioda, iz tkiva je izolovana DNK ekstrakcijom pomoću fenol-hloroform-izoamil alkohola i spektrofotometrijski je određena koncentracija i čistoća uzoraka. Stepen degradacije molekula DNK procenjen je PCR reakcijom, amplifikacijom fragmenata gena dužine 150bp (GPD1) i 262bp (β-actin). Najveća koncentracija, čistoća i očuvan integritet DNK dobijeni su iz tkiva mozga. Sa produženim vremenom inkubacije tkiva u formalinu koncentracija i integritet DNK opadaju u svim ispitivanim tkivima, posebno u mozgu, dok čistoća uzoraka ostaje konstantna. Takođe, fiksacija tkiva na +4°S doprinosi boljem kvalitetu izolovane DNK u odnosu na DNK izolovane iz tkiva fiksiranih na sobnoj temperaturi. Može se zaključiti da vrsta zdravih humanih tkiva, temperatura i vreme inkubacije tkiva u formalinu imaju značajan uticaj na koncentraciju, čistoću i integritet DNK tokom procesa fiksacije tkiva izuzetih tokom sudsko-medicinske obdukcije.

***Ključne reči:** sudsko-medicinska obdukcija, formalin, izolovanje DNK, PCR, spektrofotometrija.*

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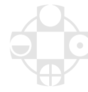
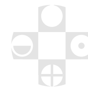


ABBREVIATIONS

AP – apurine or apirimidine sites
bp – base pairs
DNA - deoxyribonucleic acid
EDTA - ethylenediaminetetraacetic acid
FFPE – formalin fixed paraffin embedded
GPD1 - glycerol-3-phosphate dehydrogenase 1
NaCl - sodium chloride
OD – optical density
PCI - phenol-chloroform-isoamyl alcohol
PCR - polymerase chain reaction
RT – room temperature
SNP - single nucleotide polymorphisms
STR – short tandem repeats
TBE - Tris/Borate/EDTA buffer
TE - Tris/EDTA buffer
TNS – TE/NaCl/SDS buffer

INTRODUCTION

The post-mortem molecular analyses of the nuclear acids are becoming increasingly common in the epidemiological studies and in retrospective investigations, with the aim to identify genetic factors that cause death and rare diseases(1). Although blood samples are often used, formalin fixed paraffin embedded (FFPE) tissues are a suitable biological sample for the performance of a molecular autopsy (2). The different preserved tissues, excluded during a forensic autopsy, are often the only accessible samples in retrospective studies. However, in order for tissue samples to be used for molecular analysis, DNA molecules must be preserved. It is known that a large number of factors disturb the molecular profile of cells and tissues, so it is necessary as soon as possible to optimize the appropriate methodological approaches used in tissue preservation. The factors that affect the integrity of DNA in preserved tissues include factors on pre-fixation level (proper exclusion of tissue during autopsy and pre-fixation time), the type and characteristics of fixative and post-fixation parameters (paraffinization and deparaffinization procedure, DNA isolation method, storage) (3). These factors influence the yield and purity of the DNA obtained, but also lead to degradation of this molecule, which reduces its utilization in molecular analyses. The most frequently type of DNA degradation is fragmentation of polynucleotide chains of DNA into smaller fragments, sizes up to several hundred nucleotides or less (4). As a consequence, a small amount of usable DNA is obtained.

Formalin is the longest and most commonly used fixative in histopathology and protocols for formalin fixation of a tissue have changed over time. In previous years, the only requirement in tissue fixation was that the tissue morphology was preserved. However, with the emergence of immune-histochemical and molecular-biological analyses, and tissue formalin fixation protocols have become more stringent. More recently, the key requirement in forensic genetics is the preservation of the nucleic acid in formalin-fixed tissues, since large tissue archives would be available for profiling gene expression in order to generate new and reliable diagnostic and prognostic parameters (5, 6). It is known that the main effect of formaldehyde in the tissue is associated with the formation of methylol groups on the amino groups of nitrogenous bases in the DNA molecule and the establishment of cross-linking of methylene groups, leading to the correct fixation of a tissue (7). The formation of numerous strong covalent bonds in the DNA molecule leads to its fragmentation. Literature data show that the cross-linking of nucleic acids, between themselves and with proteins, cannot be solely responsible for the degradation of DNA and RNA that by maintaining a low temperature throughout the fixation process, the degree of nucleic acid degradation can be reduced (8).

Since there are not many studies analyzing the effects of phosphate-buffered formalin on the quality of isolated DNA, especially from healthy human tissues excluded during medico-legal autopsies, this study provides some important information on the usability of archived formalin fixed tissues in practice in molecular autopsy. Based on previous hypothesis, the goals of our research were also set. The aim of this study was to determine whether tissue fixation in formalin at low temperatures (+4°C) would contribute to preserving the quality of DNA molecules while preserving the morphological properties of a fixed tissue. We compared concentration, purity and integrity of DNA isolated from healthy, autopsy tissues fixed at +4°C or at room temperature (RT) in phosphate-buffered formalin for different time points.

Material and methods

Ethic statement

The Ethics Committee of the University of Kragujevac, Faculty of Medical Sciences (No. 01-4970), the Ethics Committee of Clinical Center of Kragujevac (No. 01-2798), Appeal Public Prosecutor's Office from Kragujevac (No. 79/13) and Higher Court in Kragujevac (SU-VIII-110/13) agreed to these investigations.

Tissue selection and processing

The healthy brain, lung and kidney tissues were taken from four people (two women and two men, aged between 23 and 42) who suddenly died a violent death. The corpses were on +4ºC up to 24h before autopsy was done. During the autopsy, the tissues were collected and immediately fixed in

phosphate-buffered formalin (Alfapanon 10%): one half of each tissue was fixed on +4ºC and the other half was fixed at room temperature (RT). The size of each tissue sample was about 5x5x3mm. The fixation was prepared in the formalin solution in a ratio of 20 parts fixative to one part of the tissue (v/v). The tissues were fixed 24h, 48h, 72h, 96h, 5 days, 6 days, 7 days, 14 days, 28 days and 2 months. The control tissue samples were prepared for experiments immediately after autopsy.

DNA isolation

The DNA was isolated from all tissue samples using phenol-chloroform-isoamyl alcohol (PCI) extraction method. The samples were digested in TNS buffer (TE buffer, 3M NaCl, 10% SDS, ampoules-deionized water) with proteinase K (Thermo Scientific) at 56ºC, overnight. In each sample, an equal volume of PCI solution (in ratio 25:24:1) was added and centrifuge at 4000 rpm at +4ºC, 5 min. Thereafter, the supernatant was transferred into a new tube, an equal volume of chloroform-isoamyl alcohol solution (in ratio 24:1) was added and centrifuged in the same manner. The DNA in the supernatant was precipitated using 100% ice-cold ethanol and 3M NaCl at -20ºC, overnight. After centrifugation at 15000 rpm on +4ºC, 30 min, the supernatant was removed. The 70% ice-cold ethanol was added on the pellet, centrifuged at 15000 rpm on +4ºC, 15 min, supernatant was removed and the pellet was dried at RT, for a few hours. The DNA from the pellet was resuspended in the 50 µl TE buffer (10mM Tris-HCl and 1mM EDTA) and stored at -20ºC.

Spectrophotometric quantification of DNA

The quantification of the concentration and purity of DNA were determined using spectrophotometer (UV–1800 Shimadzu UV spectrophotometer, Japan) and measuring absorbance on 260nm and 280nm. One OD260 unit corresponds to approximately 50 µg/ml DNA. The ratio OD260/OD280 indicates protein contamination and pure samples have values more than 1.5.

Polymerase chain reaction - PCR

All PCR reactions were prepared in duplicates (8 samples per each time point and each fixation method). Two primer pairs targeting two different human house-keeping genes, GPD1 - glycerol-3-phosphate dehydrogenase and β-actin – ACTB actin beta, were designed for the amplification of DNA fragments with length 150 bp and 262 bp, respectively (Table 1). The PCR reaction mix contained One Taq 2x Master Mix with Standard Buffer (New England Biolabs Inc.), primers (forward and reverse) (Invitrogen by Thermo Fisher Scientific) and approximately 1 ng/µl of isolated genomic DNA in sterile bidistilled water in the final volume of 25µl. The PCR reactions were performed in a PCR Techne, Eppendorf under the following amplification conditions: initial denaturation at 94°C for 30 seconds; 30 cycles of 94°C for 30 seconds, 60°C for 1 minute, and 68°C for 1 minute; final extension at 68°C for 5 minutes. The positive control (PCR reaction mix contained all PCR compounds with DNA isolated from adequate tissue immediately after autopsy) and negative control (PCR reaction mix contained all PCR compounds except DNA) were included in all PCR reactions.

Agarose gel electrophoresis

The PCR products were visualized by 2% agarose gel electrophoresis. The agarose powder was dissolved in TBE buffer (Tris-base, boric acid, EDTA in bidestillated water) in concentration of 2% and stained with ethidium bromide (0.5µg/ml). The agarose electrophoresis was performed in TBE buffer on 100V, 55 minutes. The results were visualized under the UV lamp and photographed.

Statistical analysis

The SPSS version 20.0 software package for Windows was used to perform the statistical significance of group differences. For comparing the differences between formalin fixation at +4°C and at RT the Mann Whitney test was used. The Kruskal Wallis test was applied to estimate the differences between the different tissues, while the Friedman test was applied to determine the differences between the different time points. P values below 0.05 were accepted as statistically significant.

Table 1. The nucleotide sequences of primers and length of the amplicons (bp)

Primer name	Primer sequence	Amplicon length (bp)
GPD1 -20 F	CAGATGCCCAGGTGAGTGAA	150
GPD1-20 R	ACTGCCTCACTCCTTACTCCT	
ACTB-20 F	TGCTAAAGACCGTGGGGAAC	262
ACTB-20 R	TGTGACCCCTTTCTCCCTCA	

RESULTS

In this study, we compared the quantities (DNA concentration, mg/μl) and qualities (DNA purity and amplifiability) of DNA isolated from different healthy human tissues (brain, lung and kidney) which were fixed up to 2 months using different formalin fixation methods (at RT as well as at +4ºC).

The concentrations of DNA varied depending on the type of tissue samples, formalin incubation time as well as on fixation method and these differences were statistically significant. The highest DNA concentration (6.51 mg/μl) was obtained from the brain tissue immediately after autopsy (Table 2). However, with prolonged incubation time in formalin, concentration of DNA significantly decreased in all examined tissues, especially in the brain tissue fixed at RT up to 2 months (2.70 mg/μl) (p=0.000). After prolonged formalin fixation at RT, the highest DNA concentration was measured in kidney (mean value was 4.32±0.74 mg/μl) and the worst result was obtained in the brain tissue (mean value was 3.84±0.97 mg/μl) (p=0.000). Also, statistically significant higher concentration of DNA was obtained from all tissue samples fixed at +4ºC in comparison with samples fixed on RT. With the increase of formalin fixation time at +4ºC, the concentration of DNA decreased significantly (p=0.000) in all examined tissues (Figure 1), with the highest DNA yield from the kidney tissue (mean value was 5.52±0.39 mg/μl) and the lowest from the brain tissue (mean value was 4.43±0.71 mg/μl) (Table 2).

Table 2. The concentration (mg/μl) and rate of purity (OD260/OD280) of DNA isolated from different healthy tissue samples fixed in phosphate-buffered formalin up to 2 months

		Concentration (mg/μl ± SD)		**Purity (OD260/OD280)**	
tissue	**Type of fixation**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
brain	**RT**	3,84±0,97	2,70-6,51	1,50±0,14	1,44-1,95
	+4°C	4,43±0.71	3,55-6,51	1,88±0,10	1,76-2,04
lung	**RT**	4,22±0,57	3,48-5,27	1,53±0,12	1,40-1,87
	+4°C	4,59±0,43	4,03-6,02	1,95±0,038	1,87-2,01
kidney	**RT**	4,32±0,74	3,29-5,51	1,56±0,11	1,47-1,90
	+4°C	5,52±0,39	4,67-6,02	1,89±0,04	1,83-1,98

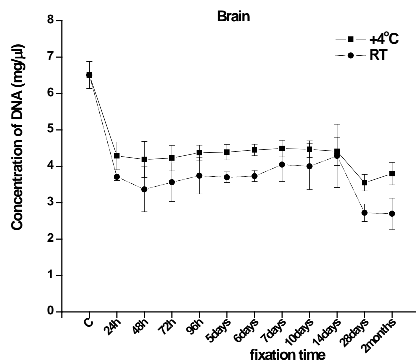
The purity of DNA extracted from human healthy tissues that were excluded 24h after death and fixed in phosphate-buffered formalin was satisfactory with range from 1.40 to 2.00. Based on the OD260/OD280 ratio, we conclude that the highest degree of purity of DNA was obtained in brain tissue samples fixed at +4ºC, but the differences among the different tissues are not statistically significant (p=0.167). Also, DNA isolated from all tissue samples has a statistically significant (p=0.000) higher purity after fixation at +4ºC in comparison with samples fixed at RT (Table 2 and Figure 1). With the increase of formalin fixation time, the purity of DNA isolated from all three examined tissues decreased, which was statistically significant (p=0.000).

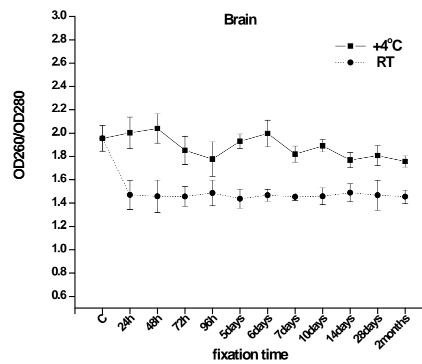
The integrity of DNA was estimated due to the amplifiability of isolated DNA. Based on PCR amplification performance, DNA isolated from tissue samples that were fixed in phosphate-buffered formalin at +4ºC had better preserved integrity than DNA isolated from tissues fixed at RT (p=0.001) (Figure 2). Amplification of the 150bp GPD1 gene fragment produced positive results in all tissue samples fixed at +4ºC up to 28 days, but only in the brain tissue fixed at RT up to 28 days. In lung and kidney tissues fixed at RT, GPD1 amplicons were visible up to 14 days. The amplification rate for 262bp β-actin gene fragment was 50% (4/8) among brain tissue samples fixed at +4ºC up to 28 days, lung tissue up to 14 days and kidney tissue up to 10 days fixation. However, after fixation at RT, reduced efficiency of the amplification of the β-actin gene fragment was obtained: 25% in brain tissue up to 14 days, 25% in lung tissue up to 10 days and 25% in kidney tissue up to only 5 days. In all examined tissue samples, the decrease of amplifiability of isolated DNA with the

prolonged incubation time in formalin (both at +4ºC and at RT) was statistically significant ($p=0.015$). Comparing the mean values of parameters for all three tissues studied, it has been shown that the brain tissue shows the best, and the kidney tissue the worst results.

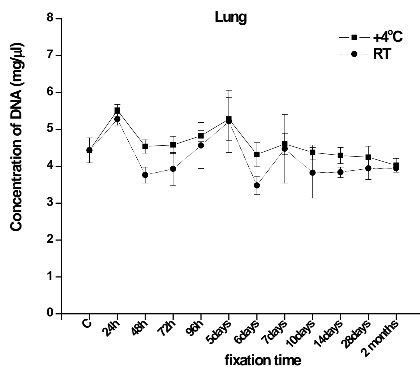
Figure 1 - Differences in concentration and purity of DNA isolated from human healthy (A) brain, (B) lung and C) kidney tissues fixed in phosphate-buffered formalin at room temperature (RT) or at +4°C. The concentration and the purity of DNA decrease with prolonged incubation time in all examined tissues ($p<0.001$). Statistically significant ($p<0.001$) higher concentration and purity of DNA were obtained from all tissue samples fixed on +4ºC in comparison with samples fixed on RT.

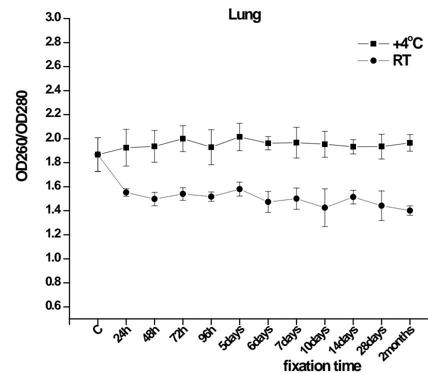
A BRAIN



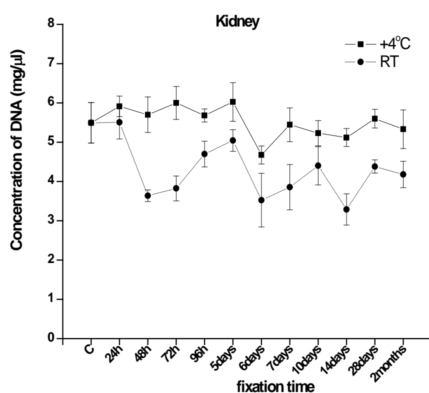


B LUNG





C KIDNEY



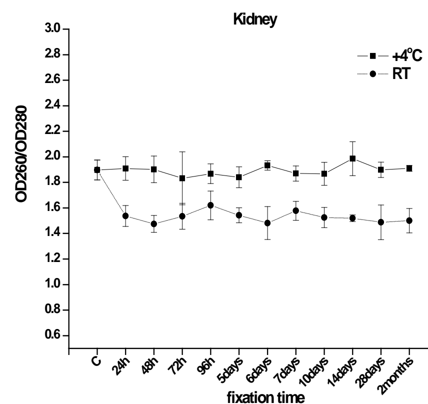


Figure 2. Number of samples in which are detected different fragments of DNA isolated from three human healthy tissues fixed in phosphate-buffered formalin for various incubation time.

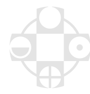
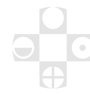
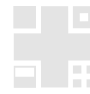
		24h	48h	72h	96h	5days	6 days	7days	10days	14days	28days	2 m
Fixation on RT												
Brain												
	β-actin	8/8	8/8	8/8	8/8	8/8	8/8	6/8	4/8	2/8	0/8	0/8
	GPD1	8/8	8/8	8/8	8/8	8/8	8/8	8/8	4/8	4/8	2/8	0/8
Lung												
	β-actin	8/8	8/8	8/8	8/8	6/8	6/8	2/8	2/8	0/8	0/8	0/8
	GPD1	8/8	8/8	8/8	8/8	8/8	8/8	8/8	4/8	4/8	0/8	0/8
Kidney												
	β-actin	8/8	8/8	8/8	4/8	2/8	0/8	0/8	0/8	0/8	0/8	0/8
	GPD1	8/8	8/8	8/8	8/8	6/8	8/8	6/8	4/8	2/8	0/8	0/8
Cold fixation on +4°C												
Brain												
	β-actin	8/8	8/8	8/8	8/8	8/8	8/8	8/8	6/8	4/8	4/8	0/8
	GPD1	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	6/8	0/8
Lung												
	β-actin	8/8	8/8	8/8	8/8	8/8	6/8	6/8	4/8	4/8	0/8	0/8
	GPD1	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	4/8	0/8
Kidney												
	β-actin	8/8	8/8	8/8	8/8	8/8	6/8	6/8	4/8	0/8	0/8	0/8
	GPD1	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	6/8	4/8	0/8

DISCUSSION

The most important step in a procedure to preserve tissues is a fixation. Since 19th century, the 4% formaldehyde solution in water (formalin) is in use for tissue fixation. In addition to formalin, there are also some other fixatives such as ethanol-based fixatives, glutaraldehyde, potassium dichromate acetic acid, etc. (9). However, these fixatives are not widely used, so the formalin is the most common fixative in histopathology. In recent years, the formalin buffered with several different buffers such as calcium carbonate, magnesium carbonate, citrate, Tris and most commonly phosphate buffers (4) has been in use, which has certain advantages over the unbuffered: longer shelf life and the possibility of prolonged tissue fixation without adverse effects on DNA degradation (10).

Formaldehyde is the simplest aldehyde with one C atom, which is rapidly dissolved in water, whereby methylene hydrate (methylene glycol, $CH_2(OH)_2$) forms. The molecules of methylene glycol react with each other and form a polymer (11). The aqueous formalin solution contains 37-40% formaldehyde in the form of short polymer (containing 2-8 monomers) and 60-63% water. Addition of 10% methanol to an aqueous formalin solution prevents polymerization and the formation of paraformaldehyde (insoluble long polymers containing more than 100 monomers). In the tissue, methylene glycol is dehydrated into carbonyl formaldehyde. Both forms of formaldehyde (both hydrated and non-hydrated) fix tissue (11). When tissues are put into formalin, they are quickly penetrated by methylene glycol and a small amount of formaldehyde. Formaldehyde reacts with amines (amino acid lysine), purines and thiols (cysteine) and forms a methylol which loses the molecule of water and makes the Schiff base (imine). The Schiff base reacts with other nucleophilic molecules, such as amino groups of DNA and proteins, and creates cross-linking between the macromolecules via the methylene bridge (11). In addition to the formation of methylol derivatives and the formation of cross-linking methylene bridges, formaldehyde induces the formation of AP sites (apurine and apirimidine) and the breakdown of phosphodiester bonds in the DNA molecule (12). Formaldehyde breaks the hydrogen bonds of the dual-chain DNA molecule in regions rich with adenine and thymine, leading to the formation of new chemical reactions, protein binding for DNA, and fragmentation of DNA molecules (13). The initial phase of this reaction is reversible, while subsequent reaction phases, when a large number of covalent bonds are created, are irreversible (3).

Process of formalin fixation helps to preserve the tissue and cell structures and morphology which makes possible the histopathological analysis of samples. However, the formalin fixation has some limitation, especially due to degradation of nucleic acids. Tissue formalin fixation process has two phases: penetration and fixation. Penetration implies the

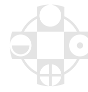
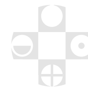
  

ability of the fixative to diffuse into the tissue and requires at least 1h per 1mm of tissue thickness (14) to reach the nethermost parts of cells. This process is a physical process which is determined by several physical factors: temperature, pH, volume of solution, length of incubation, pressure, surface area, concentration of formaldehyde, etc. (3). At the other hand, fixation is a process in which the initial formation of cross-linking between the macromolecules is completed. The fixation process is influenced by various factors, such as the incubation length, temperature, pH value of the formalin solution and viscosity. Penetration of formalin into tissue and initially cross-linking occurs in the first 24 to 48 hours, while the process of fixation and formation of stable covalent bonds can be confirmed for up to 30 days (15). Understanding the chemical and physical aspects of the fixation process, as well as the factors influencing these processes, contributes to the optimization of conditions and the selection of correct methodological approaches in histopathology and molecular autopsy.

The mechanism of action of formalin on DNA molecules as well as factors affecting the fixation efficiency was studied mainly on tumor tissues that, after formalin fixation, were embedded into paraffin, or on frozen tissue. There is no sufficient literature data about the effects of formalin on DNA molecules in healthy tissue excluded during autopsy. Archival fixed tissues excluded during autopsy are potentially large source for forensic testing especially or DNA analysis in clarification of cases of criminal activity, determination of paternity or in the persons identification. In our laboratory, different tissues (brain, lung, kidney, heart and liver) excluded during autopsies are fixed in formalin, embedded in paraffin and kept in the archives for many years. Therefore, it was of great importance to test how long these tissues may be fixed in formalin, optimal conditions of fixation, the influence of temperature and tissue type on DNA integrity and their usability in forensic practice.

DNA quality is measured in the context of concentration and purity as well as in terms of PCR fragment length. Concentrations of DNA in tissue samples were calculated using values obtained spectrophotometrically on 260nm, while purity was calculated due the OD260/OD280 ratio. The OD260/Od280 values more than 1.5 indicated low protein contamination in samples (16). The total amount of extracted DNA highly depended on the method used. In the previous studies, it was shown that phenol-chloroform-isoamyl alcohol (PCI) extraction gives higher yield and purity (17, 18) and successful gene amplification (18) than the commercial kit (10). For this reason, we used PCI method for DNA extraction. To evaluate the degree of degradation of DNA molecules, we used PCR amplification of fragments with a length of 150 bp (GPD1 gene fragment) and 262 bp (β-actin gene fragment). Forensic laboratories use STR (**s**hort **t**andem **r**epeats) as well as SNP (**s**ingle **n**ucleotide **p**olymorphism) markers as standard for DNA identification (19). To perform this method, DNA fragments of length from 150-450 base pairs are required. SNP markers are small genetic variations occurring in human DNA for every 100 to 300 base pairs along 3 trillion human genome bases. SNP markers appear in coding and non-coding parts of DNA as well as nuclear and mitochondrial DNA. SNP amplicons are shorter (shorter than 150 base pairs) than other markers, thus allowing the amplification of the damaged DNA. Babol-Pokora and Berent (19) used five SNP markers following the length of amplitudes 123, 99, 93, 85 and 71 base pairs and concluded that the SNP was ideal for forensic analysis of degraded samples. For this reason, the use of selected genes in our investigation is justified.

The quality of DNA isolated from different organs, fixed to formalin at different times, often varies due to the variation in the cellular composition of these tissues. Tissues that have non-homologous cellular composition (pancreas, colon, lungs) should be avoided because the concentration and purity of the DNA isolated from them are poor (20). In addition, the lung tissue is non-suitable for molecular analysis due to presence of mucus. Tissue samples isolated from organs which have high cellular density (brain, heart, liver, spleen) have been used for molecular analysis because they showed better PCR amplification of DNA molecule (21). At the other hand, DNA isolated from chest cavity organs (heart and lungs) shows better quality than DNA isolated from tissue originating from the abdominal cavity (pancreas, liver, kidney, colon, stomach), due to pancreatic enzymes and accelerated bacterial growth leading to faster tissue autolysis (22). Kidney tissue should not be taken from individuals older than 50, since the cell autolysis has probably began (21). In our study, we decided to use three different organs from different cavities. These organs are approachable for collection of samples in routine autopsy and appropriate for our study. To the best of our knowledge, there is only few literature data about the DNA integrity in the healthy tissues fixed in formalin (9, 10, 23). There are much more studies about DNA integrity isolated from healthy tissues (1, 24) or from cancer tissues (15, 25, 26) fixed in formalin and embedded in paraffin. In terms of cancer tissue and other diseased tissues which are embedded in paraffin, the results were lower probably due to permanent alteration in DNA and damage of DNA with aggressive reagents for deparaffinization (27). Also, reagents used for paraffin embedding of tissue could damage the quality of DNA molecule (21). There are no studies that analyzed DNA integrity isolated from cancer or healthy tissues only fixed in formalin, without paraffin embedding. The impact of our study is that we analyzed healthy tissues excluded during forensic autopsy which performed 24h after death. The optimal fixation time depends on the tissue type and specimen size (28). DNA isolated from brain formalin fixed tissue showed the best quality in terms of purity and integrity compared with other examined organs. The concentration of DNA isolated from the brain tissue immediately after autopsy was the highest which can be explained by the high cell density in this tissue (21). With prolonged incubation time, the concentration of DNA in the brain tissue decreased which indicates a high sensitivity of this organ to prolonged formalin exposure. The highest DNA concentration was measured in kidneys after prolonged

incubation time in formalin due to the specific cell composition in this tissue. The DNA integrity in the brain tissue was the highest in comparison with other examined tissues, both after fixation at RT and at +4C. The integrity of the DNA is sufficiently preserved in the brain tissue up to 28 days of fixation at RT as well as at +4°C. Miething et al. concluded that the brain tissue showed strong fluctuations in the test results which can be attributed to uneven penetration by fixative (9). There is no literature data about the successful amplification of DNA fragments isolated from healthy lung tissue extracted during autopsy and fixed in formalin. Funabashii et al. considered that lungs are not good for subsequent analyzing because of the presence of mucus in these organs (21). In our study, the DNA integrity in the fixed lung tissue was sufficiently preserved up to 14 days fixation at RT and up to 28 days fixation at +4°C. The worst integrity of DNA was in kidney tissue suggesting that the process of autolysis in kidney cells is intensive immediately after death (21). The temperature of formalin fixation had a significant effect on examined parameters of DNA isolated from different tissues. Better quality of DNA was obtained from samples fixed at a lower temperature. It was shown that low temperatures and short fixation time decreased the degree of DNA degradation (14). The speed of formalin fixation process depended on the rate of diffusion of formalin across the tissue and the rate of chemical reaction with cellular components (29). Formalin fixation process requires at least 1 hour per 1 mm of tissue thickness, so it is recommended that the process of tissue fixation by formalin lasts up to 48 hours (30). Fox et al. (31) have shown that for tissue samples of about 20mm the formalin penetration occurs 24h at 25°C, and 18h at 37°C. Our results have shown that the tissue formalin fixation incubation time can be longer, without significant consequences on DNA concentration, purity and amplificability up at least 10 days of formalin fixation. Moreover, increase in temperature of formalin fixation will favor the disassociation of formaldehyde from the polymers and its reaction with tissue macromolecules. 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 (15).

In conclusion, the proposed method of formalin fixation of human healthy autopsied tissue results in lower degree of DNA fragmentation. Fixation at low temperatures increases the degree of preservation of molecular structures in fixed formalin tissues. The possibility of obtaining high quality DNA from archival tissues gives prospects for wider molecular analysis and profiling than presently feasible. The results of this paper will contribute to better optimization of conditions during tissue fixation after autopsy and better preservation of DNA quality in tissues that can be used for forensic purposes.

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