The Quality of DNA Isolated from Autopsy FF and FFPE Tissues: Study of 1662 Samples

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Abstract

Background: There are enormous formalin fixed paraffin embedded tissue archives and constantly growing number of methods for molecular analyses but, isolation of DNA from this tissue is still challenging due to the damage effect of formalin on DNA. To determine the extent to which DNA purity, quantity and integrity depends on the process of fixation in formalin, and to what extent on the process of tissue paraffin embedding, we compared the quality of DNA isolated from fixed tissues and DNA isolated from tissues embedded in paraffin blocks after fixation.

Methods and Results: Heart, liver and brain tissues obtained from healthy people who suddenly died a violent death were fixed in 10% buffered formalin as well as in 4% unbuffered formalin 6h, 1-7 days (every 24h), 10, 14, 28 days and 2 months. Also the same tissues were fixed in 4% unbuffered formalin and embedded in paraffin block and stored from few months to 30 years. Yield and purity of the DNA samples isolated from these tissues were measured using spectrophotomer The PCR amplification of the hTERT gene was performed to evaluate the degree of DNA molecule fragmentation. Although the purity of the DNA isolated from almost all tissue samples is satisfactory, the DNA yields changes significantly.

Conclusion: The largest decrease in DNA yield was observed after tissue fixation in formalin, especially with prolonged formalin fixation, and additionally after paraffin embedding of tissue. DNA integrity also depends on time of tissue formalin fixation and the age of paraffin blocks.

Introduction

Formalin fixed (FF) and formalin fixed paraffin embedded (FFPE) tissues that have been routinely collected for decades around the world in the departments of forensic medicine and pathology are one of the most available materials for molecular analysis and clinical pathology practice [1]. Molecular analysis of FFPE tissue are becoming more common, especially in cases of sudden unexplained death, and their purpose is to identify gene mutations that may be responsible for death. However, the process of tissue formalin fixation and tissue paraffin embedding involves the application of various procedures and agents that lead to damage of DNA molecules. This results in DNA extraction of variable yield and purity which subsequently reduces the ability to perform molecular analyzes. Therefore optimizations of the methods of extracting high-quality of DNA are crucial.

When we talk about the quality of DNA molecules isolated from appropriate tissues, we mean primarily on purity, yield and integrity of isolated DNA. Purity of DNA molecules indicated the presence of proteins, carbohydrates, fats, solvents (such as phenol) and salts in sample. The presence of these molecules and compounds reduces the quality of isolated DNA and often prevents further analysis such as PCR [2, 3]. The ratio of absorbance at 260 nm, 280 nm and 230 nm are used to determine DNA purity. If the ratio of absorbance at 260 nm and 280 nm are between 1.5 and 2.0, and ratio of absorbance at 260 nm and 230 nm are between 1.7 and 2.2 is generally accepted as pure DNA [3–6]. Yield of DNA is very important to achieve complete profiles in forensic investigations [7]. Although there are enormous FFPE tissue

archives and constantly growing number of methods for molecular analyses, but isolation of DNA from this tissues is still challenging due to the damage effect of formalin on DNA. Extraction of DNA from FF and FFPE tissues include multiple steps and also a large number of parameters which affect DNA quality are involved such as choice of fixative, time of fixation, the period of storage FFPE blocks, tissue type etc [8–11]. There are data in the literature about investigation for possible improvements in various aspects of DNA extraction and DNA amplification for DNA isolated from FF and FFPE tissues to make it usable for molecular analyzes [12, 13], but there is still no agreement among scientists about adequate protocol for DNA extraction from FFPE tissues. Also there are no studies that examine the extent to which formalin affects and the extent to which paraffin affects the quality of isolated DNA.

The scope of this study was to establish the effects of buffered and unbuffered formalin fixation of tissues for various length of fixation on the yield, purity and integrity of isolated DNA from tissues that were excluded during autopsy and fixed in one or the other formalin. Also the aim of this study was to determine whether the storage period of FFPE tissue blocks had a significant influence on the quality of isolated DNA. To determine the extent to which DNA purity, quantity and integrity depends on the process of fixation in formalin, and to what extent on the process of tissue paraffin embedding, we compared the quality of DNA isolated from fixed tissues and DNA isolated from tissues embedded in paraffin blocks after fixation. DNA extraction from healthy heart, liver and brain tissues was performed using two methods (organic extraction and Commercial kit) to determine which tissues are most suitable for fixation in formalin and paraffin embedding to serve as a source of preserved DNA for further molecular analysis.

Material And Methods

Tissue selection

Tissues were excluded during medico legal autopsies on Department of Legal Medicine and Toxicology in UKC Kragujevac from healthy people, 20 to 50 years old, who suddenly died from violent death (homicide, suicide or accident). To investigate the effect of tissue fixation in formalin on the yield, purity and integrity of isolated DNA, sections of healthy heart, liver and brain tissues size per 2,5x1,5x2,5 cm were fixed in unbuffered 4% formalin or in 10% buffered formalin. Tissue samples were incubated at room temperature (RT) in formalin in hermetically sealed plastic jars. The length of tissue formalin fixation was 6h, 1–7 days (each day), 10 days, 14 days, 28 days and 2 months.

To investigate the effect of paraffin embedding on yield, purity and integrity of DNA isolated from healthy heart, liver and brain tissues, these tissues were fixed in 4% unbuffered formalin and embedded in paraffin block and stored from few months to 30 years.

Tissue preparations of FF and FFPE blocks after H&E staining were histomorphologically examined and only healthy tissues were included in the study.

Paraffin embedding of tissues

The process of tissue fixation begins by immersing tissue sections in 4% unbuffered formalin. After tissue formalin fixation, the material was processed in automated tissue processor (Leica TP 220, Germany) according to the following program: 90 min in 86% alcohol; 3 times for 90 min in 96% alcohol, 4 times for 90 min in 100% alcohol, 2 times for 90 min in xylene and 90 min in paraffin. After processing in automated tissue processor, the tissue material is spilled into special paraffin molds. FFPE tissue blocks are stored on Department of Legal Medicine and Toxicology in UKC Kragujevac blocks up to 30 years in a dark room, medium humidity, at RT.

Preparation of tissues fixed in formalin

DNA was isolated from healthy heart; liver and brain tissues fixed in 4% unbuffered formalin as well as in 10% buffered formalin. From tissue samples that were fixed in 4% unbuffered formalin or in 10% buffered formalin a small cube of tissue was first cut from the middle in diameter of 3x3x3 mm. After that the tissues were macerated with a scalpel and incubated in absolute ethanol at RT for 10 min. After careful pouring of absolute ethanol, the tissues were incubated in 70% ethanol for 10 min at RT. By careful pouring of 70% ethanol the samples were ready for DNA extraction.

Tissue deparaffinization

FFPE tissue blocks without mechanical damage and without traces of mold were selected. FFPE tissues blocks were cut on a microtome into thin tissue slides 10 μm thick. The first 2–3 tissue slides of each sample were discarded to avoid cross-contamination between samples. After that, 3 tissue slides of each FFPE block were incubated in xylene 3 times for 30 min, at room temperature where after each incubation the sample ware centrifuged for 1 min at 14,000 rpm at room temperature and then the supernatant was carefully separated. Then the tissue slides ware rehydrated in decreasing concentrations of ethanol: in 100% ethanol for 5 min, after centrifugation for 1 min at 14,000 rpm in 70% ethanol for 5 min, and after centrifugation for 1 min at 14,000 rpm in distilled water 5 min at room temperature. The samples were then centrifuged once more for 1 min at 14,000 rpm at room temperature and samples were ready for DNA extraction.

DNA extraction

The total number of DNA samples isolated from FF tissues was 576. DNA is also isolated from FFPE tissues in total number of 1086. Two different methods were used to isolate DNA: extraction with phenol-choloroform-isoamyl alcohol and with PureLink Genomic DNA Kit (Invitrogen by Thermo Fisher Scientific, Carlsbad, CA, USA).

Before DNA extraction tissue digestion was performed in tubes using 300μl of TNS digestion buffer (10mM Tris-HCl, 1mM EDTA, 3M NaCl, 10% SDS and ampoules-deionized water) and 50μl of Proteinase K (20mg/ml, Thermo Scientific), with overnight incubation at 56°C.

DNA extraction with phenol-choloroform-isoamyl alcohol

350 µl of phenol-choloroform-isoamyl alcohol in ratio 25:24:1 was added in each sample tube and centrifuged at 14 000 rpm, 5 min at + 4°C. The upper phase was transferred into new tube and added 350 µl of choloroform-isoamyl alcohol in ratio 24:1. After centrifugation at 4000 rpm, 5 min at + 4°C the upper phase was transferred into new tube. The isolated DNA was precipitated with NaCl solution and ice cold ethanol (-20°C) overnight and after that the solution was centrifuged at 15 000, 30 min, at + 4°C. The supernatant was decanted and 70% ethanol was added to the precipitate and centrifugated at 15 000, 15 min, at + 4°C. The supernatant was out flowed and the precipitate was well dried. Precipitated and well-dried DNA was solubilized in 50µl TE buffer and stored at -20°C until use.

DNA extraction with PureLink Genomic DNA Kit

DNA extraction using the PureLink Genomic DNA Kit was performed according to the manufacturer's instructions with some modifications. All tissue samples were digested and overnight incubated as said before. After that the manufactures instructions was followed. The final elution volume of each sample was 50 µl.

Quantification of yield and purity of extracted DNA

Yield and purity of the DNA samples were measured using spectrophotomer (UV-1800 Shimadzu UV spectrophotomer, Japan). 2 µl of isolated DNA was dissolved in 998 µl of TE buffer. The spectrophotometer was calibrated with 1 ml of TE buffer. To determine the purity of isolated DNA, the ratio OD260/280 was determined, which is the ratio between absorbance of nucleic acids (OD260) and proteins (OD280). The ratio of OD260/OD230 absorbances indicates contamination with salts, carbohydrates, lipids, and solvents such as phenol. The DNA concentration in the sample was determined by measuring the absorbance at 260 nm (OD260). Absorbance of one optical unit (OD) corresponds to 50 µg/ml of double-stranded DNA. The total DNA yield in 50 µl of isolates was determined by multiplying the calculated DNA concentration (µg/µl) and the volume of the DNA solution (50 µl).

C(µg/µl)=(OD260x500x50)/1000

Yield of DNA = C(µg/µl) x 50µl TE buffer

Polymerase chain reaction (PCR)

The integrity of DNA was evaluated by PCR amplification of hTERT gene amplicon length 113 bp. The PCR amplification was performed in a final volume of 25 µl containing: One Taq 2x Master Mix with Standard Buffer (New England Biolabs Inc.); primers (10 µM forward primer sequence GCCGATTGTGAACATGGACTACG and 10 µM reverse primer sequence GCTCGTAGTTGAGCACGCTGAA (Invitrogen by Thermo Fisher Scientific) and DNA sample (approximately 1ng/µl of genomic DNA) in sterile bidestilated water. The PCR amplification was performed in PCR aparatus Techne genius, Eppendorf. The following PCR conditions were used for each PCR reaction: initial denaturation at 95°C, 11 min; 30 cycles of 94°C, 30 sec, 59°C 45 sec, and 70°C 45 sec; and final extension at 60°C for 60 min.

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.

Statistical analysis

Statistical analysis for variances was performed using IBM SPSS software, version 20.0 (IBM, Armork, NY, USA). Descriptive statistics were used for all variables in this study. Before statistical analysis of data, the tests of normality were analyzed with Kolmogorov-Smirnov and Shapiro-Wilks tests. By testing the normality we observed that the experimental data were not normally distributed. Results of yield and purity are presented as mean value and standard deviation. The differences between groups ware analyzed by Post Hoch testing within the nonparametric Friedman and Kruskal Wallis tests. Differences were considered statistically significant when probability (p) was less than 0.05, while statistically very significant difference was when probability was less than 0.01.

Results

Purity, yield and integrity of DNA isolated from FF tissue

The influence of type of fixative on purity, yield and integrity of isolated DNA

Purity and yield of DNA isolated from fixed tissue depends of type of used fixative. Comparing the purity of DNA samples isolated from tissues fixed in 10% buffered or 4% unbuffered formalin, regardless with which method DNA was isolated, OD260/230 ratio were higher for DNA isolated from tissues fixed in 10% buffered formalin compared to those fixed in 4% unbuffered formalin and this difference in purity was statistically significant ($p < 0.05$). Also yield of isolated DNA was statistically significant higher in DNA samples isolated from tissues fixed in 10% buffered formalin ($p < 0.01$).

Since it has been shown that the type of formalin in which tissues were fixed prior to DNA isolation affects on DNA purity and yield, we compared the effect of formalin fixation on DNA samples that were isolated with PCI method to DNA samples isolated with Commercial kit (Fig. 1).

Also there is statistically significant differences among DNA integrity between samples isolated from tissue fixed in buffered and in unbuffered formalin ($p < 0.05$). Percentage of successful PCR amplification for hTERT gene for DNA isolated from tissue fixed in buffered formalin was 58.33% and for DNA samples isolated from tissue fixed in unbuffered formalin was 18.06%.

The influence of method used for DNA isolation from FF tissue on purity, yield and integrity of DNA

DNA samples isolated using PCI method contain statistically significant less protein (higher absorbance ratio OD260/280) ($p < 0.01$) and other impurities (higher absorbance ratio OD260/230) ($p < 0.01$)

compared to DNA isolated with Commercial kit (Table 1). Also, the yield of DNA isolated with PCI method was statistically significant higher than the yield of DNA isolated with Commercial kit ($p < 0.01$) (Table 1).

Table 1

Degree of purity (OD260/280 and OD260/230) and yield (ng DNA in 50 µl TE buffer) of DNA isolated from tissue fixed in 10% buffered formalin and 4% unbuffered formalin using two different extraction methods: PCI and Commercial kit.

Extraction method	Type of fixative	OD260/280	OD260/230	Yield (ng ± SD) Mean ± SD Range
PCI	**10% buffered formalin**	**2.06 ± 0.33**	**2.45 ± 0.70**	**257.09 ± 119.19** 121.25-612.95
	4% unbuffered formalin	2.03 ± 0.34	2.21 ± 0.79	209.91 ± 93.94 103.75–527.50
	p	*0.701*	*< 0.05*	*< 0.01*
Commercial kit	**10% buffered formalin**	1.68 ± 0.54	1.36 ± 0.08	123.35 ± 14.04 93.75-201.25
	4% unbuffered formalin	1.67 ± 0.56	1.35 ± 0.07	121.62 ± 9.66 93.75–142.50
	p	*0.249*	*0.986*	*0.964*
p		*< 0.01*	*< 0.01*	*< 0.01*

Comparing PCI method and Commercial kit statistically significant differences between integrity of isolated DNA were observed ($p < 0.01$). Percentage of successful PCR amplification for hTERT gene for DNA isolated using PCI method was 56.94% while using Commercial kit was only 16.67%.

Purity, yield and integrity of DNA isolated from different tissue type fixed in formalin

Values of purity of DNA samples isolated with PCI method (OD260/A260 and OD260/A230 ratio) were different between three different tissues ($p < 0.05$; $p < 0.01$). Post hot testing has proven that the highest OD260/A260 and OD260/230 ratio were for DNA isolated using PCI from liver tissue. DNA yield also depends on the type of tissue from which DNA was isolated when using PCI method for isolation. Differences in yield between different tissues were statistically significant ($p < 0.01$). The highest yield was obtained for DNA samples isolated from liver tissue (346.84 ± 116.06 ng DNA) using PCI method for DNA isolation (Table 2).

Table 2
Degree of purity (OD260/280 and OD260/230) and yield (ng DNA in 50 µl TE buffer) of DNA isolated from three different tissues fixed in formalin using two different extraction methods: PCI and Commercial kit.

Extraction method	Type of tissue	OD260/280	OD260/230	Yield (ng ± SD) Mean ± SD Range
PCI	**heart**	2.16 ± 0.39	1.87 ± 0.29	170.24 ± 32.64 103.75–250.00
	liver	1.97 ± 0.24	3.12 ± 0.71	346.84 ± 116.06 137.50-612.95
	brain	2.00 ± 0.34	1.99 ± 0.39	180.35 ± 38.82 115.00-255.00
	p	*< 0.05*	*0.01*	*0.01*
Commercial kit	**heart**	1.70 ± 0.57	1.35 ± 0.06	121.09 ± 9.62 93.75-136.25
	liver	1.59 ± 0.48	1.37 ± 0.08	124.75 ± 15.47 93.75-201.25
	brain	1.74 ± 0.58	1.35 ± 0.07	121.50 ± 9.70 98.75–140.00
	p	*0.422*	*0.361*	*0.567*

Percentage of successful PCR amplification for hTERT gene in DNA isolated from heart tissue was 35.42%, from liver tissue 39.58% and from brain tissue was 37.5%. Processing the data there was no statistically significant differences between the integrity of DNA isolated from three different tissues fixed in formalin ($p = 0.701$).

The influence of the length of tissue fixation in formalin on purity, yield and integrity of isolated DNA

Analyzing the purity of DNA isolated from different tissue types individually through different lengths of tissue formalin fixation statistical data processing showed that there is no statistically significant difference in OD260/280 and OD260/230 ratio between adjacent time points regardless of the applied DNA isolation method ($p > 0.05$). OD260/280 ratio of DNA samples was satisfactory regardless of the method used for DNA isolation, but OD260/230 ratio depended on the method used for DNA isolation. Namely, DNA samples isolated using PCI method had a lower concentration of salt, lipids and solvents (higher OD260/230 ratio) compared to DNA samples isolated with Commercial kit. However, the length of tissue formalin fixation affects on yield of DNA ($p < 0.01$). There was statistically significant decrease in DNA yield, between 6h and 24h as well as between 14 days and 28 days of tissue formalin fixation using PCI method for DNA isolation (Fig. 2).

Percentage of successful PCR amplification of hTERT gene in DNA samples isolated from tissue fixed from 6h to 2 months were from 2.08–91.67% depending on the length of tissue fixation in formalin

Purity, yield and integrity of DNA isolated from FFPE tissue

The influence of method used for DNA isolation from FFPE tissue on purity, yield and integrity of DNA

Results indicate that DNA purity and yield depend on the method used for DNA isolation. DNA purity and yield were higher in DNA samples isolated using PCI method compared to DNA samples isolated with Commercial kit and this difference was statistically significant ($p < 0.01$). The OD260/A230 ratio, which indicates the presence of salts, lipids and solvents in DNA samples were 2.03, within the optimal values (1.7–2.2) for DNA samples isolated with PCI method. However, for DNA samples isolated using Commercial kit, OD260/A230 ratio was significantly lower than the optimal (Table 3).

Table 3
Degree of purity (OD260/280 and OD260/230) and yield (ng DNA in 50 µl TE buffer) of DNA isolated from FFPE tissue using two different extraction methods: PCI and Commercial kit.

Extraction method	OD260/280	OD260/230	Yield (ng ± SD) Mean ± SD Range
PCI	1.98 ± 0.36	2.03 ± 1.06	197.94 ± 70.62 71.30-636.30
Commercial kit	1.69 ± 0.54	1.09 ± 0.17	106.81 ± 17.81 57.50-137.50
p	*0.01*	*0.01*	*0.01*

There was statistically significant difference between integrity of DNA isolated using PCI method compared to Commercial kit. In 87.34% was successful PCR amplification of hTERT gene in DNA samples isolated with PCI method, while just in 8.86% using Commercial kit.

Purity, yield and integrity of isolated DNA from different type of FFPE tissue

Statistical data processing showed that there was no statistically significant difference in OD260/280 ratio between DNA samples isolated from different tissues (heart, liver or brain) using both method for isolation (p 0.05). OD260/230 ratio for DNA samples isolated with PCI method from different tissues were different, i.e. DNA samples isolated from the liver tissue were the purest (the highest OD260/230 ratio 2.67 ± 1.33). Also the difference in the yield of DNA isolated with PCI method from heart, liver and brain tissues was statistically significant between individual tissues ($p < 0.01$), i.e. the highest yield was obtained for DNA isolated from liver tissue (272.96 ± 118.3 ng DNA) (Table 4).

Table 4

Degree of purity (OD260/280 and OD260/230) and yield (ng DNA in 50 µl TE buffer) of DNA isolated from three different formalin fixed paraffin embedded tissue (heart. liver and brain) using two different extraction methods: PCI and Commercial kit.

Extraction method	Type of tissue	OD260/280	OD260/230	Yield (ng ± SD) Mean ± SD Range
PCI	heart	1.96 ± 0.38	1.80 ± 1.05	166.36 ± 53.37 88.80-386.3
	liver	2.05 ± 0.33	2.67 ± 1.33	272.96 ± 118.30 107.50-636.31
	brain	1.92 ± 0.38	1.63 ± 0.82	154.50 ± 40.19 71.34-293.81
p		*0.05*	*0.01*	*0.01*
Commercial kit	heart	1.66 ± 0.54	1.10 ± 0.17	108.35 ± 17.37 58.82-130.01
	liver	1.7 ± 0.54	1.09 ± 0.16	106.12 ± 17.63 57.49–137.50
	brain	1.7 ± 0.53	1.08 ± 0.18	105.96 ± 18.42 57.48–132.50
p		*0.05*	*0.05*	*0.05*

Analyzing the differences between success of PCR amplification of hTERT gene in DNA samples isolated from different FFPE tissues it was observed statistically significant difference ($p < 0.01$). According to the percentage of successful PCR amplification of hTERT gene, DNA isolated from heart tissue (51.93%) and brain tissue (50.82%) was preserved integrity than DNA isolated from liver tissue (22.65%).

The influence of storage length of FFPE tissues on purity, yield and integrity of isolated DNA

Analyzing the purity of DNA samples isolated from heart, liver and brain FFPE tissue archived up to 30 years, indicate that OD260/280 as well as OD260/230 ratio were in satisfactory range, and that there was no statistically significant difference in absorbance ratio between adjacent time points regardless on applied DNA isolation method ($p > 0.05$). Statistical processing of data regarding the yield of DNA isolated with PCI method from FFPE heart, liver and brain tissues archived up to 30 years found that there was a statistically significant increase in DNA yield with increasing time of archiving samples starting from 7 years of storage. Post hoc testing revealed that there was a statistically significant difference in the yield of DNA isolated from FFPE heart and liver tissues aged 7–11 years and 12–16 years (p 0.01), as well as 17–21 and 22–26 years (p 0.01), while for DNA isolated from FFPE brain tissue a statistically significant difference in yield was detected between tissues aged 17–21 and 22–26 years (p 0.01) (Fig. 3).

Percentage of successful PCR amplification of hTERT gene in DNA samples isolated from FFPE tissue archived from few months to 30 years were from 10–100% depending on the length of archiving of FFPE tissue.

Comparison of purity and yield of DNA isolated from FF tissue and FFPE tissue

The purity of DNA samples is not affected either by the tissue fixation process or the paraffin embedding, but the method used for DNA isolation. There was no statistically significant difference in the OD260/A280 and OD260/230 ratio for DNA samples isolated with PCI method from control tissues, from FF tissues in 4% unbuffered formalin and FFPE tissue. The purity (OD260/280 ratio) of all DNA samples was in the optimal range of values. However, OD260/230 ratio was lower in all DNA samples isolated with Commercial kit (Table 5).

Table 5

Purity and yield of DNA isolated with PCI method or with Commercial kit from control tissues not fixed in formalin, from tissues fixed in 4% unbuffered formalin and formalin fixed paraffin embedded tissues.

	Extraction method	Type of tissue	OD260/280	OD260/230	Yield (ng ± SD) Mean ± SD Range
Control	**PCI**	**heart**	2.25 ± 0.28	2.07 ± 0.23	197.50 ± 83.26 114.24-280.76
		liver	2.30 ± 0.37	1.75 ± 0.28	738.75 ± 228.54 510.21-967.29
		brain	2.21 ± 0.34	2.27 ± 0.25	326.25 ± 120.7 205.88-446.62
Tissue fixed in 4%unbuffered formalin		**heart**	2.18 ± 0.35	1.73 ± 0.24	155.13 ± 27.37 103.75–215.00
		liver	1.93 ± 0.29	1.97 ± 0.87	300.17 ± 110.27 137.50-527.50
		brain	1.98 ± 0.32	1.95 ± 0.44	172.45 ± 38.28 115.00-235.00
FFPE tissue		**heart**	1.96 ± 0.38	1.80 ± 1.05	166.36 ± 53.37 88.75-386.25
		liver	2.05 ± 0.33	2.07 ± 1.33	272.96 ± 118.30 107.50-636.25
		brain	1.92 ± 0.38	1.63 ± 0.82	154.50 ± 40.19 71.25-293.75
Control	**Commercial kit**	**heart**	3.38 ± 1.08	1.29 ± 0.29	117.50 ± 36.70 80.80-154.20

	Extraction method	Type of tissue	OD260/280	OD260/230	Yield (ng ± SD) Mean ± SD Range
		liver	2.22 ± 0.58	1.76 ± 0.38	167.50 ± 56.80 110.70-224.30
		brain	3.57 ± 1.21	1.28 ± 0.27	116.25 ± 26.70 89.55-142.95
Tissue fixed in 4%unbuffered formalin		**heart**	1.61 ± 0.49	1.35 ± 0.06	120.95 ± 9.38 95.00-135.00
		liver	1.55 ± 0.07	1.36 ± 0.07	122.17 ± 10.32 93.75–142.50
		brain	1.85 ± 0.66	1.35 ± 0.07	121.74 ± 9.62 100.00-137.50
FFPE tissue		**heart**	1.66 ± 0.54	1.10 ± 0.17	108.35 ± 17.37 58.75–130.00
		liver	1.70 ± 0.54	1.09 ± 0.16	106.11 ± 17.63 57.52–137.50
		brain	1.70 ± 0.53	1.08 ± 0.18	105.96 ± 18.41 57.50-132.50

Yield of DNA was highest in tissue samples isolated immediately after autopsy. The highest yield of DNA is from the control liver tissue. Also yield of DNA was higher in tissue fixed in 4% unbuffered formalin compared to FFPE tissue. This applies to DNA samples isolated with both methods.

Discussion

FFPE tissues represent the largest available archives of human material [4]. The advantage of FFPE blocks is simple and relatively safe handling, cheap storage, wide availability and suitability for the application of immunohistochemical and other analyzes. Isolation of nucleic acids from archived biological material such as FFPE tissue blocks for PCR analysis is increasingly used in clinical practice [14] and is a significant source of DNA for use in forensics [15, 16]. However, in retrospective studies and

forensic analyzes, FFPE tissues are often the only and last available material for further molecular analysis [17]. For further molecular analyses it is necessary to obtain DNA of adequate purity and yield [18]. Therefore, it is extremely important to examine the yield and purity of DNA molecules isolated from FF and FFPE tissue. It is still unclear whether the reduction in yield of DNA isolated from FFPE tissue is most affected by the age of the paraffin tissue blocks, the type of FFPE tissue, the quality of the initial tissue treatment, or changes in reagents and processes of tissue fixation [19]. Tissue type, fixation process, post-fixation processes and DNA isolation are the basic steps in the process of tissue embedding in paraffin and molecular analysis where should look the possibility for optimizing and obtaining a larger amount of pure DNA.

Methods for analysis of isolated DNA require optimal DNA concentration, so the yield of isolated DNA from certain sample is very important. DNA yield depends on the type of biological material from which it was isolated, but also on the applied method for DNA isolation. There are various data in the literature about yield and concentration of DNA isolated from FF and FFPE tissues, but most of these data refer to tumor tissue. Ferruelo et al. isolated DNA from healthy FFPE liver tissue excluded during autopsy whose yield ranged from 47 ng/µl to 130 ng/µl depending on the age of paraffin blocks and the OD260/280 ratio was in the range of 1.69-1.96 [20]. Funabashi and co-workers succeed to isolated DNA from healthy autopsied FFPE liver tissue in yield range from 7.6-1045.6 ng/µl, from spleen 10.5-1009.5 ng/µl, and from the brain in range 6.3-335.2 ng/µl depending on the applied method for DNA isolation as well as the length of archiving of paraffin blocks [21].

Since, 4% unbuffered formalin was used in laboratories for tissue fixation, all tissues paraffin embedded decades ago, were fixed in unbuffered formalin [22]. Unbuffered formalin degrades rapidly and has a limited shelf life. In recent years, 10% buffered formalin has been used [19, 23]. To determine the effect of different types of formalin on the quality of isolated DNA tissues that were excluded during forensic autopsies were fixed in buffered formalin and unbuffered formalin. Results presented that purity of isolated DNA from tissue fixed in buffered formalin as well as in unbufferd formalin are within the optimal values. Yield of DNA isolated from tissues fixed in buffered formalin is higher than DNA isolated from tissue fixed in unbuffered formalin, which is consistent with data from the literature [24]. Regarding the integrity of DNA, DNA isolated from tissue fixed in buffered formalin was more preserved integrity, than DNA isolated from unbuffered formalin.

Optimization of the method for isolating high quality DNA is very important. There are contradictory data in the literature with which method for DNA isolation is obtained better quality of DNA. Isolation of DNA with Commercial kit is faster and easier but the yield of isolated DNA is lower compared to the extraction with PCI method [25]. Our results presented that DNA isolated with PCI method is superior in term of purity, yield and integrity compared to DNA isolated with Commercial kit. DNA samples isolated using Commercial kit contains much more protein, salt, carbohydrates, lipids and other impurities. Also DNA yield is significantly higher in samples isolated by PCI method compared to Commercial kit, which can be explained by the loss of DNA during washing of silica gel membranes on which is adsorbed DNA [26]. It is

also observed that the quality of DNA is more affected by the method used for isolation rather than the type of formalin used for tissue fixation.

DNA quality is significantly affected by type of FF (formalin fixed) and FFPE tissue. There are different opinions about which organs are suitable for DNA isolation that will be used for further molecular analysis. The quality of DNA isolated from different organs often varies due to changes in the cellular composition of these tissues. Tissues that have no homogeneous cellular composition (pancreas, colon, lungs) should be avoided because the yield and purity of DNA isolated from them are poor [1]. The highest yield of DNA was isolated from liver tissue, which can be explained by the high cell density as well as the presence of polyploidy cells that are presented in the liver in 30-40% [27]. In this study DNA isolated from FFPE liver tissue had the best purity and yield, but this DNA was inferior integrity compared to DNA isolated from FFPE heart and brain tissues. It is known that liver tissue is subject to rapid autolysis because it contains a large number of highly catabolic enzymes, while muscle tissue decays much more slowly [28].

DNA quality is greatly influenced by the length of tissue fixation in formalin from which DNA is isolated [29-31]. The results presented that the purity of the samples was similar in all DNA samples regardless of the duration of tissue fixation. *Nam* and coworkers also concluded that prolonged tissue fixation does not affect the purity of isolated DNA [32]. However, prolonged tissue fixation leads to decrease in yield of isolated DNA especially after 14 days of tissue fixation. Literature data presented that high yield of DNA is obtained from tissues fixed in unbuffered formalin up to 7 days. After 16 days of fixation DNA yield decreases by 50%, and after 32 days of tissue fixation DNA yield values are very small [33]. Moreover DNA integrity depends on the length of tissue fixation in formalin, i.e. with prolonged time of tissue fixation in formalin, the success of PCR amplification decreases [29, 30, 32, 34]. DNA quality also depends on the length of archiving FFPE tissue blocks. Values of absorbance ratios indicating the purity of DNA samples are within optimal limits except for DNA samples isolated with Commercial kit. The yield of DNA isolated from FFPE tissues storage up to 30 years is satisfactory and optimal for further molecular biological analyzes. However, the DNA yield in these samples is twice lower than in DNA samples isolated from the tissue immediately after autopsy, i.e. without further fixation and paraffin embedding. This points to the fact that tissue fixation and paraffin embedding processes inevitably lead to decrease of DNA yields regardless of the length of storage of paraffin blocks. In literature data DNA yield is different in relation to the length of storage of FFPE tissues, and this is related to tumor tissues [26, 35]. The conclusion is that the age of paraffin blocks has no significant effect on the yield and purity of isolated DNA [36, 37]. The success of PCR amplification of DNA fragments isolated from FFPE tissues and archived over a long period of time is different. The results of some studies for tumor FFPE tissues had revealed that the success of PCR amplification depends significantly on the age of paraffin blocks [38], while other studies have made known that the increase in amplification success does not depend on their age [10, 39]. The success rate of PCR amplification of DNA fragments isolated from autopsy FFPE tissue does not decrease linearly with age of paraffin blocks [40]. In this study it was observed that DNA integrity yet depends on the age of paraffin blocks.

To determine the extent to which DNA quality depend on the formalin fixation process, and to what extent on paraffin embedding we compared the yield, purity and integrity of DNA isolated from healthy FF tissues and DNA isolated from FFPE tissues. If we compare the quality of DNA isolated from fresh tissues immediately after autopsy with DNA isolated from FF and FFPE tissues, we can conclude that both the process of fixation and the process of paraffin embedding affect the quality of isolated DNA. Although the purity of the DNA isolated from almost all tissue samples is satisfactory, the DNA yields changes significantly. In all examined FF or FFPE tissues samples DNA yield was significantly lower than in control tissue samples (DNA isolated immediately after autopsy before formalin fixation of tissue). The largest decrease in DNA yield was observed after tissue fixation in formalin, especially with prolonged formalin fixation, and additionally after paraffin embedding of tissue. It can be concluded from our results that the length of tissue formalin fixation has a greater effect on yield of isolated DNA than the length of archiving of FFPE blocks. The process of tissue fixation in formalin and paraffin embedding of tissue also affects on integrity of DNA. Formalin leads to fragmentation of DNA molecules, due to the formation of cross-links between proteins and DNA molecules as well as the breaking of phosphodiester bonds in nucleic acids [40, 41]. In process of embedding tissue into paraffin, if residual water is not replaced with paraffin it can lead to further degradation of DNA molecule [14]. Also inadequate storage conditions of paraffin blocks (humidity, temperature and mold) over time can lead to further DNA degradation [14].

Conclusion

For fixation of healthy autopsy tissues, it is the best to use buffered formalin and the length of formalin fixation up to 28 days. The method used for DNA isolation has a significant impact on DNA quality, ie. DNA isolated with PCI method is significantly better in term of quality than the DNA isolated with Commercial kit. Although yield of DNA isolated from liver tissue is higher than the yield of DNA isolated from heart and brain tissue integrity of DNA is better preserved in FFPE brain and heart tissue. Despite the challenges represented FFPE tissues, if the tissues are proper formalin fixed and paraffin embedded they remain a valuable source of DNA in retrospective molecular research.

Declarations

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Katarina Vitošević, Miloš Todorović and Danijela Todorović. The first draft was written by Katarina Vitošević and Miloš Todorović and all authors commented on previous version of the manuscript. All authors read and approved the final manuscript.

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Competing interests Allauthors declare that they have no conflict of interest.

Ethics approval Institutionalethical approval was obtained in compliance with the Declaration of Helsinki. Authorization to use human biological samples for research was obtained. The Ethics Committee of the University of Kragujevac, Faculty of Medical sciences, the Ethic Committee of University Clinical Centre of Kragujevac (No. 01-2798), Appeal Public Prosecutor's Office from Kragujevac (A No. 79/13) and Higher Court in Kragujevac (SU-VIII-110/13) agreed to these investigations.

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Figures

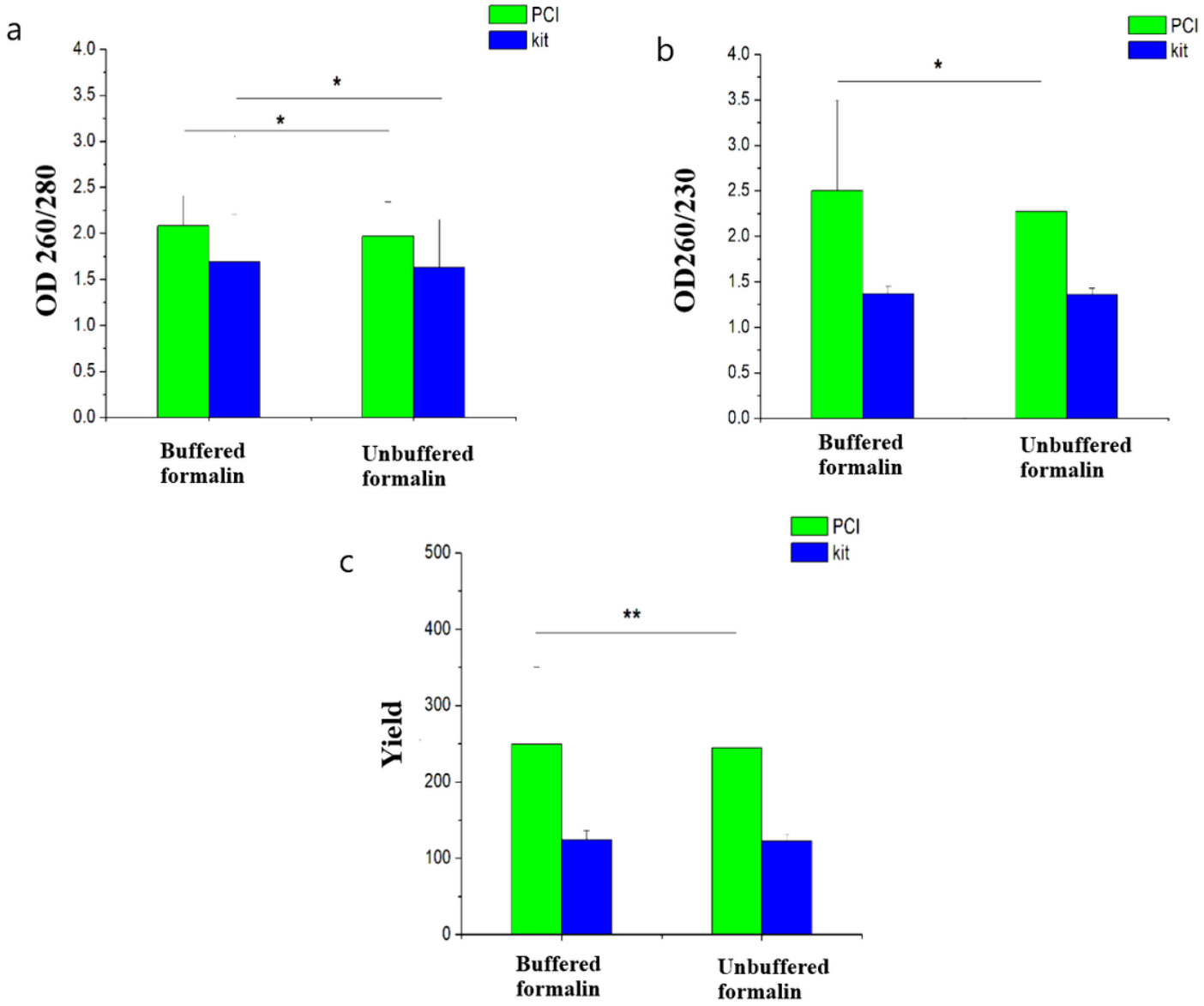

Figure 1

The influence of type of formalin used for tissue fixation on purity (a, b) and yield (ng DNA in 50 µl TE buffer)(c) of DNA isolated using two different extraction methods: PCI and Commercial kit.

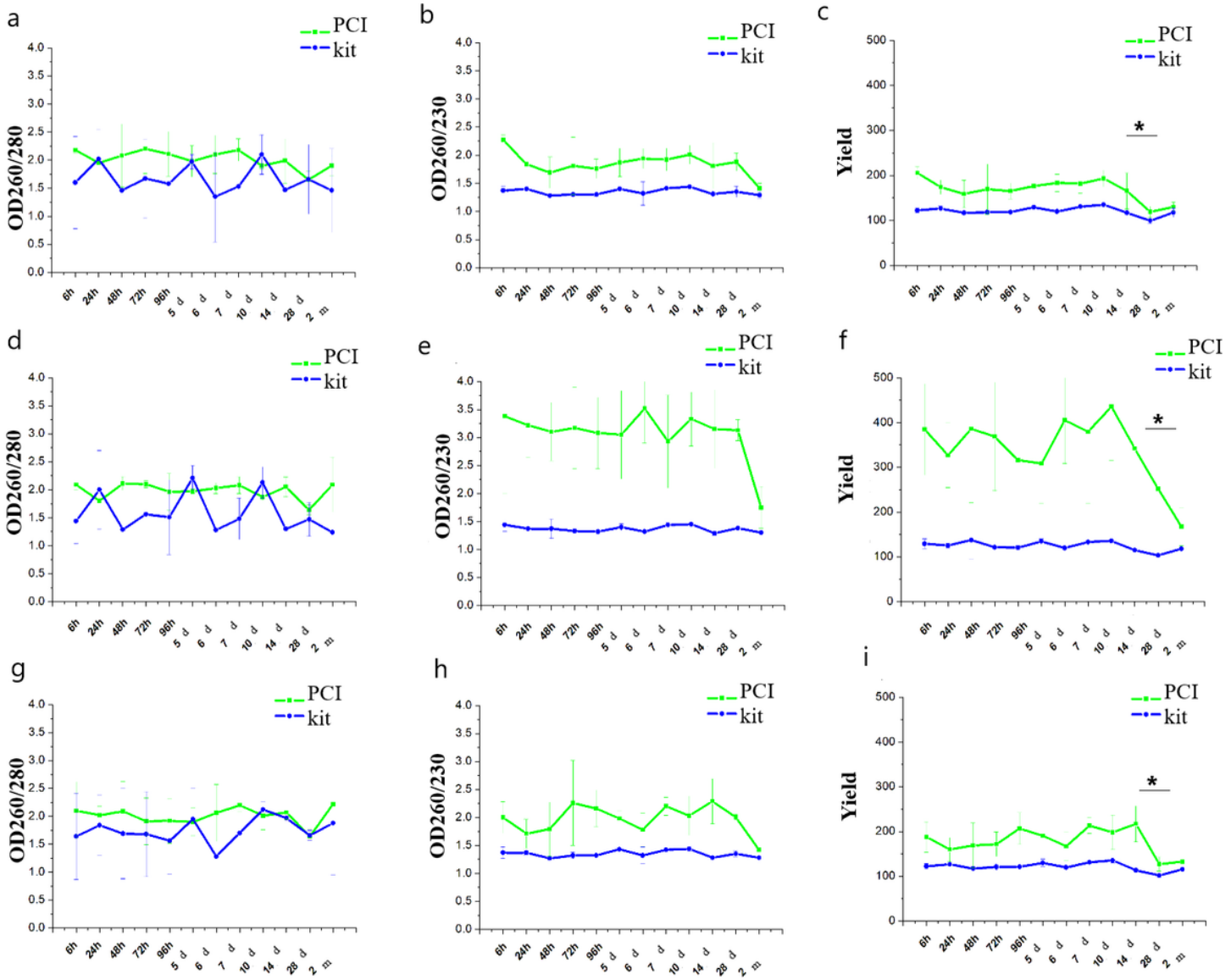

Figure 2

The influence of length of tissue formalin fixation on purity (OD260/280 ratio: a, d, g and OD260/230 ratio: b ,e, h) and yield (ng DNA in 50 µl TE buffer) (c, f, i) of DNA isolated from heart (a ,b, c), liver (d, e, f), brain (g, h, i) tissue.

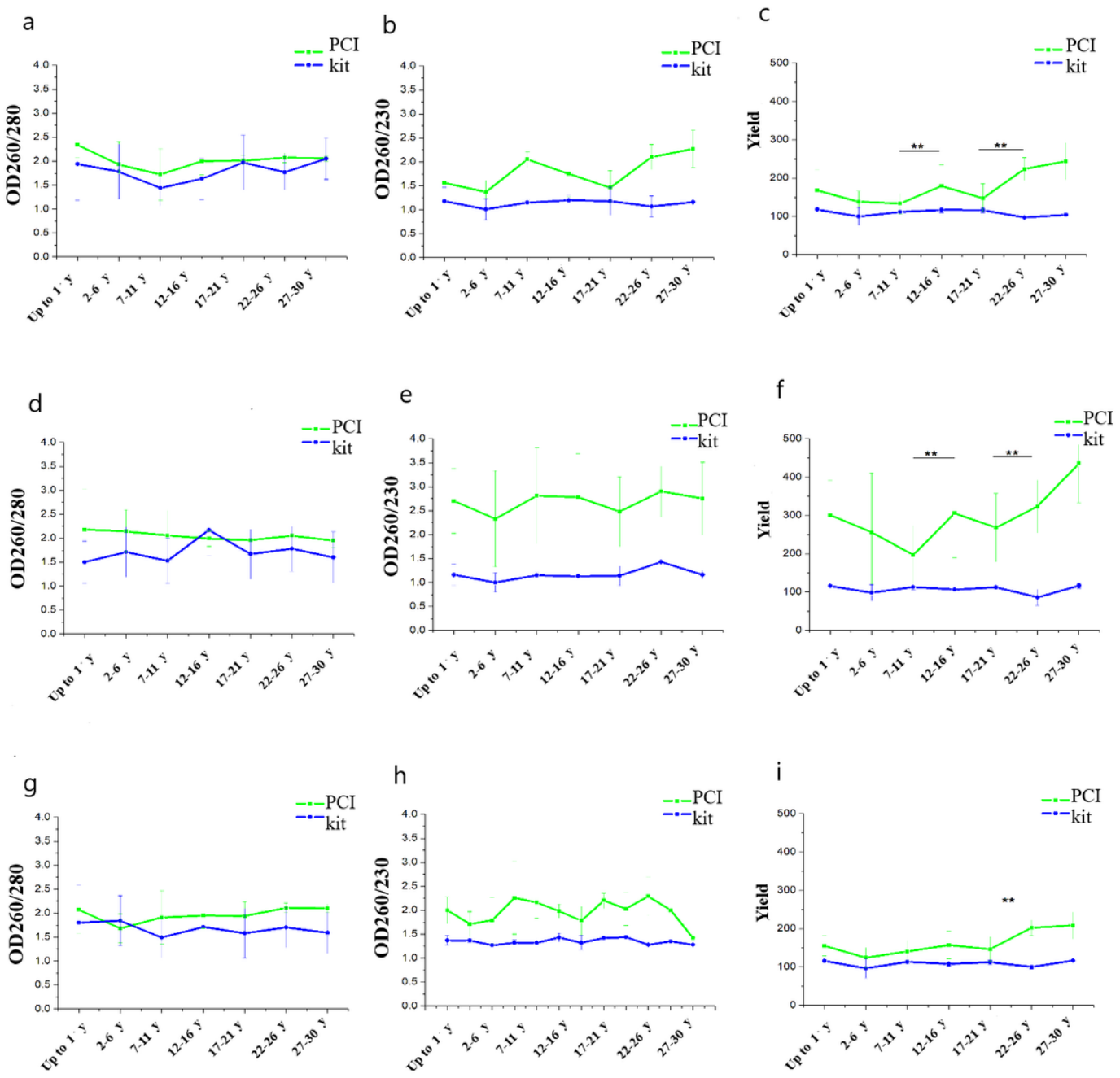

Figure 3

The influence of FFPE tissue storage period on purity (OD260/280: a, d, g and OD260/230: B, E, H) and yield (ngDNA in 50 µl TE buffer) (c, f, i) of DNA isolated from heart (a, b, c), liver (d, e, f) and brain tissue (g, h, i).