The Effects of Suboptimal Formalin Fixation on DNA Content of Formalin Fixed Paraffin Embedded Tissues

Formalinde Fikse Edilmiş Parafine Gömülü Dokularda Suboptimal Formalin Fiksasyonunun DNA İçeriği Üzerine Etkileri

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ABSTRACT Objective: Tissue fixation is the most important step in morphologic preservation. While pro-tein preservation is essential in histopathology and immunohistochemistry, nucleic acid pres-ervation is important for molecular techniques. In daily practice, 10% formalin is prepared from 37-40% formaldehyde stock solution. Meanwhile, the pH of the fixative needs to be kept between 6-8. In this study, the effect of variable concentrations and pH of formalin on DNA preservation was investigated with polymerase chain reaction (PCR) and fluorescein in situ hybridisation (FISH). **Material and Methods:** Samples with a 3-milimeter diameter were obtained from colon, thyroid and breast after resection. Samples were fixed in ten different formalin solutions prepared in variable concentrations and pH values. Genomic DNA was extracted from paraffine blocks, and the quantity and purity of the DNA were analyzed. To test the reliability of DNA fragments, PCR amplification was carried out using human β-globin gene (HBG). FISH method was applied to search the effects of varying formalin fixa-tives on hybridization kinetics using her-2/neu-17CEP dual fluorescein probes. **Results:** DNA was successfully extracted from all 30 samples of three different tissues fixed in 10 different concentrations and/or pH values of formalin solution. However, alterations in the formalin concentration and the pH value negatively affected FISH quality. However, alterations in pH was more effective on FISH results. **Conclusion:** In conclusion, during preparation and usage, adverse changes in pH and/or concentration of formalin do not affect DNA preservation sig-nificantly. However, changes in both pH and concentration of the formalin fixative may widely alter hybridization properties, so as FISH results.

Key Words: Formaldehyde; polymerase chain reaction; in situ hybridization, fluorescence

ÖZET Amaç: Doku fiksasyonu, morfolojik muhafazada en önemli basamaktır. Protein prezervasyonu histopatoloji ve immünohistokimyada, nükleik asit prezervasyonu da moleküler uygulamalarda önemlidir. Günlük pratikte %10 formalin %37-40 formaldehit stok solüsyonundan hazır-lanır. Bu arada fiksatifin pH'sının 6-8 arasında tutulması tercih edilir. Bu çalışmada, forma-linin değişik konsantrasyonlarda ve pH düzeylerinde DNA prezervasyonu üzerine olan etkisi polimeraz zincir reaksiyonu (PZR) ve floresan in situ hibridizasyon (FISH) ile değerlendirildi. **Gereç ve Yöntemler:** Üç milimetre çaplı örnekler, rezeksiyonun ardından kolon, tiroid ve memeden elde edildiler. Örnekler, değişik konsantrasyonlarda ve pH'da hazırlanmış on farklı formalin solüsyonunda fikse edildi. Parafin bloklardan genomik DNA çıkarıldı ve DNA'nın miktar ve saflığı analiz edildi. DNA fragmanlarının güvenilirliğini test etmek için, human β-globin geni (HBG) kullanılarak PZR amplifikasyonu yürütüldü. Değişik formalin fiksatiflerin hibridizasyon kinetikleri üzerine olan etkilerini araştırmak için her-2/neu-17CEP dual floresan probları kullanılarak FISH metodu uygulandı. **Bulgular:** On faklı konsantrasyon ve/veya pH'da formalin solüsyonunda tespit edilen 30 ör-neğin tamamından başarıyla DNA izole edildi. Ancak formalin konsantrasyonu ve pH değer-indeki değişiklikler FISH kalitesini negatif yönde etkiledi; pH değerindeki değişiklikler FISH sonuçları üzerinde daha fazla etkiliydi. **Sonuç:** Sonuç olarak, hazırlanma ve kullanma esnasında, formalinin pH ve/veya konsan-trasyonundaki zıt değişiklikler DNA prezervasyonunu etkilemiyor olabilir. Fakat formalin fiksatifteki hem pH hem konsantrasyon değişiklikleri, hibridizasyon özelliklerini ve bu bağlamda FISH sonuçlarını geniş ölçüde değiştirebilir.

Anahtar Kelimeler: Formaldehid; polimeraz zincir reaksiyonu; in situ hibridizasyon, flöresans

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Tissue fixation is the most important step for morphologic preservation in routine microscopic diagnostic approaches. It is essential to examine the tissues fixed in appropriate fixative and at optimal fixation time for accurate morphologic details to make the correct diagnosis. Recently, many ancillary diagnostic methods have been increasingly used in pathology practices.[1] Especially molecular methods facilitating the diagnosis or guiding the therapeutic approaches have been implemented for the last two decades. Therefore, the samples submitted to the pathology department need to be fixed in appropriate fixatives for routine histochemical and immunohistochemical studies as well as further molecular applications.[2,3] While the proteins are the main target for immunohistochemical applications, nucleic acids are the basic elements of the molecular techniques. To determine the ideal fixative, it is essential to prefer the one preserving not only the protein content but also the nucleic acids. The fixatives such as ethanol, formalin, Zenker, B5, Bouin's, Hollande are used in routine pathology practices. In previous studies evaluating the nucleic acid preservation of the fixatives, the best choices were reported to be formalin and ethanol.[4,5]

In routine pathology practices, 10% formalin solution is used for fixation. Instead of using ready to use buffered 10% formalin, formalin is usually prepared from 37-40% formaldehyde stock solution in our country similar to some other countries. In our institution, the final fixative solution is prepared in ideal concentration and pH, in the pathology department or in clinics transferring the sample for pathologic examination. The final pH of the fixative should be between 6 and 8. Therefore, 10% formalin solution is suggested to be buffered. Sodium salts of hydrogen phosphate are used for this purpose. However, in daily practice, preparing the ideal formalin fixative is not always possible due to incorrect procedures in buffering and dilution. Additionally, keeping the fixative in optimal quality may not be possible, because the pH of the solution may change in time.

The aim of this study was to assess the effect of variable concentrations and pH levesl of formalin fixative on deoxyribonucleic acid (DNA) preservation to understand how inapproprate 10% buffered formalin solution preparations influence the DNA preservation for further molecular approaches.

MATERIAL AND METHODS

General informed consent was taken from patients upon hospitalization regarding further research analysis on surgically resected diagnostic samples sent to the pathology department. Samples with 3-mm diameters were obtained with punch-biopsy from normal appearing areas of the colon, thyroid and breast immediately after resection. The biopsy specimens were fixed in room temperature in ten different compositions of formalin solutions (Lachema, Czech Republic catalog no: 30251) prepared in variable concentrations and pH values as shown in Table 1. The pH value of the solutions were adjusted with sodium salts of hydrogen phosphate. Each specimen was fixed in 100 ml fixative solution for 24 hours.

Following the fixation step, samples were embedded in paraffin after routine tissue processing procedure. Factors that might damage DNA such as heat, pressure and microwave during the process were avoided. Five 10-µm thick sections from each of the 30 sample blocks were collected in sterile tubes. Later, total genomic DNA of each sample was extracted according to the procedures of formalin fixed paraffin embedded (FFPE) tissue extraction kit (QIAamp DNA mini kit, QIAGEN,

TABLE 1: Concentration and pH documentation of fixatives used in the study for tissue fixation.

	Tissue Type		
Formalin	**Colon**	**Breast**	**Thyroid**
Concentration: 10% pH: 3.65	C1	B1	T1
Concentration: 10% pH: 4.38	C2	B2	T2
Concentration: 10% pH: 6.03	C3	B3	T3
Concentration: 10% pH: 8.88	C4	B4	T4
Concentration: 10% pH: 7	C5	B5	T5
Concentration: 11% pH: 7	C6	B6	T6
Concentration: 12% pH: 7	C7	B7	T7
Concentration: 20% pH: 7	C8	B8	T8
Concentration: 30% pH: 7	C9	B9	T9
Concentration: 40% pH: 7	C10	B10	T10

Hiden, Germany). The quantity and purity of the extracted genomic DNA were analyzed with spectrophotometer (BioPhotometer, Eppendorf AG, Hamburg, Germany). The purity was detected by measuring the OD260/OD280 ratio (Table 2) and the quality was tested by gel electrophoresis and polymerase chain reaction (PCR). For gel electrophoresis, 2% agarose gel plates were prepared using agar (Sigma Catalog no: A9539) and Tris Borate Ethylenediaminetetra-acetic acid (TBE, pH 8.0). By diluting or concentrating, each sample was standardized to 15 µl containing 0.5 µg DNA and samples were transferred on the gel plate. Each gel plate was run in TBE buffer at 100V constant current for 35 minutes. After electrophoresis, gel plates were stained with 1% ethidium bromide (Sigma catalog no. 8750). DNA fragments, bands or smeared pattern were evaluated in gel image analysis system (Kodak Gel Logic200, Rochester, NY). As a size marker, 100 bp DNA ladder was used.

After fixation of breast, thyroid and colon tissues in various formalin solutions, the fragment size of the extracted DNA was evaluated. To test the reliability of DNA fragments for further possible molecular tests, PCR amplification of the extracted DNA from each sample was carried out using human β-globin gene (HBG), which is a housekeeping gene. PCR cycle was set as 30 cycles after incubation in 95°C for 5 minutes for DNA polymerase enzyme activation. Each cycle consisted of denaturation in 94°C for 30 seconds, annealing in 55°C for 30 seconds and elongation in 72°C for 30 seconds. Final extension was in 72°C for 10 minutes (Apollo ATC 401, USA). The primers and tube mixture were designed as previously reported.[5] PCR amplicons were electrophoresed and after etidium bromide staining, DNA band patterns were evaluated in gel image analysis system. Successful amplification of HBG was confirmed by the presence of 260 base pair (bp) band.

Fluorescent in situ hybridization (FISH) method was used to search the effects of varying formalin fixatives on hybridization kinetics of the tissues.[6] In all 30 tissues, her-2/neu-17CEP dual fluorescein probes were used for the FISH test. Before the FISH test, 30 tissue blocks were re-blocked as four macroarray tissue blocks, 3 blocks containing 9 samples each and one block containing 3 samples. Nine samples from breast, thyroid and colon were blocked in three separate macroarrays and one sample from each tissue was blocked in the fourth macroarray. Five-micrometer sections from each microarray block were taken on the positive charged slides.

For the FISH procedure, deparaffinization (SkipDevax, Insitus Biotechnologies, NM) and digestion (Tissue Digest Reagent, Insitus Biotechnologies) of the sections were carried out according to the instructions in the kit manuel. PathVysion Her-2 Kit (Vysis, IL) instructions were carried out for FISH. Each section was examined under fluo-

TABLE 2: Quantity and purity of DNA extracted from 30 samples fixed in formalin with varying concentrations and pH.

	DNA					
	QUANTITY (µg)			PURITY (OD260/OD280)		
FIXATIVE	Thyroid	Colon	Breast	Thyroid	Colon	Breast
10% pH 3.65	6.00	2.00	2.00	2.11	2.05	2.13
10% pH 4.38	4.50	2.70	1.00	1.98	2.07	2.11
10% pH 6.03	2.00	6.00	1.20	2.01	2.10	2.10
10% pH 8.88	7.50	1.70	1.00	2.00	2.05	2.00
10% pH 7	3.50	7.00	3.10	1.86	1.90	1.87
11% pH 7	1.20	4.00	3.50	1.88	1.90	1.86
12% pH 7	10.00	2.50	4.70	1.87	1.87	1.87
20% pH 7	8.00	1.70	1.50	1.90	1.86	1.86
30% pH 7	5.50	6.00	8.00	1.86	1.87	1.83
40% pH 7	7.50	11.20	2.00	1.85	1.87	1.89

DNA: Deoxyribonucleic acid.

rescein microscope (Leica DM 2500, Germany). DAPI/FITC (Filter Cube: DAPI/FITC, ordernumber 11532287), S-Green (Filter Cube: Spectrum Green, ordernumber 11532223) and S-Gold (Filter Cube: Spectrum Gold, ordernumber 11532206, Leica) filtercubes were used. Images containing 100 cells were taken and recorded using the image analyse system (Leica, DFC 300FX, Germany). From the images, the number of orange signals (Her-2/neu) and green signals (CEP 17) in each cell was counted and the presence of nonspecific background noise signal was evaluated.[7]

RESULTS

DNA was successfully extracted from all 30 samples obtained from three different tissues fixed in 10 different concentrations and/or pH values of the formalin solution. The total amount of DNA extracted from similar sizes of colon, thyroid and breast tissue was between 0.10 and 11.2 μg. The DNA amount was 1.7-1.2 μg for colon, 1.2-10 μg for thyroid, and 1-8 μg for breast. The effect of formalin concentration and pH on the amount of extracted DNA was statistically insignificant. OD260/OD280 ratio of extracted DNA was between 1.71 and 2.01.

The agarose gel electrophoresis patterns of 30 different DNA samples were evaluated (Figure 1). Detected gel pattern differences about extracted DNA was found ineffective for HBG amplification (Figure 1). Using HBG gene specific primers, 260bp fragment was amplified in all samples.

In the FISH analysis, orange (Her-2/neu probe) and green signals (CEP17 centromeric probe) were intensively seen in sections of breast,

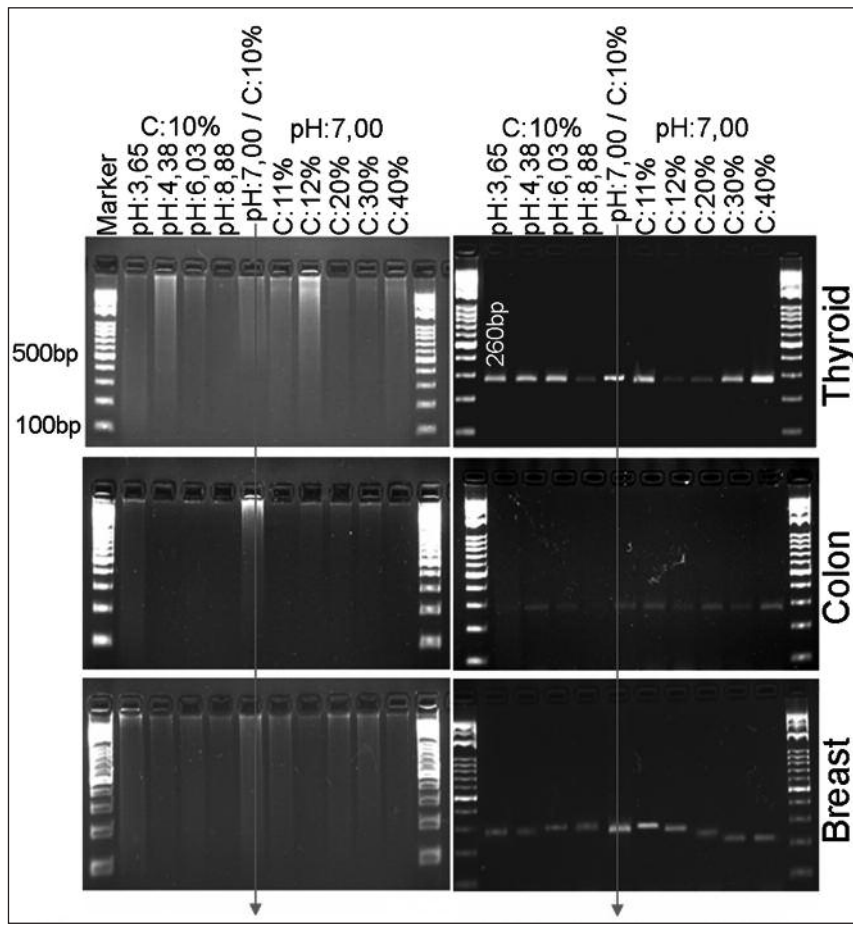

FIGURE 1: Agarose gel patterns of extracted deoxyribonucleic acid (DNA) (left hand side) and the amplified 260bp fragment sized human β-globin (HBG) gene from extracted DNA (righ hand side) of 30 samples are shown with relation to concentration and pH variation of the formalin fixative used. Size markers are situated at both ends of the gel plates.

thyroid and colon tissues fixed in 10% buffered formalin (Figure 2). Two green and 2 orange signals per cell were counted in 40 cells. Alterations in formalin concentration negatively influenced FISH quality such as background noise signalling and unidentifiable cell borders (Figure 2). Negative effects on FISH quality were more prominent with pH alterations of the fixative.

DISCUSSION

Recently, molecular methods have frequently been used in pathology practices for either diagnostic or prognostic approaches. Identification of genetic alterations such as point mutation, deletion, translocation and amplification may provide invaluable information over conventional histopathologic diagnosis, patient follow-up, disease prognosis and treatment responses. Target molecules used for identification of genetic alterations are mainly DNA and/or RNA. Type of test material for extraction of these molecules may be fresh or frozen tissue and paraffin embedded tissues.[5,6] Since many molecular techniques are ancillary methods that aid histopathologic diagnosis, paraffin blocks are the most common source for molecular tests. Another reason to prefer paraffin embedded tissue blocks in molecular techniques is the difficulty and cost to establish fresh tissue banking for retrospective studies. Consequently, tissue fixation is still one of the most important factors influencing the results of molecular testing.

Formalin is the most common fixative used in pathology practice. In previous studies assessing the effects of fixatives on nucleic acids, ethyl alcohol and 10% formalin were reported to be ideal fixatives.[5] Fixation time, fixative concentration and pH should be optimized to protect both nucleic acids and proteins; optimal conditions should be main-

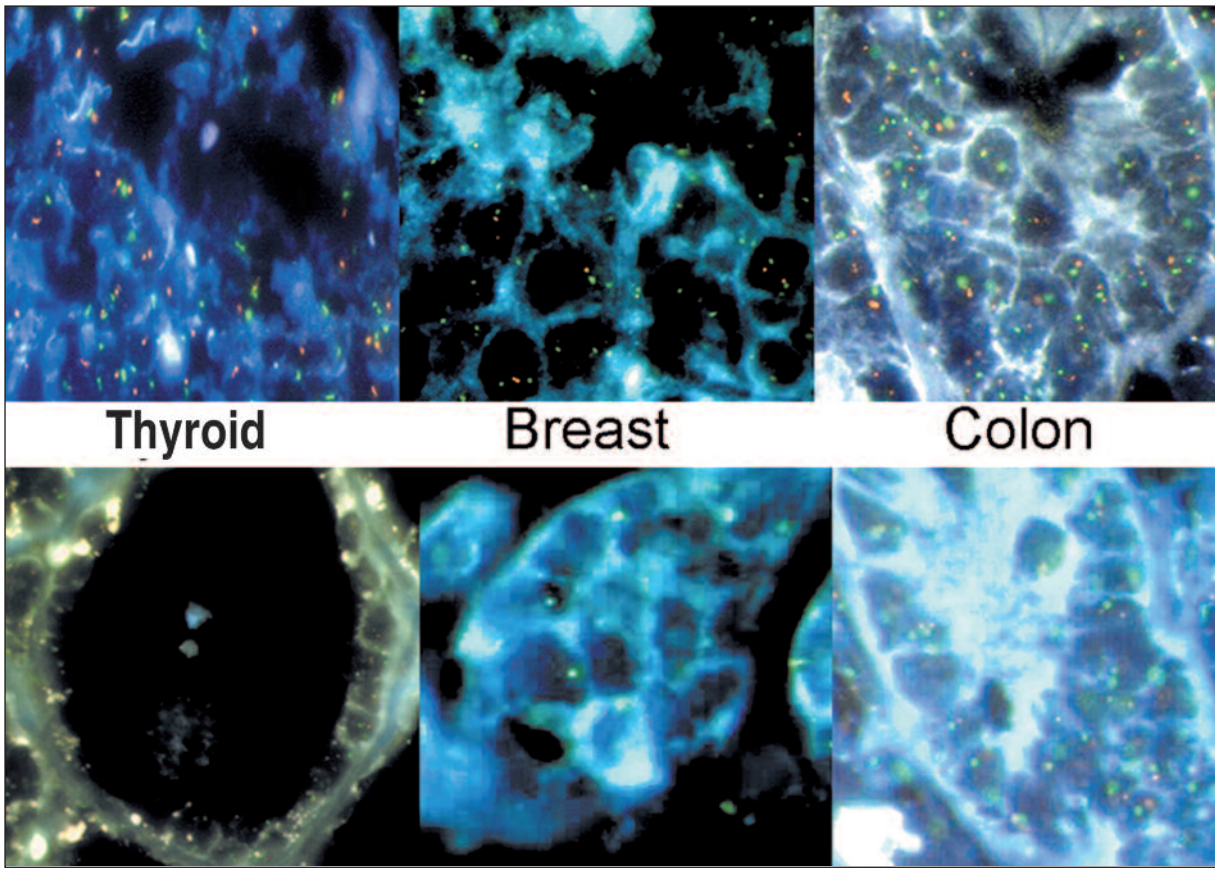

FIGURE 2: Her2/neu fluorescent in situ hybridization (FISH) images of thyroid, colon and breast tissues. Tissues fixed in 10% neutral buffered formalin are shown in the upper row and tissues fixed in 10% formalin with pH 3.65 are seen in the lower row.
(See for colored form http://tipbilimleri.turkiyeklinikleri.com/)

tained in daily practices. In routine histopathologic examinations, the most important reaction to preserve the tissue and cell morphology is the stabilization or fixation of the proteins. However, histomorphologic examination alone is becoming inadequate for accurate diagnosis parallel to the improvement in understanding the diseases and their etiopathologies. Fixatives should preserve the antigenic features of proteins because immunohistochemical examinations may also be required. Moreover, nucleic acids, which are the main targets for molecular methods, must be preserved. Compared to protein fixation, the effects of fixatives on nucleic acids are yet to be clarified.

Until the fixation process is completed, tissues are recommended to be kept in optimal circumstances with appropriate hydrogene ion concentration to preserve the structural and functional properties of many molecules, especially proteins. Therefore, hydrogene ion concentration of the fixative is suggested to be between pH 6-8. The formaldehyde solution, which is the most common fixative in daily practice, should be buffered since inappropriate pH values may especially cause alterations in ultrastructural features. However, chemical properties of 10% formalin solution may change in time.

CONCLUSION

In conclusion, in the pathology practice, incorrect procedures while preparaing buffered 10% formalin solution manually from 37-40% stock solution should not influence DNA preservation in terms of PCR based amplifications arround 260bp DNA fragment size, which is the mostly preferred amplicon fragment size for formalin fixed paraffin embedded tissue samples for PCR tests.[8] However, changes in the pH value and concentration of the formalin fixative may widely alter hybridization properties and consequently FISH results.

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