A new fixative for molecular pathologic evaluation

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감사의 글

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그리고 한국 땅을 밟을 때부터 가족처럼 따뜻하게 대해주시고 병리전문지식뿐만 아니라 인생의 참뜻도 알려주신 정순희 교수님, 조미연 교수님, 또 자신의 일처럼 도와주신 엄민섭 선생님, 이미정 선생님과 병리과 여러 선생님들께도 감사의 마음을 전합니다.

마지막으로 저를 낳아주시고 애지중지 키워주시고 항상 곁에서 지켜 주시고 아낌없이 사랑을 주신 사랑하는 양가부모님, 사랑하는 남편에게 작지만 마음을 담은 선물로 드립니다.

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ABSTRACT

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Purpose of the study: Formalin is the most widely used fixative because of its low price and good capacity of preserving morphology of a fixed tissue. However the cross-linking effect of formalin can cause some damage on macromolecules (DNA, RNA, protein) of fixed tissues. The purpose of this study is to develop a new ideal and safe fixative that will have nearly the similar grade of morphological shape to that of formalin, which could be used without problems not only in many clinically required special chemical and immunohistochemical stainings, but also in molecular biological tests.

Methods: After developing environment friendly and nontoxic fixatives (WONFIX) through the comparative analysis of several fixatives in alcohol series, the fixation characteristics of developed fixatives have been compared with other fixatives by using rat or human tissues. By selecting the frozen tissue and 5 different fixatives (1.WONFIX 2.Carnoy's fixative 3.methacarn 4.Wolman's solution 5.formalin), the tissue was fixed for 24hours by using each fixative and then embedded in paraffin. By using an optical microscope, the morphological pattern in each fixative was evaluated to read the quality of morphological finding in A~E grades and the immunohistochemical staining was performed to compare the antigen expression patterns. After extracting DNA, RNA, and protein from the paraffin blocks of fixed tissue, the concentration of macromolecules and the statistical significance were compared by using the Wilcoxon signed rank test method. Regarding the housekeeping gene, PCR, real-time PCR, and Western blot analysis were performed.

Results: In the optical microscopic evaluation of fresh tissue and the tissues fixed at 5 different types of fixatives, the best tissue morphology was observed in the tissue fixed at formalin, which was followed by WONFIX. Although the immunohistochemical staining results by using 14 antibodies resulted to show the same positive and negative expression frequency, the staining intensity observed in the tissue fixed at WONFIX was the strongest and showed nearly the similar staining pattern to that of formalin. The largest amount of DNA extraction was made in the tissue fixed at WONFIX ($p<0.05$), which enabled to perform PCR upto 1.5kb. The extracted amount of RNA at the time of using WONFIX fixative was placed at the fourth grade, but the amount was significantly larger than the amount that was extracted from formalin ($p<0.05$). The mean threshold cycle number (CT) value acquired by performing the real-time PCR was found to be 11.3 in fresh tissue and 12.3 in WONFIX. The mean CT value was acquired by performing real-time PCR for the extracted RNA after the microdissection of 1000 cells, which resulted to show 19.4 in fresh tissue, 23.2 in WONFIX, and 30.6 in formalin. The RNA preservation state of the tissue fixed at WONFIX was better than the tissues fixed at other fixatives. When the protein quality was compared by conducting the Western blot analysis, the protein extracted from the tissue fixed at WONFIX resulted to show similar state that could be observed at the fresh tissue extraction.

Conclusions: WONFIX is the ethanol based fixative, which is environment friendly and safe without having heavy metals or toxic chemical substances. At the same time, the excellent morphological findings could be its characteristic, which enables to perform clinically required molecular biology tests.

Key words: Fixative, WONFIX, formalin, DNA, RNA, protein, PCR, real-time PCR, immunohistochemistry, Western blot analysis

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(Directed by Professor Kwang Hwa Park)

I. INTRODUCTION

Fixative could be defined as the liquid that can preserve the morphology and biochemical state of tissue as much as to that of live body. As the ideal condition of fixative, Firstly, it has to maintain its original morphology without contraction and deformation of tissue while processing the procedures for microscopic examination. Secondly, the components of tissue and cell should be preserved without losing. Thirdly, the chemical compositions of cells, such as protein and fat should be well preserved as in the original state to be reacted in special staining.[1] The use of such fixatives is the essential tool for the pathological diagnosis for the appropriate therapies and prognosis evaluation through the morphology test and analysis of nucleic acids and proteins. Although the studies on fixatives have been made for a long time, the perfect fixative that satisfy all of the above 3 requirements has not been found yet.

Regularly at the pathology department, the biopsy tissue is fixed in a fixative and embedded in paraffin, and then hematoxylin-eosin (H&E) slides are prepared for diagnosis. The remaining blocks could be used as the very important resources to perform the studies of using molecular biological techniques and to confirm the treatment responses in rare disease, in etiological studies, or in the long term track down studies. In addition, such method allows to acquire tissue and cell samples in different stage of a disease from a patient, which enables to elucidate the pathological mechanism by a disease stage.

When the currently widely used fixatives were classified according to the chemical properties, it could be classified into aldehydic, alcoholic, and heavy metal series. The largest part of the fixation is conducted by using an aldehydic series of 10% neutral buffer formalin (40% formaldehyde 100ml, distilled water 900ml, sodium dihydrogen orthophosphate 4g, disodium hydrogen orthophosphate 6.5g).

Formalin was found by Butlerow in 1859, and it has been used as germicides, tissue fixatives, and as preservatives when the formal production was started in the companies of France and Germany. Other than that, it has been also used in the manufacture of glues and synthetic fibers.[2] The advantages of formalin could be listed as its cheap price and availability of performing fast and effective treatment to pathogens (parasites, fungus, and bacteria).[3] But its colorless transparent and strong volatility can stimulate eyes to shed tears when its content in air reaches more than 0.1ppm and have bad effects on the upper respiratory track, and its strong corrosiveness can cause hypersensitivity, dermatitis, and eczema when it is directly contacted to human body. The small amount of formaldehyde requires short time to be metabolized within in a human body. Since 35% is metabolized as formic acid to be excreted as urine and the last of 65% is continuously metabolized to be excreted as in the form of carbon dioxide and water, its accumulation in human body is small. However, formalin can cause gene mutations in the cells separated from human body or in animal cells. So, it is considered to be the carcinogen and the chance to cause abnormality in organisms should not be disregarded. Therefore, the development of safe and useful formalin alternatives is necessary for the purpose of industrial safety.

As the disadvantages of formalin, its action on varying the volume or mass in fixed tissue could be listed. So, the tumor size in fresh tissue and in fixed tissue looks different and a difference could be observed by the types of organ or by the fixation time.[4-6] Pathologists receive a tissue after a surgery and describe the size, weight, and shape of the tissue at the gross examination. After then, the pathogenic lesions are selected to prepare slides for microscopic examination and all findings are integrated to make a diagnosis. In case of malignant tumor, it is evaluated by following the system established by the American Joint Committee on Cancer (AJCC), and clinical physicians decide the prognosis and therapy based on the evaluation. In the definition of AJCC, the disease stages are divided by the size of tumors. The tumor size evaluation standardized by AJCC did not yet clearly state whether the evaluation of the size has to be made based on fresh tissue state or formalin fixed tissue state, or on the size measured by microscopic findings. As an example, the non-small cell carcinoma developed in lung is divided into disease stage 1 and disease stage 2 based on the tumor size of 3cm by following the AJCC system. But, the therapy and prognosis for both stage of the disease are distinctively different. Hsu et al. (2007) reported the existence of statistically significant difference by comparing the tumor size before fixation and after fixation in 401 patients who were diagnosed with non-small cell carcinoma.[7] It could be attributable to the strong contractility of formalin fixed tissue, which changed the disease stage according to the

tissue condition at the time of measuring the size. Furthermore, tumor size is used to differentiate the disease stage, and the size change before and after the fixation can change the disease stage.[6] In addition, some of studies reported that the prognosis of colon cancer is related to the distance between tumor and surgical resection margin, and reported that the distance of safety margin was contracted twice the distance of original one by formalin fixation.[8] However, the measurement of tumor size from the fresh tissue after a clinical surgery is a very difficult task. Therefore, the development of fixatives which can preserve the tissue size nearly as the original size could be told as the very important task.

The staining technique that was initiated from the mid 19 century has been advanced to the surprising level of technique that is used at the current century through the continuous and steady studies, and it has been widely used not only in the field of research studies, but also in the diagnosis of pathological tissues.

In the histochemistry, the most effective enzyme activity could be observed in fresh or frozen tissue section. As an example, when studying the skeletal muscle-related enzyme in a patient suspected with myopathy, the staining of fresh tissue can make a precise diagnosis.

The immunohistochemical staining method is the technique of detecting antigens in tissue, which visualizing the antigen by the immune reaction of antigens and antibodies. However, the tissue embedded paraffin after the fixation in formalin can cause the change in protein compositions, and the primary structure of protein is destroyed most rapidly through the cross-linking with formaldehyde, and all other structural changes are made in the secondary, third, and fourth structures of protein.[9] Therefore, the use of paraffin embedded tissue shows the limitation in immunohistochemical staining method compared to that of using frozen tissue sections.

Based on the development of molecular biology, numerous diseases have been found to be related to the gene changes, which required the extraction of genes from human body tissues for the purpose of diagnosis and study. As an example, when the clonality of lymphoma originated from B cells has to be evaluated compared to that of previous immunohistochemical staining, the more reliable immunoglobulin gene rearrangement technique is used.[9, 10] In case of tuberculosis and infectious diseases caused by viruses, the Polymerase Chain Reaction (PCR) technique is the frequently used diagnostic method, and the development of diagnostic methods by applying RT-PCR and real-time PCR have been made continuously.[11, 12] However the commonly used technique of performing PCR by using paraffin embedded tissues after the fixation in formalin shows a low success rate due to the inability of conducting the Southern blotting and Northern blotting, which is caused by the

destruction of DNA and RNA by formaldehyde. Moreover, the acquisition of amplified product more than 400bp is difficult in case of performing PCR test.[13] When the PCR test was applied as the gene testing method to amplify about 100bp of genes, the possibility to have false positive results could be increased. Therefore, the necessity to acquire larger PCR products for precise diagnosis has been gradually increased.

The study of Wong et al. (1998) confirmed the change of BRCA1 in the tissue of a patient diagnosed with ovary cancer by examining the gene mutations in two different slices of paraffin embedded normal tissue after the formalin fixation, which detected different gene mutations between the slices.[14] Such contrary result is considered to be attributable to the occurrence of gene mutation during the fixation procedure by using formalin. So, blood was collected from two patients to examine the gene mutations in fixed tissue and fresh tissue, which resulted in showing the occurrence of different gene mutations in fixed tissue compared to the fresh tissue samples. According to the study results, formalin has an effect on the genes of tissues.[14, 15] But the currently reported 38% of somatic mutations have been confirmed by using formalin fixed tissues.[15]

For the precise molecular biology examinations and studies, the frozen tissue bank has been used. But, the operation of the frozen tissue bank in general hospitals has to face many difficulties because of the difficulties to acquire enough amount of fresh tissue that could be preserved immediately after a surgery and the operation of the bank require large expenses economically. Most of all, in the cases of using RNA, the RNA of tissue stored in a frozen tissue bank is destroyed sometimes after the storage, and the utility of the tissues is vanished. Although the RNA preservation solution of RNA later (Qiagen, Germany) has been used for the storage, it can cause protein denaturation which won't be helpful in preserving morphologic findings.

Even if the tissue quality does not reach to that of frozen tissue, it is absolutely necessary to develop new fixatives which can preserve the cellular morphology to that of live body to satisfy a clinically required molecular biological diagnosis, which could be applied in the study techniques that are helpful in conducting study or therapy. It can contribute not only to the development of clinical diagnosis, study, and therapy, but also to the development of biochemistry, molecular biology, and health and medical industry. Also, its industrial value is very high since the most of fixatives used in many hospitals and research institutions could be replaced with it.

For that, pathologists made steady efforts to develop new fixatives. Vicek et al.(2003) reported the development of a fixative of UMFIX, which can preserve the DNA upto 450bp

and show better RNA preservation characteristics compared to that of formalin.[16] Since the major composition of the fixative is methanol, it was found to be safer than formalin, but the risk of industrial hazard has not been reduced. Delfour et al.(2006) reported the acquirement of satisfactory result by examining RNA with the laser-capture microdissection method onto the previous study by preparing the RCL2 fixative. However, the method still based on methanol as the UMFIX and required to preserve at 4℃, which is uncomfortable to use in general purposes.[17] Until so far, the satisfactory fixatives that can be used widely in pathology tests have not been found, and the already developed fixatives are expensive and have limited purposes of use, which only allowed them to use in few studies.[18, 19]

The purpose of the current study is to develop new types of fixatives for the rapid fixation of tissue and to preserve the maximum morphological findings compared to that of formalin fixed tissues, which are safe and cheap priced one that can simultaneously satisfy several special chemical staining , immunohistochemical staining and molecular pathologic tests.

II. MATERIALS AND METHODS

1. Materials

1) The study reports on fixatives have been investigated to compare the already developed several available fixatives, and the WONFIX which resulted to show the most similar morphological shape to that of formalin fixed tissue was prepared.
2) The fresh samples that were collected from March, 2008 during surgical operations performed at the Wonju Christian Hospital of the Wonju Medical College of Yonsei University were subjected for the current study.
3) On February 27, 2008, the current study submitted a study application to the Institutional Review Board of the Wonju Christian Hospital of the Wonju Medical College of Yonsei University and acquired the approval. (CR107067)

2. Methods

1) Histomorphologic findings

Tissue was cut regularly (2cm) and fixed at the selected 6 types fixatives (1.Frozen tissue 2.WONFIX 3.Carnoy's fixative 4.methacarn 5.Wolman's solution 6.formalin) and paraffin embedded. The paraffin embedded tissue was thin sliced in the thickness of 5 μm, and the H&E stained slides were distributed to 3 pathologists, 2 cytologists, and to 7 medical college students to read the morphological findings of the slides in 5 grades (A~E) without knowing the information about the fixatives.

2) Immunohistochemical staining

The immunohistochemical staining was conducted for the following antigens of carcinoembryonic antigen (CEA), pan-cytokeratin (CK), vimentin, CD3, CD4, CD5, CD8, CD20, CD45, estrogen receptor (ER), progesterone receptor (PR), c-erbB2, p53, and ß-HCG to see the expression difference between the fixatives (Table 1).

The immunohistochemical staining was done by attaching 5μm thin sliced paraffin embedded tissue that was fixed at formalin to the coating slides and dried at 50℃ for 2 hours. Then the paraffin was removed by 4 times of washing with xylene for 5 minutes and stepwisely treated with 100% alcohol for 3 minutes and allowed to hold water in DW. After washing the slides with flowing tap water and the slides were dipped in DW. The PT module (LAB VISION, CA, U.S.A.) was filled with TE buffer (Tris-EDTA buffer pH 9.0) and preheated. The water absorbed slides were heated by putting into the rack of the PT

module. After dipping in tap water for 5 minutes and immersed in DW, the perimeter of tissue was marked with Elite PAP pen (DBS, CA, U.S.A). Then, the slides were reacted with a primary antibody for 1 hour and washed with Tris buffered saline plus tween 20 X (TBST buffer), which was treated with a primary antibody enhancer for 20 minutes and treated with horse radish peroxidase (HRP) polymer secondary antibody (Labvision, CA, U.S.A.) for 30 minutes. After then, the slides were washed with TBST buffer and stained with 3-amino-9-ethylcarbazole (AEC) for 10 minutes and counter stained with Mayor hematoxylin before dipped in DW and mounted with a soluble mount medium.

3) DNA extraction and PCR

The paraffin block was sliced in 10μm of thickness and removed the paraffin by adding xylene and by treating with absolute alcohol. The procedure was repeated for 3 times and dried. After then, the slices were stored for 1 night by immersing at 60℃ of warm water after adding Mg^{++} free buffer and protein kinase. At the next day, they were centrifuged at 13200 rpm and the supernatant was recovered to acquire DNA and the amount was measured. The PCR was conducted by using the HotStar Taq kit (Qiagen, Germany). The target gene sequences for PCR were listed in Table 2, which included human thromboxane synthase gene (100bp), human recombination activating gene (200bp), human promyelocytic leukemia zinc-finger gene (300bp), human AF4 gene (400bp), human AF4 gene (600bp), human recombination active-ting gene (1kb), and human recombination active-ting gene (1.5kb). The final volume of 20μl was acquired through the reaction by adding 10pmol primer, 15mM $MgCl_2$, 200uM dNTP, 100ng DNA, and 1U Tag DNA polymerase. The PCR product was electrophorized after repeating the following reaction step for 35 times, which included initial activation step at 95℃, denaturation at 94℃ for 15min, annealing at 60℃ for 1min, extension at 72℃ for 30sec, and final extension at 72℃ for 10min.

4) RNA extraction and real-time PCR

From the paraffin embedded tissue blocks which were fixed by using different fixatives, RNA was extracted and the comparison for the extracted amount and quality of RNA was made.

The paraffin blocks were sliced in the thickness of 10μm and RNA was separated by using the RNA RNeasy Mini kit (Qiagen, Germany). The concentration of the extracted RNA was measured at 260nm by using a spectrophotometer (Bio-Rad, CA, U.S.A.). The separated RNA was used to synthesize cDNA by using the 1st Strand cDNA Synthesis Kit

(Roche, Germany). If the amount of RNA was small, the concentration was measured after synthesizing cDNA. The real-time PCR for the synthesized cDNA was performed by using the Quantitect SYBR Green PCR kit (Qiagen, Germany). SYBR green HotStart Tag DNA polymerase, HotStart Tag PCR reaction buffer, 25mM $Mgcl_2$, β-actin primer (primer sequence: 5'-TAAGCGCGG CTACTT-3' and 5'-TCCT TAATGTCACGCACGATTT-3), dNTP, and DW were mixed to formulate the final reaction volume of 25μl for the reaction. The following reaction step, which included PCR Initial activation step at 95℃ for 15min, denaturation at 94℃ for 20sec, annealing at 60℃ for 30sec, and extension at 72℃ for 30sec was repeated for 45 times to compare the amount of mRNA.

5) Microdissection

RNA was extracted from 1000 cells that were acquired by conducting microdissection of frozen tissue, WONFIX, and formalin fixed tissue to perform real-time PCR and the amount and quality of RNA were compared by using the mean threshold cycle number (CT).

The WONFIX fixed paraffin embedded tissue and formalin fixed tissue were sliced in the thickness of 5μm and removed the moisture by placing at a slide dryer. After then, paraffin was removed by treating with xylene for 3 minutes for 3 times and sequentially dipped in 100%, 95%, 92%, and 80% alcohols, and DEPC diluted DW for 1 minute to remove the xylene, and the RNase was removed by dipping into DEPC solution for 1 minute for twice, and dipped in eosin for 1 minute for staining. The stained slides were dipped into 95% alcohol for 30 seconds, dipped twice into 100% alcohol for 30 seconds, and dipped into xylene for 1 minute for 3 times to finish the staining. The fresh tissue stored at the tissue bank was placed on a slide and dried at a dryer to remove the moisture, and the above described staining procedure was repeated. The stained slides were mounted on an optical microscope and the lesions without having necrosis or inflammation were selected to separate approximately 1000 tumor cells. The collected tumor cells were placed in a tube and RNA was extracted by using the PicoPure RNA isolation kit (ARCTURUS, CA, U.S.A.). The extracted RNA was used for the synthesis of cDNA by using the 1st Strand cDNA Synthesis Kit (Roche, Germany) and the real-time PCR for β-actin was progressed to compare the mean CT values.

6) Protein and Western blot analysis

After extracting protein from the tissue blocks fixed at each fixative, the quality of ß-actin protein was compared by using the Western blot analysis.

Onto the fixed tissue, 1x SDS sample buffer (50mM dithiothreitol (DTT), 2% w/v SDS, 62.5mM Tris-HCl (pH 6.8, 25℃), 10% glycerol, and 0.01%w/v bromophenol blue) was

added and the tube was placed in a vessel filled with ice and the viscosity of the sample was reduced by breaking down the tissue sample with a homogenizer for 15~20 seconds. After then, the sample preparation was heated at 95℃ for 10 minutes and cooled down by placing at the top of ice. The cooled sample was microcentrifuged at the speed of 13200 rpm to extract the proteins, and transferred after performing SDS-PAGE. Transfer method observed the following protocol which initially balanced the electrophorized gel with 50ml transfer solution (25mM Tris-Cl, pH8.3, 1.4% glycine, 20% methanol) for 10 minutes, and the immobilion-P membrane (Millipore, Bedford, MA, U.S.A.) was cut in appropriate size to place it in methanol solution for 2 minutes and then immersed in a buffer solution for 10 minutes. After the procedure, the Western blot cassette was placed in a vessel that filled with the transfer buffer solution, and 2 sheets of dacrosponge and Whatman 3mm filterpaper soaked with the transfer buffer solution were placed on top of the cassette. The balanced gel with the transfer buffer solution was carefully placed on the whatman 3mm filter paper avoiding the formation of air bubbles. On the top, 2 sheets of gel filter paper and Dacron sponge were mounted. After fixing the cassette carefully by avoiding the formation of air bubbles, the clips at both sides of the cassette were tightly secured. The cassette was transferred into a blotting chamber and the transfer buffer solution was added to the marked position to fully immerse the membrane inside of the cassette, and facilitated the electrodes to adjust the membrane side to be the anode and gel side to be the cathode. The transfer was conducted at 100mA for 90 minutes. After finishing the transfer, the transfer state of protein into membrane was confirmed by using the Ponceau S solution (Ponceau S 0.2g, Trichloroacetic acid 3g, Sulfosalicylic acid 3g) before performing immunohistochemical stainings.

The immunohistochemical procedure could be listed as in below. The protein transferred membrane was immersed and stirred in TBST solution (10mM Tris-Cl, pH8.0, 150mM NaCl, 0.05% Tween20) containing 1% bovine serum albumin and placed at room temperature for 1 hour to block the unattached side of membrane with protein. After removing the buffer solution that was used for blocking, the membrane was washed with TBST buffer solution 3 times for 10 minutes. 10ml of 1:1000 antiserum solution diluted with TBST buffer solution for the primary antibody of β-actin (Cell signaling technology, MA, U.S.A.) was added and placed at 4℃ for 3 hours to bind with the primary antibodies transferred onto the membrane. After removing the antiserum dilution solution, the membrane was washed thrice with TBST buffer solution for 10 minutes, and the occurrence of antibody-antigen reaction was confirmed by attaching the secondary antibody of anti-

rabbit IgG horseradish-peroxidase (HRP) (NOVUS, CO, U.S.A.) that was diluted with TBST buffer solution in 1:1000. After then, the membrane was washed thrice with 15ml of TBST buffer solution for 10 minutes and reacted with film by using development and fixation solutions.

7) Statistical analysis

The amount of DNA and RNA extracted from fresh tissue and from different fixatives was evaluated to confirm significance of the DNA and RNA amounts extracted from WONFIX to the other results acquired by using other fixatives by using the Wilcoxon signed rank test (SPSS 12.0, Chicago, U.S.A.) and the statistical significance was set as 0.05.

Table1. Characteristics of primary antibodies for immunohistochemical staining.

Antibody	Clone	Target protein (kDa)	Dilution	Source	Site
CEA	Col-1	55	1:1000	Zymed Laboratories Inc.	Colon cancer
Pan-CK	AE1/AE3	65-67	1:500	Dako	mucosal epithelium of gallbladder
Vimentin	V9	57	1:500	Dako	mesenchymum of gallblader
CD3	F4,2,38	16~28	1:200	NeoMarkers	T-cell
CD4	4B12	59	1:50	Thermo	T-cell
CD5	4C7	67	1:200	Novo castra	Thymocyte
CD8	SP16	32	1:500	Dako	T-cell
CD20	L26	33	1:500	Thermo	B-cell
CD45	PD7/26	180	1:500	Dako	Leukocyte
ER	SP1	67	1:400	Thermo	Leukocyte
PR	SP2	412~526	1:500	Thermo	Breast carcinoma
cerbB2	SP3	185	1:500	Thermo	Breast carcinoma
p53	D07	53	1:500	NeoMarkers	Colon cancer
ß-HCG	CG04+CG05	22	1:100	NeoMarkers	Placenta

Table. 2. DNA primer sequences for polymerase chain reaction.

	Forward	Reverse
100 bp	GCC CGA CAT TCT GCA AGT CC	GGT GTT GCC GGG AAG GGT T
200 bp	TGT TGA CTC GAT CCA CCC CA	TGA GCT GCA AGT TTG GCT GAA
300 bp	TGC GAT GTG GTC ATC ATG GTG	CGT GTC ATT GTC GTC TGA GGC
400 bp	CCG CAG CAA GCA ACG AAC C	GCT TTC CTC TGG CGG CTC C
600 bp	GGA GCA GCA TTC CAT CCA GC	CAT CCA TGG GCC GGA CAT AA
1 Kb	TGT TGA CTC GAT CCA CCC CA	ATC TGT CTC CCT GTG ATG GC
1.5 Kb	TGT TGA CTC GAT CCA CCC CA	GTC TCG TGG TCA GAC TCA TC

III. RESULTS

1. Morphological evaluation of fixed tissues

The fixed gallbladder tissues were used to prepare slides and examined under the optical microscope. The WONFIX fixed tissue and the tissue fixed by Wolman's solution resulted to show very similar pattern to that of formalin, and the tissues fixed by Carnoy's fixative and by methacarn showed excessive dehydration that caused the contraction of the tissues and revealed excessive staining pattern (Figure 1). When colon cancer tissue was fixed at different types of fixatives, the morphological findings of the tissue fixed at WONFIX resulted to show the most similar morphological pattern to that fixed at formalin, and the tissue fixed at methacarn revealed the contraction of tissue due to excessive dehydration and stained excessively in red color (Figure 2). When the 12 participants evaluated the morphological findings, the result mostly showed the following grading pattern that the formalin fixed tissue was graded with A (best), the tissues fixed by WONFIX and Wolman's solution were graded with B, the tissue fixed by methacarn was graded with C, and the tissue acquired from frozen tissue section was mostly graded with E (worst) (Table 3).

2. Evaluation of immunohistochemical staining

For the fresh tissue and the tissues fixed at 5 different fixatives were tested for the immunohistochemical staining for CEA, pan-cytokeratin, vimentin, CD3, CD4, CD5, CD8, CD20, CD45, estrogen receptor, progesterone receptor, c-erbB2, p53, and ß-HCG. The staining results of the tissue fixed at WONFIX did not show significant difference between positives and negatives in case of staining was done for CEA, pan-cytokeratin, vimentin, progesterone receptor, c-erbB2, and for p53. However, the strongest staining intensity was observed from the tissue fixed at WONFIX by showing the similar intensity to that of formalin (Figure 3, 4). The staining intensity for CD3, CD4, CD5, CD8, CD20, and CD45 resulted with the lower intensity than those results acquired by formalin fixation. The expression for ER and ß-HCG resulted with the satisfactory staining by formalin fixation, but the tissues fixed at the rest of the fixatives came up with the negative results.

3. DNA analysis

After extracting DNA from frozen tissue and from the tissues fixed at 5 different fixatives, each extracted amount of DNA was compared. The mean DNA extraction value in fresh tissue resulted with 659.8±15.5 μg/ml, WONFIX fixed tissue resulted with 920.4±57.5 μg/ml, the tissues fixed at Carnoy's fixative resulted with 464.4±34.0 μg/ml, methacarn fixed tissue resulted with 723.7±108.8 μg/ml, Wolman's solution fixed tissue resulted with 482.5±60.6 μg/ml, and formalin fixed tissue resulted with 416.9±54.8 μg/ml by showing the largest extraction amount of DNA when tissue was fixed by WONFIX ($p<0.05$) (Figure 5). The largest amount of DNA was preserved in fresh tissue, which was followed by the tissues fixed by WONFIX and Wolman's solution (Figure 6). PCR was performed by using the extracted DNA, which enabled to progress the reaction upto 1.5 kb when the DNA extraction was progressed with fresh tissue, and with the tissues fixed at WONFIX or Wolman's solution. However, the DNA extracted from tissue, fixed at formalin resulted to show only the band at the size of 300bp (Figure 7).

4. RNA analysis

After extracting RNA from the tissues fixed at different types of fixatives, the mean extracted amount of RNA was compared. The fresh tissue resulted with 319.4±32.6 μg/ml, WONFIX fixed tissue resulted with 229.1±73.5 μg/ml, the tissue fixed at Carnoy's fixative resulted with 213.4±76.6 μg/ml, the methacarn fixed tissue resulted with 246.4±32.2 μg/ml, the Wolman's solution fixed tissue resulted with 349.4±54.3 μg/ml, and the formalin fixed tissue resulted with 96.8±20.0 μg/ml. The largest RNA extraction was available when the tissue was fixed at Wolman's solution, and the tissue fixed at WONFIX enabled to acquire the fourth largest amount of RNA (Figure 8). The amount of RNA extraction made by using the WONFIX fixed tissue was compared to the amounts that were extracted from different fixatives. When the fixation was made by using WONFIX, the amount of extracted RNA was significantly larger than that was extracted from the tissue fixed at formalin ($p<0.05$). The mean CT values acquired by conducting real-time PCR by using the extracted RNA recorded 11.3±1.7 for fresh tissue, 12.3±2.7 for the tissue fixed at WONFIX, and 24.0±1.2 for the tissue fixed at formalin. The result showed that the tissue fixed at WONFIX resulted to show nearly similar CT value to that was recorded by using frozen tissue (Figure 9).

5. Microdissection and real-time PCR

Approximately 1000 cells were collected from the fresh tissues of colon cancer, thymus, and liver along with the tissues fixed at WONFIX and formalin to extract the RNA and real-time PCR was performed. The mean CT value of fresh tissue recorded 19.4±4.2, WONFIX fixed tissue recorded 23.2±2.5, and the formalin fixed tissue recorded 30.6±4.2. The WONFIX fixed tissue resulted with lower CT value than that was acquired by the tissue fixed at formalin (Figure 10).

6. Protein analysis

The proteins extracted from the tissues by using different fixatives were tested for the staining with Coomassie brilliant blue, which resulted with the largest amount in fresh tissue, and followed by WONFIX fixed tissue, and nearly no extraction was observed in the tissue fixed at formalin (Figure 11). By using the extracted protein, the Western blot analysis was performed for ß-actin and the protein band was most distinctively observed from the fresh tissue, followed by WONFIX, and Wolman's solution (Figure 12).

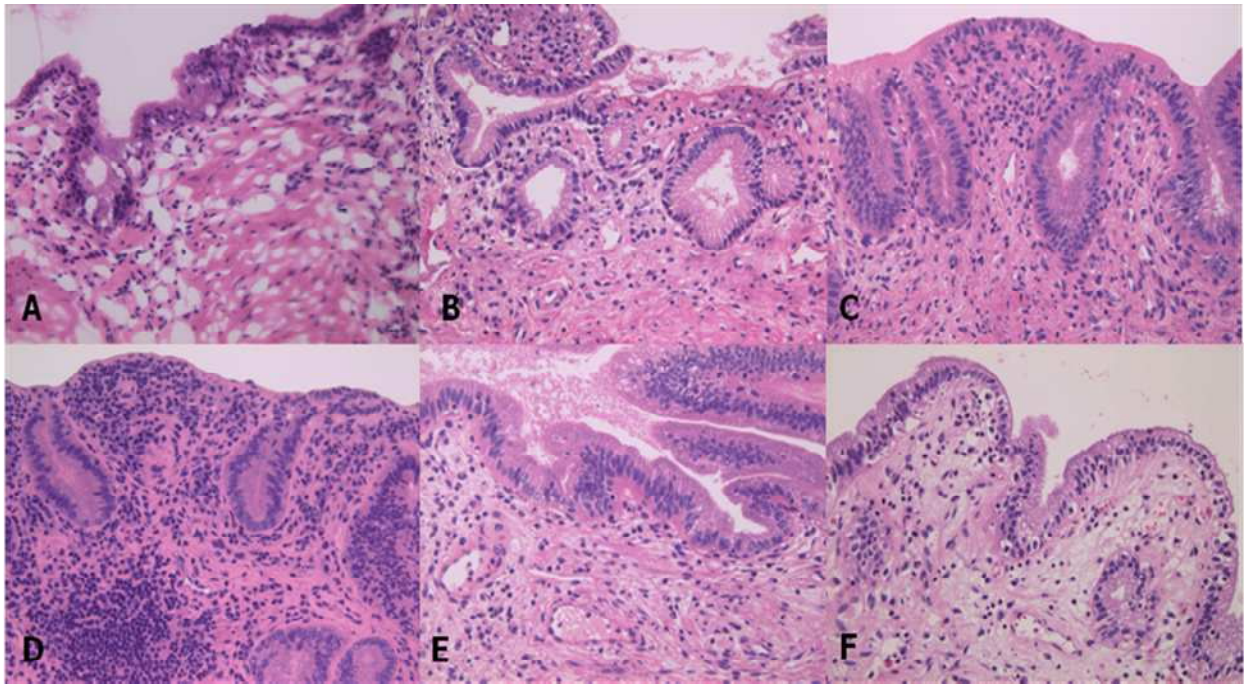

Fig.1. Morphology of gallbladder mucosa tissue. (H&E, X400) A.Frozen B. WONFIX C.Carnoy's fixative D. Methacarn E. Wolman's solution F. Formalin

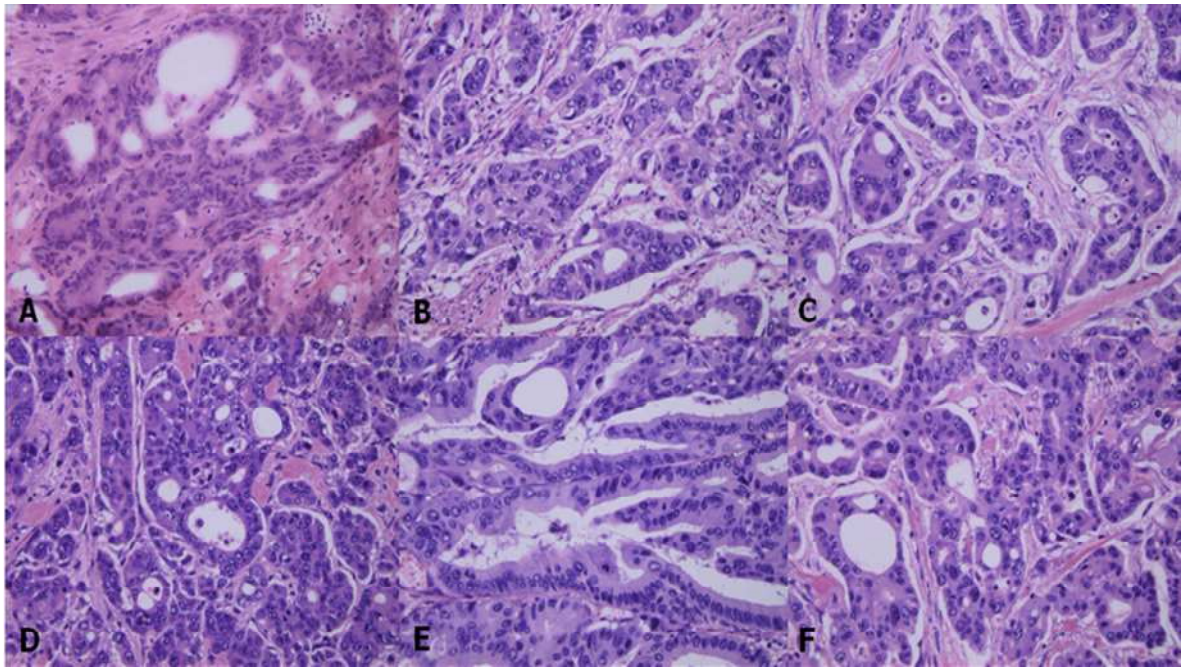

Fig.2. Morphology of colon cancer. (H&E, X400) A.Frozen B. WONFIX C.Carnoy's fixative D. Methacarn E. Wolman's solution F. Formalin

Table. 3. Morphological evaluation of fixed tissues

	Frozen	WONFIX	Carnoy's fixative	Methacarn	Wolman's solution	Formalin
A	0	2	1	2	1	5
B	0	8	6	2	8	5
C	0	2	2	7	3	1
D	4	0	3	1	0	1
E	8	0	0	0	0	0

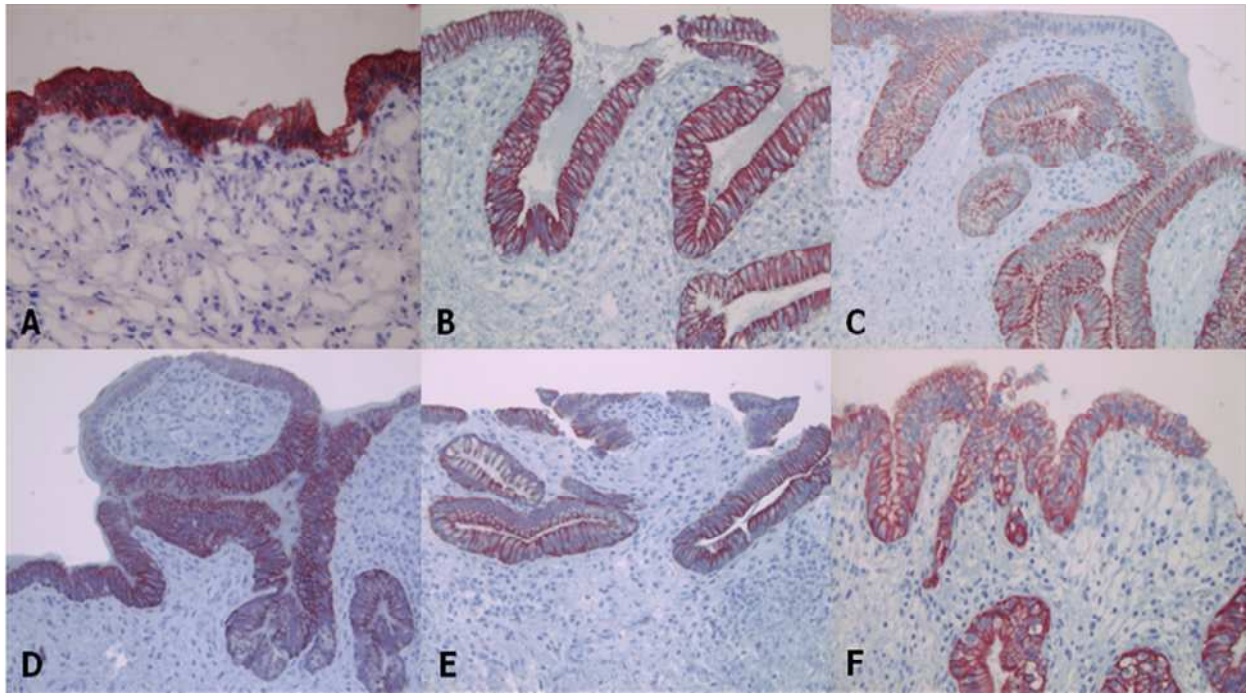

Fig.3. Immunohistochemical staining (CK) of gallbladder mucosa tissue. (X400) A.Frozen B. WONFIX C.Carnoy’s fixative D. Methacarn E. Wolman’s solution F. Formalin

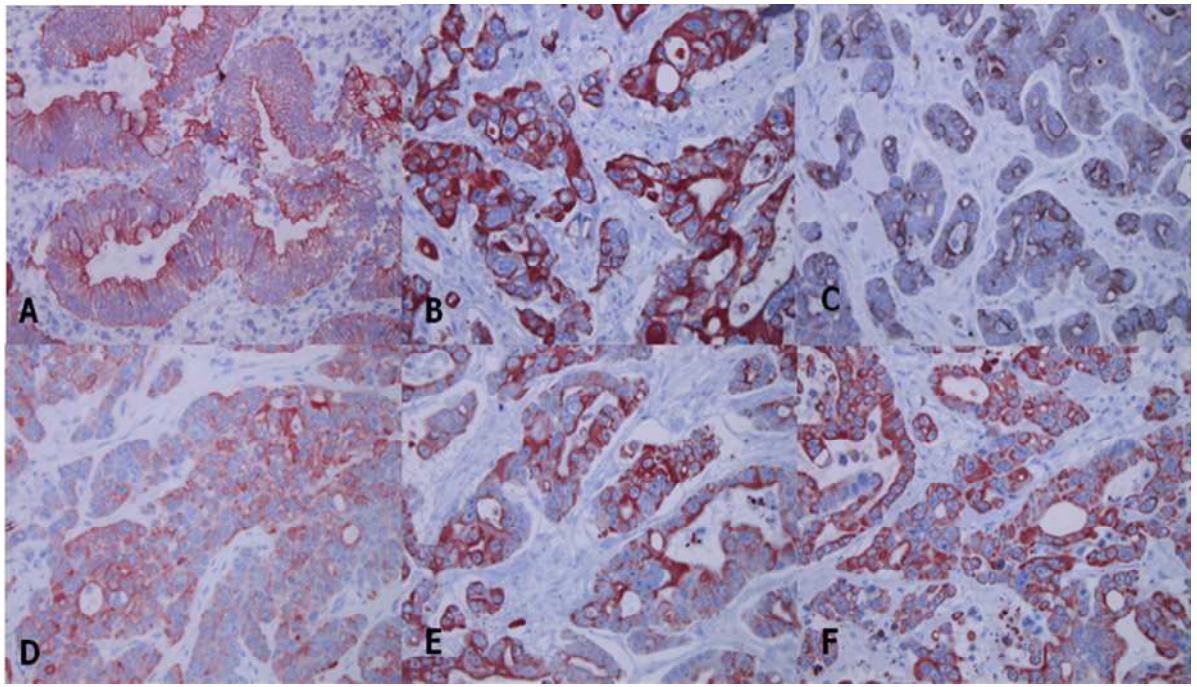

Fig.4. Immunohistochemical staining (CEA) of colon cancer. (X400) A.Frozen B.WONFIX C.Carnoy's fixative D. Methacarn E.Wolman's solution F. Formalin

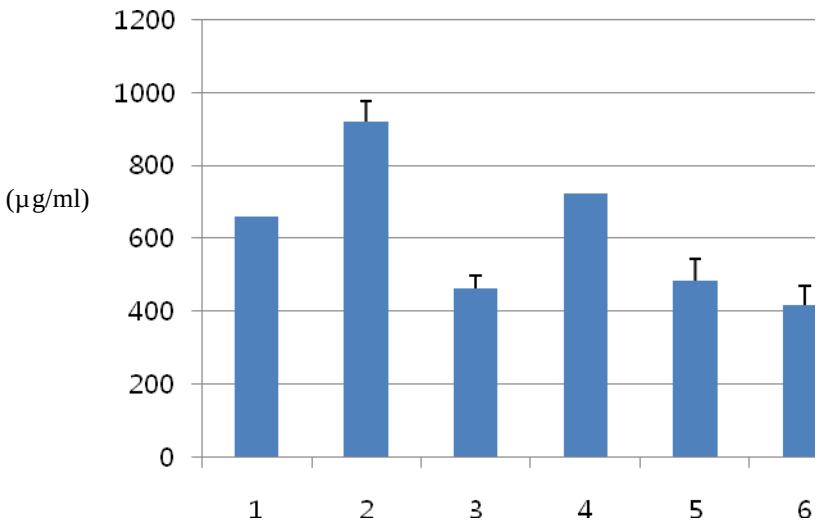

Fig.5. DNA concentration measurement results after extracting from the different fixatives. 1) Frozen tissue (659.8±15.5 μg/ml) 2) WONFIX (920.4±57.5 μg/ml) 3) Carnoy's fixative (464.4±34.0 μg/ml) 4) Methacarn (723.7±108.8 μg/ml) 5) Wolman's solution (482.5±60.6 μg/ml) 6) Formalin (416.9±54.8 μg/ml)

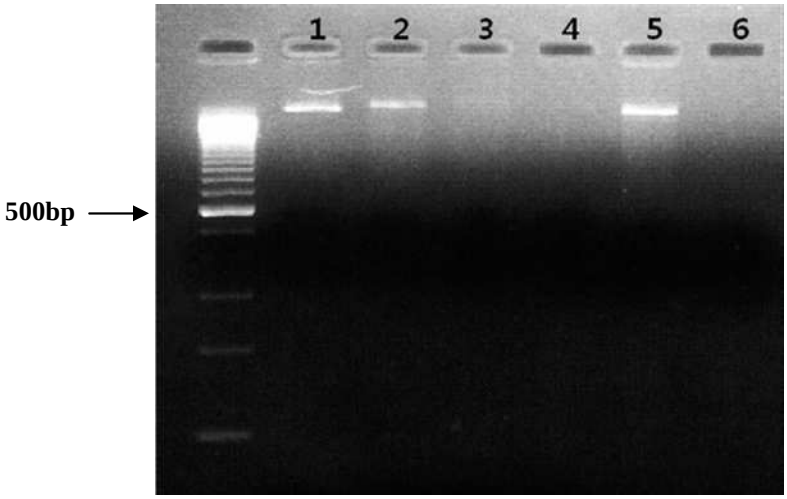

Fig.6. Agarose gel electrophoresis of DNA isolated from the normal human gallbladder mucosa tissue. 1) Frozen tissue 2) WONFIX 3) Carnoy's fixative 4) Methacarn 5) Wolman's solution 6) Formalin

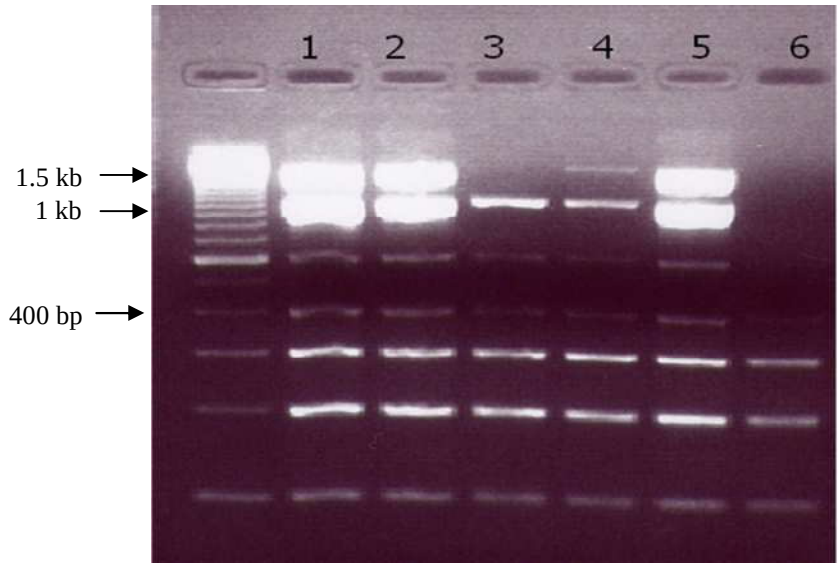

Fig.7. Results of DNA PCR on paraffin embedded normal human ovary samples fixed at different fixatives. 1) Frozen tissue 2) WONFIX 3) Carnoy's fixative 4) Methacarn 5) Wolman's solution 6) Formalin

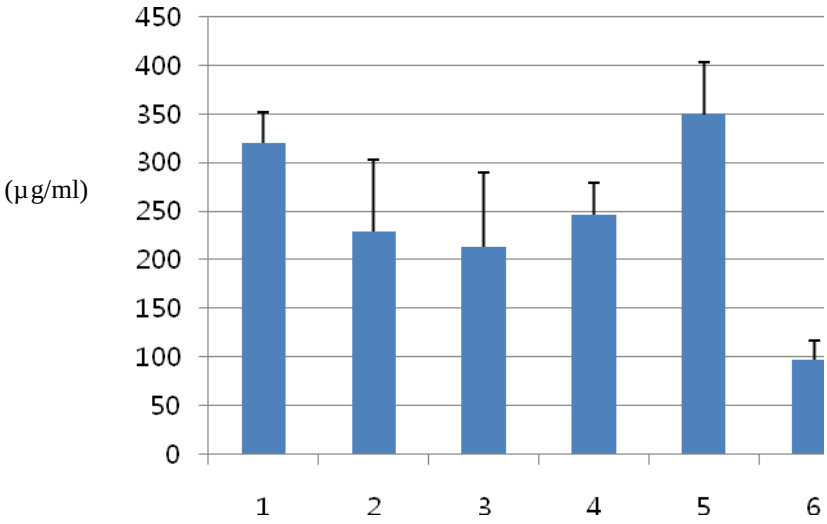

Fig.8. RNA concentration measurement results after extracting from the different fixatives. 1) Frozen tissue (319.4±32.6 µg/ml) 2) WONFIX (229.1±73.5 µg/ml) 3) Carnoy's fixative (213.4±76.6 µg/ml) 4) Methacarn (246.4±32.2 µg/ml) 5) Wolman's solution (349.4±54.3 µg/ml) 6) Formalin (96.8±20.0 µg/ml)

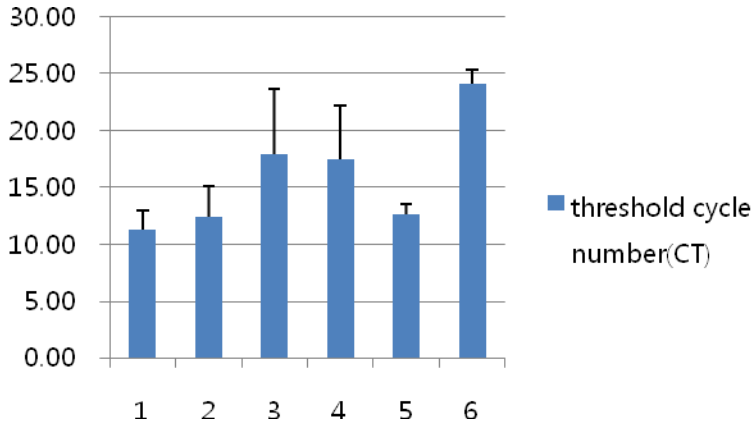

Fig.9. Mean CT value of real-time PCR on paraffin embedded normal human thyroid gland samples fixed at different fixatives. 1) Frozen tissue (11.29±1.70) 2) WONFIX (12.34±2.72) 3) Carnoy's fixative (17.92±5.75) 4) Methacarn (17.42±4.71) 5) Wolman's solution (12.64±0.93) 6) Formalin (24.04±1.23)

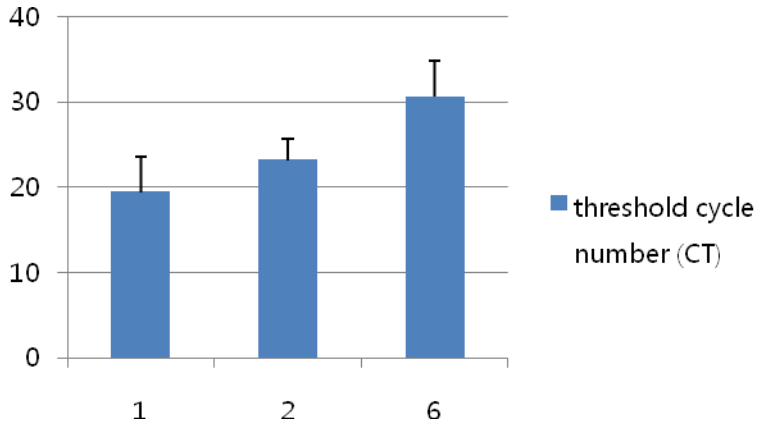

Fig.10. Mean CT values of real-time PCR from microdissected tissue with three different fixatives. 1) Frozen tissue (19.44±4.20), 2) WONFIX (23.16±2.48), 6) Formalin (30.59±4.16)

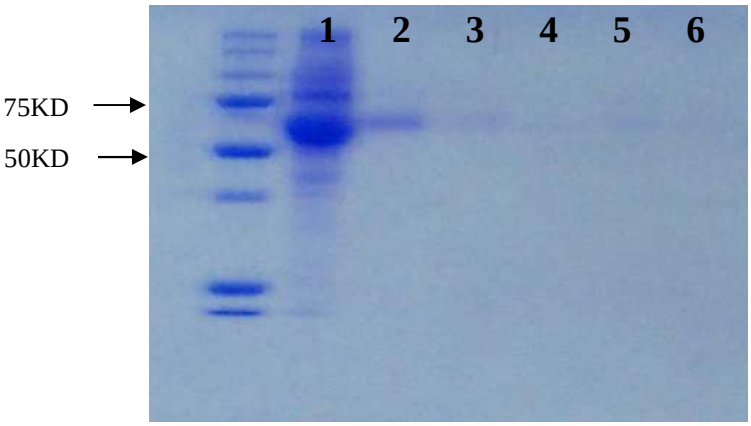

Fig.11. Coomassie brilliant blue staining of total protein isolated from the normal human ovary tissue. 1) Frozen tissue 2) WONFIX 3) Carnoy's fixative 4) Methacarn 5) Wolman's solution 6) Formalin

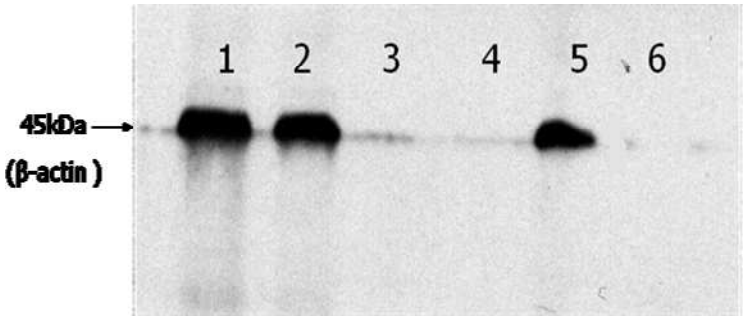

Fig.12. Results of Western blot of paraffin-embedded normal human thyroid gland samples fixed at different fixatives. 1) Frozen tissue 2) WONFIX 3) Carnoy's fixative 4) Methacarn 5) Wolman's solution 6) Formalin

IV. DISCUSSION

The commonly used basic fixative of formalin is the carcinogen that can cause cancer when contacted for long term, and has the stimulating volatility. So, it is not appropriate in industrial environment. Although Glacial acetic acid could be added into the list of fixatives by showing the similar morphological findings as the formalin, its strong volatility that causes unpleasant feeling is the disadvantage of it. In case of chloroform, it has corrosiveness and more importantly, act as the risk factor that can cause liver toxicity when it was contacted for long time. However, since WONFIX is the alcohol series fixative based on ethanol and other additives have no toxicity, it is the safe fixative that does not cause environmental contaminations or industrial hazards. In addition, since it does not require special treatment steps for the fixation, it is the fixative that can be easily used in general laboratories.

For centuries, researchers have been attempted to find the alternatives of formalin by accepting the fact that it can cause protein degeneration and becomes the risk by itself, and alcoholic fixatives or the fixatives based on zinc fixative have been suggested as the alternatives.[3, 20] Unlike formalin, ethanol does not form cross linkages during the fixation procedure and only removes the water surrounding the protein to preserve the three-dimensional morphology and function of the protein. The chemical molecules included in the fixative and the water molecules surrounding the protein exchange each other to fulfill the purpose of the fixation, and the functions of several enzymes including proteinases are inactivated within a shortest time. However, since the increase of alcohol concentration can cause stronger dehydration effect along with severe tissue hardening, it is important to adjust the alcohol concentration appropriately.[21] In the preparation of cytologic specimens, 50% ethanol is the most widely used at the prefixation of lyophilic samples, which allows to preserve the samples without degradation for several days or for several months in case by cases.[22] But since most of cytologic samples are lyophilic samples, such as sputum, urine, and body cavity fluids, the protein in the samples are coagulated and precipitated when the alcohol concentration exceeds more than 50%, and the cell transforms into globular type and chromatin tends to be concentrated. In the tissue fixation, there are many disputes on the appropriate alcohol concentration. Warmington et al.(2000) reported that excellent cytologic findings could be found when the alcohol concentration was adjusted as 60% when the ethanol-based fixative F13 (60% ethanol+ 20% methanol +7% polyethylene glycol) was used.[23] Gillespie et al.(2002) compared the results of tissue fixation by using alcohol fixatives at several different alcohol concentrations and the results of using aldehyde fixatives, and

reported that 70% of alcohol fixatives resulted to show the most similar findings as that of formalin.[18]

Beckstead et al.(1994) reported that zinc-based fixative can preserve the good reactivity at the time of immune staining since it maintains good morphology without promoting cross-linking or coagulation,[24] Wester et al.(2003) reported the contribution of zinc-based fixative in the preservation of DNA in some level, but only enabled to preserve upto 792bp.[3] The author also conducted many experiments by using the zinc based fixative (data not shown). However, the zinc based fixative allowed to have similar morphological shape to that could be found by using formalin by requiring short time for the fixation, but showed a disadvantage of causing tissue decomposition due to the lack of tissue fixation capacity.

At the current study, the morphology of tissue fixed for 6 hours, 24 hours, 48 hours, 72 hours, and 144 hours (6 days) has been compared. The WONFIX fixed tissue resulted to show no significant difference to that was fixed at formalin and well preserved without tissue decomposition (data not shown). It seems necessary to have further studies regard on the stability of fixed tissue for long time in a future.

By employing the immunohistochemical staining method, irrelevant antibodies have been used to verify the origin or to make a diagnosis of not easily differentiated tumors, lymphoma, and neuroendocrine tumors and the tumors developed at soft tissues, such as adipose tissue, fibrous tissue, smooth muscle, and skeletal muscle etc. In case of breast cancer, the scoring is made based on the staining intensity and distribution after performing the immunohistochemical staining for c-erbB2, and the fluorescent in situ hybridization (FISH) is recommended in case of the scoring is 1+ or 2+. During the procedure, it is doubtful that whether the molecular biological state of the formalin fixed tissue could show the same tissue quality as that of fresh tissue.[18, 25-27] Shi SR et al.(2008) reported the staining intensity and distribution of immunohistochemical staining by using tissues fixed at various fixatives.[28] When acetone and formalin fixatives were compared, 60% of antibodies came up with a different staining result and 31% of antibody expression showed better expression characteristics in acetone than alcohol fixatives.[28] Such results indicate that different fixatives can have different influences on the results. The current study performed immunohistochemical staining for 14 types of antibodies by using the tissues fixed at different fixatives. Similar to the antibody expression of formalin fixed tissue, the WONFIX fixed tissue showed the expression of most of antibodies. The staining intensity of WONFIX was observed to be stronger than the tissues fixed at other types of fixatives and showed the same pattern to that of formalin fixed tissue. However, the staining intensity for ER and ß-HCG was

found to be negative other than the formalin fixed tissue. This could be attributable to the following facts that the antibodies have been developed based on formalin or the immune response capacity was lowered by the delay of fixation procedures. The reading results of immunohistochemical staining are used in therapy and prognosis as well as in diagnosis. As the current study result shows, the tissues fixed at different fixatives revealed to show contrary expression and intensity characteristics in some antibodies. For that part, it is considered to be necessary to conduct further studies to set the standardized staining method or reading parameter regard on immunohistochemical staining.

With the rapid development of molecular biological techniques, the pathology studies that were limited to make diagnosis based on microscopic observations have been improved to make differentiated diagnosis of a disease by adopting various molecular biological study techniques, and it plays an important role in providing information for the treatment and prognosis of a patient. As an example, the molecular biological techniques of FISH and PCR could be applied for the diagnosis of breast cancer as well as immunohistochemical staining method to deliver therapeutic protocols and information to clinician.[29] To adopt such study techniques, the development of ideal fixatives which will not destroy DNA, RNA, and protein of a tissue is the prerequisite of the study. The tissue fixed at WONFIX enabled to preserve DNA upto 1.5kb when PCR was performed.

Compared to DNA, RNA is more easily destroyed. Although there have been cases that performed RT-PCR by using the RNA extraction acquired from paraffin embedded formalin fixed tissue. The amplification is difficult due to the destruction of RNA. In some studies, the addition of ethylenediamine-4N'-tetraacetic acid to formalin has been reported to improve the quality of RNA.[18] But the degradation of RNA is still remained as a fundamentally unsolved problem. For that matter, several solutions have been suggested, and the currently used tactic is to preserve it by using a RNA preservation solution. Although it is advantageous in the preservation of RNA, it causes a severe cytomorphologic deformation which is disadvantageous for diagnosis. Therefore, it has to face many limitations in the study of comparing the molecular biological differences of precancerous and cancerous lesions by using microdissection technique.[30-32] The current study fixed colon cancer, thymus, and liver tissues in WONFIX and paraffin embedded before conducting microdissection for RNA extraction to perform real-time PCR, which resulted with CT value of 23.2. Although the value was larger than the CT value of 19.4 in fresh value, it was smaller than the CT value of formalin that resulted with 30.6. It indicates that the tissues fixed at WONFIX can preserve enough RNA necessary for further studies and much better morphological findings could be

observed than the staining of fresh tissues. Because WONFIX can achieve good preservation of RNA while having a good morphology of tissue, it is considered to be an ideal fixative from the perspectives which enables to conduct relevant studies.

Not only as the constituent of a living body, protein carries out all of physiological functions and becomes the target of drug action and acts on the development of a disease. But the expression of mRNA alone could not precisely predict a protein, and the gene sequence alone could not tell a protein deformation. For that matter, the studies that evaluate the phenomenon caused by genes and the phenomenon caused by multigenic/epigenic factors have been performed along with the studies that evaluated the protein expression difference between normal tissue and diseased tissue.[33] These study approaches have been employed in disease diagnosis and in the development of therapeutic agents, but the approaches have to face many limitations when the study was performed by using fresh tissues since it could not distinguish normal lesion from cancerous lesion. Therefore, the fixation in a fixative which can well preserve the constituents of a tissue before the paraffin formatting could be an advantage in the protein studies.

When reviewing the research techniques of protein, Western blot analysis and Enzyme-Linked Immunosorbent Assay (ELISA) techniques could be listed, but the protein extraction from the currently used formalin fixed tissue was nearly impossible and the above listed techniques were not able to perform by using fixative fixed tissues. But the protein quality extracted from the tissue fixed at WONFIX was not good enough to the quality extracted from fresh tissue, but the performance of Western blot analysis for β-actin revealed to show nearly the same pattern to that of fresh tissue from the aspect of amount and quality of the protein. This indicates that WONFIX have small or no effects on the structural change of protein. When the WONFIX fixative was used, the operation of tissue bank for the purpose of preparing blocks and storing the blocks is not needed during a regular pathological laboratory, which can save human resources and can be helpful in disease studies.

Conclusively, WONFIX is an excellent helpful fixative not only in the field of clinical diagnosis, but also in the molecular biological studies. Especially, excellent effects could be expected than other fixatives in the field of studying RNA by using microdissection or studying proteins. In a future, additional studies have to be done to confirm the stability of biomolecules in WONFIX fixed paraffin block long time after the fixation. Furthermore, the study for the presence of gene mutation is considered to be necessary.

V. CONCLUSION

WONFIX is the ethanol based fixative in alcoholic series. It is the environment friendly safe fixative since its additives does not contain heavy metals without having toxicity. In addition, WONFIX can maintain better morphology than other fixatives and it even satisfies the requirements of the molecular pathology tests for DNA, RNA, and protein used in the field of pathologic diagnosis.

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국문요약

분자병리 검사를 위한 새 고정액

연구목적: 포르말린은 가격이 저렴하고 고정한 조직의 형태를 그대로 잘 유지하여 고정액으로 가장 많이 사용되고 있다. 그러나 포르말린은 cross-linking작용으로 인해 고정한 조직의 macromolecule (DNA, RNA, protein)을 손상시킬 수 있다. 이 연구의 목적은 포르말린과 거의 유사한 수준의 형태적 소견을 유지하고 동시에 임상에서 필요로 하는 각종 특수화학 염색과 면역조직 화학염색뿐 만 아니라 분자생물학적 검사를 시행하는데 아무 이상이 없는 이상적이고, 안전한 새 고정액을 개발하는 것이다.

방 법: 알코올 계통의 각종 고정액을 비교 분석하여 친환경적이고 비독성인 고정액 (WONFIX)을 개발한 후, 쥐 조직과 인체 조직을 대상으로 다른 고정액과 비교하였다. Frozen tissue와 5개의 서로 다른 고정액 (1.WONFIX 2.Carnoy's fixative 3.Methacarn 4.Wolman's solution 5.Formalin)을 선택하여 각각의 고정액에 조직을 24시간 고정시킨 후 파라핀에 포매하였다. 광학현미경을 이용하여 고정액 별로 조직의 형태양상을 평가한 후 각 형태소견의 질을 A~E급으로 구분하여 판독하였고, 면역조직화학 염색을 시행하여 항원 발현 양상도 비교하였다. 고정한 조직의 파라핀 블럭에서 각각 DNA, RNA, protein을 추출한 후 macromolecule의 농도를 비교하고 Wilcoxon signed rank test방법으로 통계학적 유의성을 비교하였다. Housekeeping gene에 관하여 PCR, real-time PCR, Western blot analysis를 함께 시행하였다.

결 과: 신선 조직과 다섯 가지 고정액에 고정한 조직의 현미경적 소견에 관한 평가는 포르말린에 고정한 조직이 가장 우수한 형태를 보였고, 그 다음으로 WONFIX가 좋은 조직학적 형태를 유지하였다. 14개의 항체를 이용하여 시행한 면역조직화학 염색 결과는 양성과 음성발현빈도는 같았으나, WONFIX에 고정한 조직에서의 염색강도가 제일 강하였고 포르말린에 고정한 조직과 거의 유사한 양상을 보였다. 추출된 DNA의 양은 WONFIX에 고정한 조직 경우 가장 많았고 (p<0.05), β-actin에 관하여 PCR를 시행하였을 때 1,5Kb까지 PCR이 가능했다. 추출된 RNA 양은 WONFIX고정액을 이용한 경우 네 번째로 많았고, 포르말린에서

추출한 것보다 유의하게 많았다 ($p<0.05$). Real-time PCR을 시행하여 얻은 평균 threshold cycle number (CT) 값을 보면, 신선조직은 11.3, WONFIX는 12.3였다. 1000개의 세포를 microdissection하여 추출한 RNA를 이용하여real-time PCR를 시행한 후 평균 CT 값을 구한 결과, 신선 조직은 19.4, WONFIX는 23.2, 포르말린은 30.6로 WONFIX에 고정한 조직의 RNA가 다른 고정액보다 보존상태가 더 좋았다. Western blot analysis를 시행하여 단백질의 질을 비교한 결과 WONFIX에 고정한 조직에서 추출한 단백질은 신선한 조직에서 추출한 것과 비슷한 수준으로 상태가 좋았다.

결 론: WONFIX는 에탄올을 기반으로 개발된 고정액으로 중금속이나 유독한 화학물질을 포함하지 않은 친환경적이고 안전한 고정액이다. 동시에 형태학적 소견이 우수한 점이 특징이며 임상적으로 필요한 분자생물학적 검사를 가능하게 해준다.