EVALUATION AND COMPARATIVE ANALYSIS OF NEWER COMPOUND FIXATIVES

DISSERTATION

SUBMITTED TO TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY, CHENNAI

in partial fulfilment of

the requirements for the degree of

M.D. (PATHOLOGY)

BRANCH - III

TIRUNELVELI MEDICAL COLLEGE HOSPITAL,

TIRUNELVELI- 627011

APRIL-2017

CERTIFICATE

I hereby certify that this dissertation entitled **"EVALUATION AND COMPARATIVE ANALYSIS OF NEWER COMPOUND FIXATIVES"** is a record of work done by **Dr.S. MUTHUSELVI**, in the Department of Pathology, Tirunelveli Medical College, Tirunelveli, during her postgraduate degree course period from 2014- 2017. This work has not formed the basis for previous award of any degree.

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I solemnly declare that this dissertation titled **"EVALUATION AND COMPARATIVE ANALYSIS OF NEWER COMPOUND FIXATIVES"** submitted by me for the degree of M.D, is the record work carried out by me during the period of 2014-2016 under the guidance of **Prof.Dr. J. SURESH DURAI, M.D**, Professor of Pathology, Department of Pathology, Tirunelveli Medical College, Tirunelveli. The dissertation is submitted to The Tamilnadu Dr. M.G.R. Medical University, Chennai, towards the partial fulfilment of requirements for the award of M.D. Degree (Branch III) Pathology examination to be held in April 2017.

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NAME OF PRINCIPAL INVESTIGATOR: Dr. S.MUTHUSELVI, MBBS.,
DESIGNATION OF PRINCIPAL INVESTIGATOR: Post Graduate in MD Pathology
DEPARTMENT & INSTITUTION: Department of Pathology, Tirunelveli Medical College

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THE FOLLOWING DOCUMENTS WERE REVIEWED AND APPROVED

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ABBREVIATIONS

Amex	-	Acetone Methylebenzoate –Xylene
CD	-	Cluster of Differentiation
CK	-	Cytokeratin
DNA	-	Deoxy Ribonucleic Acid
EPA	-	Environmental Protection Agency
ER	-	Estrogen Receptor
FFPE	-	Formalin Fixed Paraffin Embedded
GC	-	Gas Chromatography
GC- MS	-	Gas Chromatography And Mass Spectrometry
GIT	-	Gastrointestinal Tract
HER 2	-	Human Epidermal growth factor Receptor– 2
HOPE	-	Hepes-Glutamic Acid Buffer Mediated Organic Solvent Protection Effect
HPLC	-	High Performance Liquid Chromatography
IARC	-	International Agency For Research On Cancer
IHC	-	Immunohistochemistry
MPNST	-	Malignant Peripheral Nerve Sheath Tumor
NBF	-	Neutral Buffered Formalin
NIOSH	-	National Institute Of Occupational Safety And Health Administration
PANCK	-	Pancytokeratin
PAS	-	Periodic Acid Schiff

PASD	-	Periodic Acid Schiff and Diastase
PCR	-	Polymerase Chain Reaction
ppm	-	parts per million
RNA	-	Ribonucleic Acid
RT PCR	-	Real Time Polymerase Chain Reaction
STEL	-	Short Term Exposure Limit
TWA	-	Time Weighted Average
UMFIX	-	Universal Molecular Fixative
ZBF	-	Zinc Based Fixatives

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INTRODUCTION

Fixation is one of the most important step in the practice of diagnostic pathology. Even in this modern age where many things have changed, formaldehyde is most commonly used for tissue fixation. Formaldehyde is a gas which is commercially available in the form of concentrated solution of 37%. A 10% dilution of concentrated formalin is named as 10% formalin.

The reasons which make formalin popular are; its low cost, its ability to help in long term storage of tissue, its ability to help preserve morphological features, and the fact that it allows special histological stains. Also notable is the fact that in combination with antigen retrieval it allows for reliable immunohistochemical analysis. The laboratory preparation of formalin is straightforward procedure. Moreover the use of formalin is a longstanding tradition and it is used internationally. There is a general opinion that formalin is the best fixative available and that there is no need to look for an alternate fixative.

However toxicity of formalin is emerging as the main reason for its abolition as the commonest fixative used in laboratories[1]. More recently from initial reports from IARC(International Agency for Research on Cancer) link formaldehyde exposure and leukemia. These reports were further highlighted in a report issued in 2012 by the same agency.

The agencies that monitor formaldehyde exposure in the national and international level set stringent limits for formaldehyde exposure. The above said

limit ranges from 0.016 ppm TWA(time weighted average) to 2 ppm for STEL (short term exposure limit)[1].

The formation of DNA protein cross links denotes a permanent signature of exposure to formalin. Recent research shows chromosomal alterations in health workers and these have been found to be related to formalin use in pathology laboratories. An attempt has been made in this study to minimize formalin exposure by reducing formalin concentration in compound fixatives.

AIMS AND OBJECTIVES

- To compare and analyze the fixation characteristics of a group of formalin containing compound fixatives.
- To assess the optimal efficacy of a compound fixative.
- To minimize formalin exposure in histopathology laboratory.
- To reduce fixation time and improve staining characteristics.

REVIEW OF LITERATURE

FIXATION:

It is a process by which the constituents of the cells and therefore of the tissues are fixed in a physical and partly also in a chemical state so that they will withstand subsequent treatment with various reagents with a minimum of loss, significant distortion, or decomposition.

IDEAL FIXATIVE:

It should

- Penetrate a tissue quickly.
- Be isotonic.
- Cause a minimum loss of physical and chemical alteration of the tissues and its components.
- Be rapid in action and shelf life of at least one year.
- Allows the isolation of macromolecules including proteins, m RNA and DNA without significant biochemical alterations from fixed and embedded tissues.
- Useful for diverse variety of specimen including fatty tissue, lymphoid structures and large specimens and support histochemical as well as immunohistochemical analyses and other specialized procedures.
- Not bind those reactive groups upon which specific staining of the tissue elements will depend.

- Be cheap and stable.
- Be compatible with modern automated tissue processors.
- Have toxicological and flammability profiles such that use of the fixative is safe.
- Be readily storable for long term, easily disposable or recyclable and also give excellent microtomy of embedded blocks.

TYPES OF FIXATIVES:

I. Microanatomical fixatives

II. Cytological fixatives

III. Histochemical fixatives

METHODS OF FIXATION:

1.Physical Fixation:

- Heat fixation
- Microwave fixation
- Freeze drying and freeze substitution.

2.Chemical Fixation:

- Coagulant fixatives
- Cross linking fixatives
- Compound fixatives.

MICROANATOMICAL FIXATIVES:

They preserve the anatomy of the tissue, in correct relationship of tissue layers and large aggregates of cells. All routine histopathological fixatives come under this group.

CYTOLOGICAL FIXATIVES:

These fixatives help in the preservation of intracellular structures.

Eg. Carnoy's fluid, Clarke's fluid, Muller's fluid and formal saline etc.

HISTOCHEMICAL FIXATIVES:

They are used when histochemical tests were applied. They produce only minimal changes in the elements have to be demonstrated. Freeze drying method is almost ideal for this purpose.

HEAT FIXATION:

Boiling of tissue materials in normal saline can produce adequate morphology of the tissue. It is the simplest form of fixation. Nowadays heat is mainly used to speed up other forms of fixation. And it also used in the steps of tissue processing.

MICROWAVE FIXATION:

Microwave heating speeds fixation. It can reduce fixation time from 12 hours to 20 minutes.

FREEZE DRYING AND FREEZE SUBSTITUTION:

In freeze drying, tissues are cut into thin sections and immersed in liquid nitrogen. Then the water molecules are removed in a vacuum chamber at – 40˚c. The tissues can be post fixed with formaldehyde vapor. It is useful for studying soluble materials and small molecules.

In freeze substitution , tissues are immersed in fixatives at – 40˚c, such as acetone or alcohol which slowly removes water molecules through dissolution of ice crystals. The proteins are not denatured. Then the temperature gradually rised to 4˚ c and the fixation process is completed.

EFFECTS OF FIXATION IN TISSUES:

Fixatives inhibit autolysis and putrefaction of tissues.

It preserves tissue architecture and induces hardening of tissues and solidifies the cellular material.

It alters the varying degree of refractive indices of the cells and tissue components. Fixatives induce loss of tissue materials followed by fixation. Eg. protein and mucopolysaccharides loss followed by formalin fixation.

EFFECTS OF FIXATION ON STAINING:

Histochemical reactions of the tissues are usually affected after fixation because fixatives usually disrupt the secondary and tertiary structures of proteins. As they bind to reactive groups in the tissues to achieve fixation, they affect routine staining adversely. Sometimes they act as mordant and improve staining. Eg . picric acid fixation enhances trichrome staining. A fixative which either eliminates

cationic amino groups (formaldehyde) or creates the anionic amino group or both (osmium tetroxide) will decrease tissue acidophilia and enhance basophilia. And a fixative which eliminates anionic amino groups or generates cationic amino groups or both (chromic acid) will reduce basophilia and enhance tissue acidophilia.

FIXATION ARTEFACTS:

Shrinkage artefacts:

Tissues fixed in 10% NBF and embedded in paraffin wax shrink by 33%. Alcohol fixed tissues show excessive shrinkage artefacts.

Glycogen streaming artifact:

It results from diffusion of unfixed tissue materials .It is evidenced in PAS staining of glycogen containing tissues.

Pigment artefacts:

Formalin pigment- Brown/ brownish black pigment produced under acidic conditions. They are commonly seen in blood rich tissues . Eg. spleen, liver, hemorrhagic lesions etc. formalin pigment can be removed by treating sections with 10% sodium or potassium hydroxide in 70% alcohol for 5 to 15 minutes or treating unstained sections in alcoholic saturated picric acid.

Mercury pigment – mercuric chloride containing fixatives produce varying amount of dark brown or grey coloured deposits through out the tissue. It can be removed by oxidation with iodine to mercuric chloride and subsequently removed with sodium thiosulfate.

Chromate deposits- if the specimens are not washed well after fixation in chromate containing fixatives, the salts react with alcohol forming a yellow brown to black precipitate. They are removed by treating sections in 1% hydrochloric acid in 70% alcohol for 30 minutes.

EFFECTS OF PROLONGED FIXATION:

Prolonged fixation of tissues leads to shrinkage artefacts. Prolonged fixation of tissues in formalin results in decreased antigenicity. Studies showed that immunoreactivity of CK 7 (cytokeratin), HMW CK (High molecular weight cytokeratin) and laminin was decreased in prolonged formalin fixation. But nowadays this is overcome by enzymatic digestion and heat induced antigen retrieval methods [75].

Fixation of specific tissues:

Brain

Conventional fixation of brain takes at least 2 weeks[47]. Perfusion technique of the brain through the middle cerebral arteries reduce fixation time. Fixation is also enhanced by the use of microwave technology. For biotin-avidin method of immunohistochemistry, alcoholic formalin should not be preferred.

Eyes

To obtain good sections, the globe must be firmly fixed. Eyes are usually fixed in NBF for 48 hours. After gross description, iris and optic nerve are removed and fixed for an additional 48 hours. For the canal of Schlemm and the aqueous outflow pathway studies, perfusion fixation of the eye is recommended.

Breast

Mastectomy specimens should be sliced at 5mm intervals and fixed in conventional 10% neutral buffered formalin for a minimum of 6–8 hours and a maximum of 72 hours. To prevent lysis of biomarkers, such as progesterone receptor, estrogen receptor and the human epidermal growth factor receptor-2 (HER2), fixation should be as short as possible.

Lungs

NBF is the preferred fixative for lung biopsies. Specimens received from autopsies may be inflated through the trachea or major bronchi and should be fixed overnight .

Lymphoid tissue

Excised lymph node is usually fixed in NBF or in B5 or zinc based fixatives.

Testis

Small biopsies of testis and penectomy specimens should be fixed in conventional buffered formalin.

Muscle biopsies

For histopathological examination, a portion of muscle should be fixed in NBF and the remaining portion should be kept in normal saline for further enzyme studies.

Renal biopsies

Core biopsies of kidney are usually received in three pieces with one in NBF, other in buffered glutaraldehyde for ultra structural studies and last sent snap frozen in liquid nitrogen for immunofluorescence study.

FACTORS AFFECTING FIXATION:

1. BUFFERS AND pH:

Acidic pH reduces the formation of reactive hydroxymethyl groups and cross linking resulting in improper fixation[5,7]. Therefore buffers are added to maintain a pH of 7.2 to 7.4. Commonly used buffers are phosphate, bicarbonate, Tris, cacodylate and acetate .

2. DURATION OF FIXATION AND SIZE OF SPECIMENS:

In 1941 , Medawar et al in his study observed that the depth reached by the fixative is directly proportional to the square root of duration of fixation and expressed this relation as;

$$d = k\sqrt{t}$$

where d= depth, t= duration of fixation, k= constant

The constant represents the coefficient of diffusability which is specific to each fixative. Eg. k is 0.79 for formalin, 1.0 for 100% ethanol and 1.33 for potassium dichromate[6].

To allow proper penetration of fixatives from all directions , the bottom of the container should be wadded by fixative-soaked cotton or cloth and the specimens placed over that. The specimen should be cut not be thicker than 0.5 cm. Bloody gross specimens should be washed before put into fixative.

The volume of the fixatives should be at least 10 times the volume of the gross specimen for optimal fixation.

3. TEMPERATURE:

The diffusion of molecules increases with rising temperature due to their more rapid movement and vibration. So nowadays microwaves are used to speed fixation and tissue processing.

4. CONCENTRATION OF FIXATIVE:

Effectiveness and solubility primarily determine the appropriate concentration of fixatives. Concentration of formalin above 10% will cause increased hardening and shrinkage[8]. Ethanol below 70% do not remove free water from tissues efficiently.

5. OSMOLALITY AND IONIC COMPONENTS:

Hypertonic and hypotonic solutions lead to cell shrinkage and swelling respectively. The better morphological details obtained with solutions that are slightly hypertonic (400- 450 mOsm).The ionic compositions of solutions should be isotonic as possible to the tissues.

6. ADDITIVES:

They are used to improve the morphology of the fixed tissue. Eg . electrolytes include calcium chloride , potassium thiocynate ammonium sulphate and potassium dihydrogen phosphate, non electrolyte substances include sucrose, dextran and detergent.

CHEMICAL FIXATION:

COAGULANT FIXATIVES:

Alcohols are the commonly used coagulant fixatives eg,. methanol, ethanol etc., They act by denaturing and precipitating protein by removing free water molecules and disrupting hydrogen bonds. Free water molecules usually surrounds hydrophobic areas of proteins and by repulsion, the water molecules drive hydrophobic areas into closer together with each other and hence stabilize hydrophobic bonding. When alcohol removes water, this hydrophobic bonding weakens. Like this, water molecules also take part in hydrogen bonding in hydrophilic areas of proteins. So water removal in alcoholic fixation destabilizes this hydrogen bonding. All these changes disrupt the tertiary structure of proteins. Protein denaturation depends upon, the choice and concentration of alcohol, the presence of organic and non-organic substances, and the pH and temperature of fixation. Eg. ethanol denatures proteins>phenols >>monocarboxylic acids >dicarboxylic acids[9] .

Commonly used alcohol based fixatives are clarkes solution, carnoy's fixative and methacarn fixative.

PICRIC ACID FIXATIVES:

It contains saturated aqueous solution of picric acid.

Bouin's solution:

It is composed of

Saturated solution of picric acid 1500 ml

Concentrated formalin 500 ml

Glacial acetic acid	100 ml

It is the best fixative for connective tissue stains. Lithium carbonate, 70% ethanol or another acid dye can be used to remove yellow colour precipitates of picric acid.

Hollande's solution:

It is composed of

Distilled water	1000 ml
Concentrated formalin	100 ml
Acetic acid	15 ml
Picric acid	40 g and
Copper acetate	25 g

This fixative is preferred for endocrine tissues and gastrointestinal biopsies.

MERCURIC FIXATIVES:

Mercuric chloride ($HgCl_2$) containing fixatives acts by coagulating tissue proteins. Lillie's B5 fixative is composed of 4% aqueous formaldehyde with 0.22M $HgCl_2$ and sodium acetate. Mercuric chloride establishes rapid structural stabilization and also enables bright staining by many of the dyes. B 5 fixative enhances nuclear detail and it is preferable for hematopoietic specimens. Mercury based fixatives produce brown crystalline precipitate, probably mercurous chloride (Hg_2Cl_2) through out the tissues. This mercury pigment can be removed by sequential treatments with iodine and sodium thiosulfate solutions, before staining. As mercury is toxic, all mercury based fixatives should be subjected to toxic waste disposal regulations.

Zenker's solution:

It is composed of

Mercuric chloride 12.5 g

Sodium sulfate 2.5 g

Potassium dichromate 6.3 g

Distilled water 250 ml

5 ml of glacial acetic acid is added to 95 ml of above solution just before using this fixative. This fixative is recommended for bloody specimens and for trichrome stains.

Helly's solution:

The constituents of the Helly's fixative is similar as Zenker's solution but in the last step 5 ml of concentrated formalin is added to 95 ml of stock solution. It is advisable for bone marrow, intercalated discs and extramedullary hematopoiesis specimens.

Schaudinn's fixative:

Distilled water 50 ml

Mercuric chloride 3.5 g

Absolute ethanol 25 ml

Ohlmacher's fixative:

This is composed of

Absolute ethanol 32 ml

Glacial acetic acid 2 ml

Chloroform 6 ml

Mercuric chloride 8 g

This fixative penetrates rapidly.

Carnoy-Lebrun solution:

Absolute ethanol	15 ml
Chloroform	15 ml
Glacial acetic acid	15 ml
Mercuric chloride	8 g

This fixative penetrates rapidly.

DICHROMATE AND CHROMIC ACID FIXATION:

Dichromate fixatives acts by oxidation of proteins with interaction of reduced chromium ions. Chromium ions in +6 valence state coagulate proteins and nucleic acids. To some extent the ions also form cross links. They particularly react with carboxyl and hydroxyl side chains of proteins. As they leave amino groups of proteins free, they help staining with acid dyes. Chromate interacts with unsaturated lipids and make them insoluble, so it is considered as good fixative for mitochondria. 24 hours fixation is essential for dichromate fixatives. Tissues fixed in dichromate fixatives should be washed thoroughly after fixation in order to prevent chromate deposits in sections. Usually these fixatives were used to demonstrate amine containing chromaffin granules in endocrine tissues. Nowadays it is replaced by immunohistochemistry to demonstrate chromaffin granules. Potassium dichromate is used as a component of many compound fixatives (eg. Helly's fixatives, Zenker's solution)[73,74].

Miller's or Moller's fixative:

It is composed of potassium dichromate 2.5 g and sodium sulfate 1 g dissolved in 100 ml of distilled water.

Regaud's fixative:

In this solution, potassium dichromate 3 g is dissolved in 80 ml of distilled water. 20 ml of 37% formaldehyde is added just before using this solution.

Orth's fixative:

It is composed of

Potassium dichromate	2.5 g
Sodium sulfate	1 g
Distilled water	100 ml

10 ml of concentrated formalin is added to above solution.

COMPOUND FIXATIVES:

Combination of coagulant fixatives with non coagulant cross linking fixatives is called as compound fixatives. When formaldehyde added to dehydrant ethanol, there will be less shrinkage and hardening comparing to pure dehydrants and it is efficient in preserving molecules like glycogen. Fixation of tissues in alcoholic formalin may helps to distinguish lymph nodes embedded in fat. Even though alcoholic formalin preserves antigen immunorecognition well, it increases the background staining in immunohistochemistry[50].Compound fixatives can also be used for post fixation.

Alcoholic formalin

It is composed of 895 ml of 95% ethanol and 105 ml of concentrated formalin.

Alcohol-formalin-acetic acid fixative

It is composed of

Ethanol (95%)	85 ml
Formaldehyde (37%)	10 ml
Glacial acetic acid	5 ml

Alcoholic Bouin's solution:

The constituents of this fixative is similar to Bouin's except it is less aqueous and therefore it gives better preservation of some carbohydrates (e.g. glycogen).After fixation tissues should be washed in 70% ethanol, followed by 95% ethanol (several changes). This fixative improves upon aging [52].

Gendre's fixative :

It is composed of

Saturated picric acid in 95% ethanol	800 ml
Concentrated formalin	150 ml
Glacial acetic acid	50 ml

Rossman's fixative:

It is composed of

Concentrated formalin	10 ml
Tap water	10 ml
100% ethyl alcohol	80 ml
Lead nitrate	8 g

24 hours fixation is needed. This fixative is preferable for connective tissues and umbilical cord.

NONCOAGULANT CROSS LINKING FIXATIVES:

FORMALDEHYDE:

It is a naturally occurring organic compound with the formulae CH2 O.

NOMENCLATURE:

Chemical Abstract Service Register No.: 50-00-0

Chemical abstract name: Formaldehyde

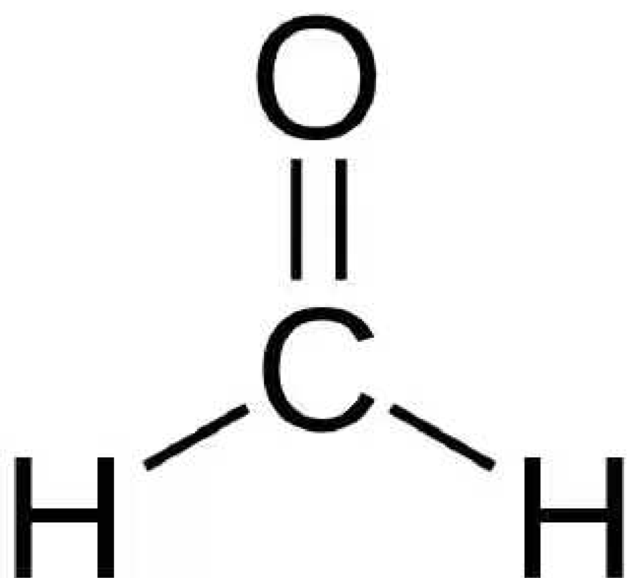
IUPAC Systematic Name: Methanal[2].

Synonyms: Formaldehyde, gas; formic aldehyde methaldehyde;

methyl aldehyde; methylene oxide; oxomethane; oxymethylene[2].

Structural formulae- CH2 O

Relative molecular mass- 30.03



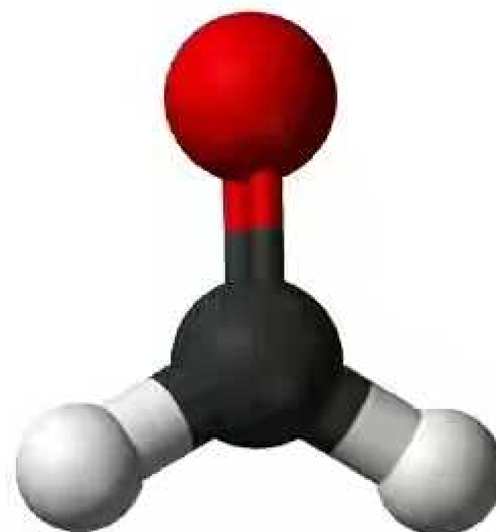


Fig 1: formaldehyde structure

Physical and Chemical properties of formaldehyde:

It is a colour less gas with a pungent odour with boiling point -19.1˚C, melting point -92˚C and density 0.815 at –20 °C. It is soluble in chloroform, water, ethanol and miscible with benzene, acetone ,diethyl ether. Formaldehyde gas is stable in the absence of water, incompatible with oxidizers, alkalis, acids, phenols, urea [2,9,10]. It reacts explosively with peroxide, nitrogen oxide and performic acid[9,10].

HISTORY:

Formaldehyde was first discovered by Butlerov in 1859. Van Hoffman established the method for synthesis of formalin from methanol and established its properties in 1868. Initially formalin was considered as an antiseptic, either to treat or prevent wound infections.

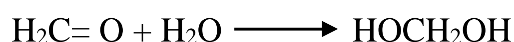
In 1892, Meister, Lucius and Brunig went to a young physician Ferdinan Blum with a proposal to test the antiseptic properties of formaldehyde. Formaldehyde was supplied by the manufacturer as a 40% aqueous solution, which is the concentration resulted from bubbling formaldehyde gas through water. Blum diluted the commercial solution of formalin with 9 parts of water to give a 4% weight/volume solution. He tested the bactericidal properties of this diluted solution and found out that it was an effective but slow agent for killing bacteria[12].

In his second paper , Blum reported that in the process of studying disinfection, he noticed that the skin of his fingers that come in contact with the diluted solution became hardened , as much as with alcohol, that is one of the commonest reagent used for fixation of tissues in olden days. Then he experimented the anthrax infected mouse tissue with diluted formalin and observed that tissues preserved in formalin had the same consistency as alcohol fixed tissues and better staining results were obtained using hematoxylin and aniline dyes. And he also observed that formaldehyde produces less shrinkage and distortion of tissues than alcohol fixed tissues[12] .

Pure formaldehyde is when completely dissolved in water, forms solution containing 37- 40% formaldehyde; this aqueous solution is known as concentrated formalin. So the usual 10% formalin contains about 4% weight to volume of formaldehyde.

MECHANISM OF ACTION:

Fraenkel-Conrat and his colleagues identified and explained the most of the reactions of formaldehyde with amino acids and proteins [13, 14]. In aqueous solution formaldehyde forms methylene hydrate. Formation of methylene hydrate is the first step in formalin fixation .



$$H_2C{=}O + H_2O \longrightarrow HOCH_2OH$$

The formed methylene hydrate acts with the side chains of proteins to form reactive hydroxymethyl side groups(–CH2–OH). The formation of hydroxymethyl

side chains formation is the fundamental and distinct reaction of formaldehyde fixation.

Formaldehyde reacts with nuclear proteins and nucleic acids [18] and it also alters nucleotides by interacting with free amino acids. The cross-linking reactions start at adenine-thymidine (AT)-rich regions of free and naked DNA. And these reactions increases with increasing temperature. Formaldehyde interact with C=C and–SH bonds in unsaturated lipids, but it does not react with carbohydrates. The side chains of lysine, arginine, tyrosine, cysteine, histidine, serine and threonine are more reactive with methylene hydrate, so they have greater affinity for formaldehyde.

REACTIONS OF FORMALDEHYDE WITH PROTEINS:

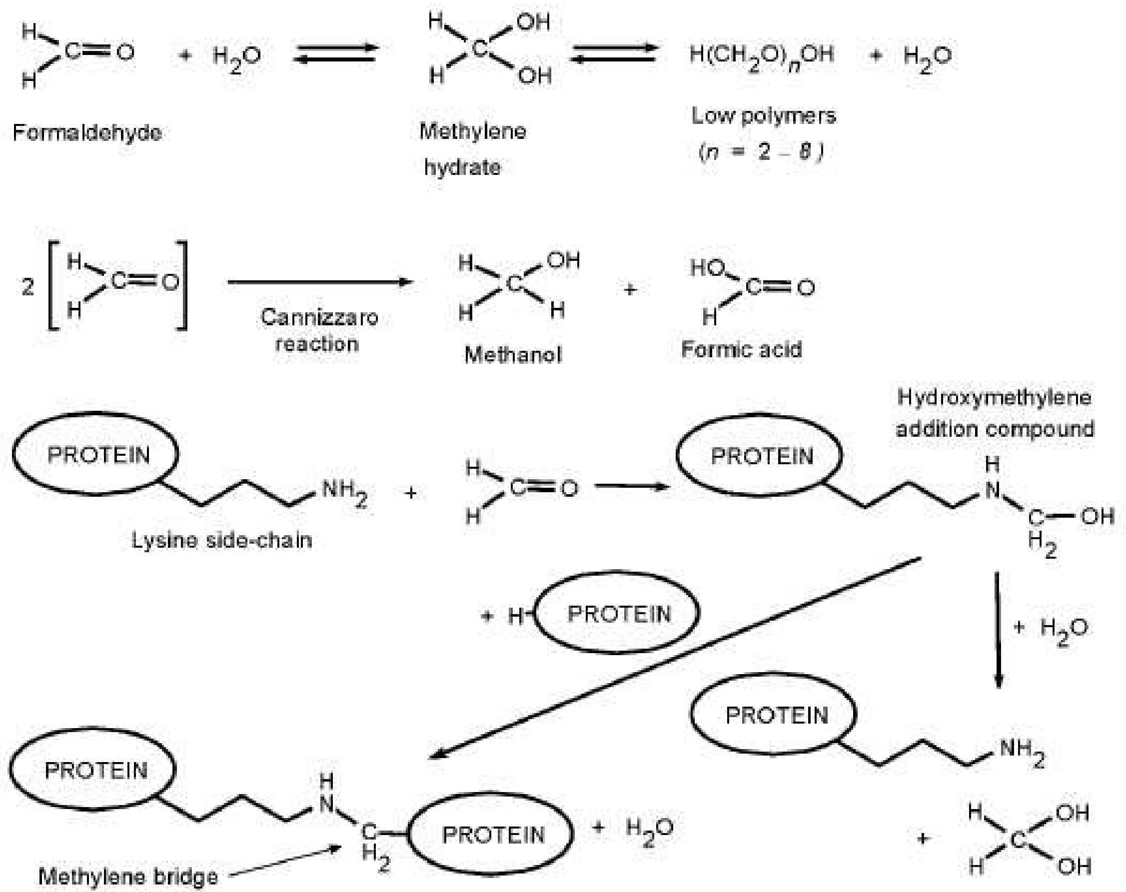


Fig 2: Reactions of formalin with proteins

COMMONLY USED FORMALIN FORMULAE:

10% Neutral buffered formalin:

It is composed of

Tap water	900 ml
Formalin (37% formaldehyde)	100 ml
Sodium phosphate,monohydrate	4 g
Sodium phosphate, dibasic, anhydrous	6.5 g

The pH should be 7.2–7.4.

Carson's modified Millonig's phosphate buffered formalin:

It is composed of

Concentrated formalin	110 ml
Tap water	90 ml
Sodium phosphate, monobasic	1.86 g
Sodium hydroxide	0.42 g

The pH should be 7.2–7.4.

This combination is better for ultra structural preservation than NBF.

Formal (10% formalin), calcium acetate:

It is composed of

Concentrated formalin	100 ml
Tap water	900 ml
Calcium acetate	20 g

It is better in preserving lipids.

Formal saline:

It is composed of

Concentrated formalin	100 ml
Tap water	900 ml
Sodium chloride	9 g

Formal (10% formalin), zinc, unbuffered:

It is composed of

Tap water	900 ml
Formaldehyde (37%)	100 ml

Sodium chloride 4.5 g

Zinc chloride or (zinc sulfate) 1.6 g.

Zinc formalin is an excellent fixative for immunohistochemistry.

Formalin, buffered saline:

It is composed of

Concentrated formalin 100 ml

Tap water 900 ml

Sodium chloride 9 g

Sodium phosphate,dibasic 12 g

Formalin, buffered zinc:

It is composed of 1000 ml of 10% neutral buffered formalin and 1.6 g of Zinc chloride.

ADVANTAGES OF FORMALIN FIXATION:

- It is cheap.
- Easy to prepare.
- Relatively stable.
- Allows the subsequent application of most staining techniques without preliminary procedures.
- Frozen sections can be prepared from formalin fixed material.
- Fat staining can easily carried out on tissues fixed in formalin.
- It penetrates well and does not cause excessive hardening or renders them brittle.
- Natural tissue colours can be restored after formalin fixation.

DISADVANTAGES:

- Allergic dermatitis.
- Irritant to nose.
- In tissues unbuffered formalin leads to dark brown artifact pigment.
- It is unsuitable for electron microscopy.

FORMALIN TOXICITY:

Head ache, burning sensation in throat, difficulty in breathing and exacerbation of asthma are the known toxic effects of formalin.

A study conducted on Finnish women working in a laboratory at least 3 days a week found that there is a significant correlation between spontaneous abortion and formaldehyde exposure[2].

And another study on Chinese women also reported that abnormal menstrual cycles in 70% of the women with occupational exposure to formaldehyde which is 17% in control group[2]. Many researches are going on through out the world to determine the teratogenic effect of formaldehyde.

In 1987, the U.S. Environmental Protection Agency (EPA) classified formaldehyde as a **probable human carcinogen** under conditions of unusually high or prolonged exposure and it limits the permissible exposure level from 1 ppm to 0.75 ppm.

In 2005-06, formaldehyde was found to be seventh most common allergen in patch tests. It was banned in cosmetics in many countries.

IARC (International Agency for Research on Cancer) also reported that there is a strong association between formaldehyde exposure and human nasopharyngeal carcinoma. And there is successive link between formaldehyde exposure and myeloid leukemia was established in 2009[1]. With evaluation and information of all known data led the IARC to reclassify formaldehyde as **known human carcinogen** that is from group 2A to group 1 carcinogen. These statements were reinforced in 2012 by the same agency[1].

In June 2011, the 12th edition National Toxicology program changed the lining status of formaldehyde from "reasonably anticipated to be human carcinogen" to "known to be a human carcinogen".

MEASUREMENT OF FORMALDEHYDE EXPOSURE:

The methods used for the determination of the concentration of formaldehyde in air are based on spectrophotometry, with sensitivity of 0.01–0.03 mg/m3 can be achieved. Other methods include colorimetry, polarography, gas chromatography (GC), fluorimetry, high-performance liquidchromatography (HPLC), infrared detection and gas detector tubes. Most of the methods require the formation of a derivative for separation and detection. HPLC is the most sensitive method (limit of detection, 2 μg/m3 or less). Formaldehyde has been measured in blood by gas chromatography–mass spectrometry (GC–MS) after derivatization to pentafluorophenylhydrazone. Formic acid or formate is produced from formaldehyde and it can be measured in blood and urine [20,22].

EXPOSURE LIMITS:

The usual mean concentration during exposure in histopathology laboratory is 0.5 ppm [44,45,46].

The NIOSH (National Institute of Occupational Safety and Health Administration) U.S defines

Permissible Exposure Limits as

0.75 ppm time weighted average limit (for 8 hoursTWA)

2 ppm short term exposure limit (STEL 15 minutes exposure) [3].

Recommended exposure level :

0.016 ppm TWA to 0.1 ppm for STEL [3].

Individual formaldehyde exposure was monitored by using Malondialdehyde- deoxyguanosine adducts on leukocytes and the alkylation of hemoglobin to form a terminal N-methylene valine residue.

NON FORMALIN FIXATIVES:

CARNOY'S FIXATIVE:

It is composed of 6 parts of ethanol, 3 parts of chloroform and one part of glacial acetic acid. It provides good preservation of nucleic acid in tissues. It is used for glycogen preservation. RNA stains like methyl green pyronine yields better results in carnoy's fixed tissues. However the main drawback is the loss of high molecular weight RNA. Also it shrinks and harden the tissue, this can be reduced by avoiding overfixation and processed for low melting point wax embedding [23].

CLARKE'S SOLUTION:

It is composed of 6 parts of ethanol and one part of glacial acetic acid.

MODIFIED METHACARN:

Components:

6 parts of methanol

3 parts of chloroform

1 part of glacial acetic acid.

Modified methacarn was found to be excellent in preserving tissue RNA[43]. It acts very gently on tissue membranes. As against formalin, immunohistochemistry can be carried out with lesser duration of incubation, greater dilution of antibodies and little requirement for antigen retrieval[24].

ZINC BASED FIXATIVES:

It is a mixture of zinc acetate, zinc chloride and calcium chloride in Tris buffer.

Z 7 contains

Zinc trifluroacetate

Zinc chloride

Zinc acetate

Calcium acetate in Tris-Hcl

The pH is maintained between 6.4 and 6.7.

It has been found to be cost effective, reliable and nontoxic in contrast to NBF. They are superior in DNA and protein extraction analysis in many types of tissue and does not need heat pretreatment for antigen retrieval[25]. The RNA, DNA and protein integrity was found to be good, therefore molecular analysis on Z-7 fixed paraffin embedded tissue samples is better compared to conventional

formalin fixed tissues[26]. DNA sequences up to 2.4 kb and RNA fragments up to 362 bp in length could be properly amplified. But zinc fixation also results in tissue shrinkage and may alter histology.

HOPE fixation (Hepes-glutamic acid buffer mediated organic solvent protection effect):

It is a good technique for comprehensive pathological analysis including immunohistochemistry and molecular pathology. This fixative consists of a mixture of amino acids at pH 5.8 to 6.4. By diffusion this fixative penetrates tissues. After fixation, the tissues incubated in acetone at 0-40ºC for dehydration. Next the specimens are transferred into low melting point paraffin wax and embedded. HOPE fixed sections show formalin like morphology and give a very good preservation of proteins and antigenic structures for analysis by immunohistochemical and enzyme histochemical techniques. Fairly good amount of good quality DNA and RNA can be extracted from these specimens even after a period of five years[27]. These specimens are also suitable for molecular analysis by PCR, RT-PCR and *in-situ* hybridization even after five years[28]. Absent cross-linking and better yield of nucleic acids shows that HOPE fixation can emerge to be an alternative method for tissue banking.

Acetone-methyl benzoate-xylene(AMeX) **fixative**:

It gives good morphology and good quality of high molecular weight DNA [29]. This method, tissues were fixed in acetone at -200˚C for overnight. Then clearing was done in methyl benzoate and xylene and finally the tissues were embedded in paraffin. Good morphology, immunoreactivity are obtained.

Extraction of good quality of higher molecular weight DNA is seen in this technique. RNA extraction is also comparable to fresh frozen tissues [30,31].

Universal molecular fixative (UMFIX):

It is composed of methanol and polyethylene glycol. It is always combined with microwave assisted rapid tissue processing. UMFIX is greatly useful for amplifying small amplicons by RT-PCR in small biopsies. It was introduced by Vincek et al recently to extract high molecular weight RNA from laser captured micro dissected samples. These samples are prepared from paraffin embedded blocks used for histologic diagnosis [32]. Tissue can also be fixed at normal room temperature with UMFIX. Immunoreactivity is similar to formalin fixed tissue. The quality and amount of mRNA and DNA extracted from UMFIX fixed tissues is also comparable to frozen sections.

FINEFIX:

It is composed of ethanol (65- 75% w/v), polyvinyl alcohol, glycerol and monomeric carbohydrates. The special feature of ethanol based fixatives is that they do not form covalent bonds between proteins; they remove the water molecules which surrounds proteins and as a result, protein coagulation occurs and enzymatic functions are brought to halt, thereby making the tissue amenable to conventional proteomic techniques. The major problem in proteomic analysis in fresh tissues is the difficulty in getting a homogenous population of cells. This is because normal structures are often intermixed with pathological structures. In FineFIX fixed, paraffin embedded tissues the morphology is preserved well. This facilitates mechanical or laser capture microdissection which helps to obtain a

small cluster of specific cell types. The integrity of DNA and RNA is well preserved, and hence FineFIX treated tissue aids in better DNA (Formalin upto 350 bp, FineFIX over 2400 bp) and RNA (formalin between 100 - 200 bases, FineFIX upto 600 bases) analysis. The proteins which are obtained from samples treated with FineFIX are of a quality similar to those got from fresh frozen sections thus helping extraction of proteins and conventional proteomic analysis[33]. It fixes the tissue in a shorter duration and so the histological artefacts seen in alcohol based fixative are absent.

RCL 2:

It is formalin free, alcohol based fixative. Its constituents are ethanol, acetic acid and complex carbohydrate. Tissue fixed by RCL2 can be kept at room temperature. They may be kept at -20˚C when a high molecular quality is needed. Morphology, quality and immunoreactivity is comparable to formalin fixed tissue. Quality of RNA and protein profile obtained from RCL2 fixed and paraffin embedded tissue is similar to frozen tissues[34].

UPM:

It is consists of ethanol , methanol, 2 propanol and formalin.

PAGA:

It contains polyethylene glycol, acetic acid, ethanol and glycerol. Nuclear details are better preserved. However shrinkage artifacts are evident while using PAGA [1].

ALTERNATIVES TO FORMALIN FIXATION:

Macroscopic analysis:

Cathy.B.Moelans et al in their study, they found that tissues fixed in RCL 2 and Finefix were of a lighter colour when compared to NBF fixed specimens. Tissues fixed in Finefix and F-solv were rigid, however tissues fixed by RCL2 was soft and slippery, making cutting a difficult task[3].

Cristina Zanini et al reported that the colour change in tissues fixed by ZBF, Z7, PAGA, , RCL2 and CellBlock (alternative fixatives) was not the same as in formalin. There was no difficulty encountered in tissue handling and sectioning when tissues were fixed in RCL2[1]. Mahdiieh Ghoddosi et al also reported that tissues fixed in RCL2 were suitable for microtomy.

Fixation time:

Penetration speed of Finefix and RCL2 slightly faster than NBF- Cathy.B.Moelans et al[3].

Morphological analysis:

Cristina Zanini et al in their study found that an increased affinity for dyes to tissue sections. Greater affinity for eosin was observed in alcohol and zinc based fixatives compared to formalin. Alcohol based fixatives showed better preservation of nuclear details. However shrinkage artifacts are observed and that was more common when alcohol is at a concentration higher than 50%. Zinc based fixatives also had shrinkage artifacts[1].

Cathy B Molens et al in their study, “ Formalin Substitute Fixatives - Analysis of Macroscopy, Morphological Analysis and Immunohistochemical

Analysis” tested three new fixatives (F-Solv, RCL2 and FineFIX) in comparison with neutral buffered formalin. They observed the physical qualities of the sections (section thickness, disruption, cracking and adhesion) was comparable in all fixatives with NBF having the best score. Quality of tissue preservation was assessed based on nuclear and cytoplasmic features, extracellular components and tissue specific features. It was best for NBF and FineFIX came last. Quality of staining was assessed based on the following elements, uniformity of staining, appearance of nucleus, cytoplasm and extracellular components. It was found to be good in alcohol based fixatives which had results similar to NBF[3].

L.Benerini Gatta et al did a study, “Application of alternative fixatives to formalin in diagnostic pathology”. Forty specimens were fixed for one day in various fixatives including formalin, Bouin and Hollande fixatives and Greenfix, CyMol and UPM. They reported that UPM, Greenfix and Cymol gave morphological information which was akin to that obtained using formalin. No differences was found in cell architecture , cytoplasmic and nuclear morphology. Bouin fixative showed higher resolution in the nucleus and nuclear matrix and the background was lightly stained. Colour contrast analysis showed hematoxylin intensity is lower in Bouin, UPM, and Cymol fixation. Eosin affinity is higher in all the alternate fixatives[4].

Ghoddosi M et al.,did a study “RCL 2: A potential formalin substitute for tissue fixation in routine pathological specimens.” They have studied 49 cases fixed in 5 volumes of RCL2 diluted with 100% ethanol , 5 volumes of RCL2 diluted with 95% ethanol and 10 volumes of neutral buffered formalin. They

observed that more than 90% cases in their study received good score for morphological features. RCL2 fixed tissues provided better cytoplasmic assessment[40].

Daniel Groelz et al, did a study “Non-formalin fixative versus formalin-fixed tissue: a comparison of histology and RNA quality.” They found that there was higher eosinophilic staining in PAX gene fixed tissues that facilitates better cytoplasmic details. Nuclear staining was good in PAX gene fixation and was similar to formalin fixation. Red cell lysis was found to be characteristic of PAX gene fixed tissues and limited the assessment of vascular congestion. However distinction between various cells in the mucosa in stomach is better in PAX gene[39].

M. Kap et al in their study, “. Histological assessment of PAXgene tissue fixation and stabilization reagents,” reported that PAX gene fixed tissue show increased eosinophilia but not sufficiently to limit diagnosis[38].

Histochemical analysis:

L.Benerini Gatta et al in their study- bronchial wall stained with PAS and tissues fixed with formalin showed good staining and clear cartilage identification. Greenfix, UPM, Cymol and Hollande allowed to fix secretion.

With alcian blue, Hollande was found to be the best. Tissues fixed using Bouin, UPM and Cymol were showing alcian blue staining which spread into cartilage and matrix. UPM and Cymol fixed tissues showed very selective staining for chondrocytes. Greenfix showed less intense staining.

With Masson trichrome stain, formalin showed good results with regard to colour, contrast and positivity. Greenfix and Hollande were also better as they showed greater definition. While tissues fixed in Bouin showed lesser definition for trichrome staining.

High iron diamine staining, Greenfix showed results equivalent to formalin. Whereas UPM and Cymol were better than Bouin and Hollande.

Cathy B.Molens et al, they studied histochemical property with PAS , PASD, Alcian blue, Azan, G & S and Jones stain were scored 0 to 2 (0- insufficient staining, 1- intermediate and 2- optimal staining). For all stains NBF offered good results with a score of 100%. This was followed by FineFIX which gave 93% and was followed next by RCL2 with 89%.

Immunohistochemistry analysis:

Cathy B.Molens et al , they studied cytokeratin AE 1/3, CAM 52, CD45, ER, PR, S100,Chromogranin A, p63 and vimentin. Results were scored 0 to 2. Overall NBF scored 100% followed by RCL2 70% without pretreatment, FineFIX 68%, F solv 60%. Chromogranin A was optimal for all fixatives. S100 was suboptimal for all alternatives. RCL2 and FineFIX resulted in less than adequate staining of ER regardless of pre-treatment[3].

Nadji et al showed 33% (23 out of 70 total antibodies) of clinical antibodies showed better results using UMFIX than standard NBF[41].

Van Essen et al did a study by using 85 in vitro clinical antibodies. They showed that fixation using 10% NBF provided better staining in 84% antibodies, while RCL2 provided better staining in 66% [42].

L. Benerini et al studied PANCK, CD31, Ki 67, S100 and CD68 in ovarian cancer specimen, they observed that immunoreactions with tissue fixed in 10% NBF for pancytokeratin resulted very good positive diffuse as well as dense staining. Greenfix produced very good diffuse positivity while better focal positivity was obtained with Hollande fixation. Bouin produced weaker focal positivity. UPM gave selective membrane staining while Cymol was less selective.

For Ki 67, Greenfix produced intense positivity while Bouin and Hollande fixation gave weaker positive staining than formalin.

For CD 31, colour intensity was weaker with Bouin and Cymol.

CD 68 staining was found to be superior with alternative fixatives compared to formalin. However the alternative fixatives showed greater background staining than 10% NBF except UPM which gave less background staining[4].

Ghoddosi M et al showed that there was no statistical difference between tissues fixed using formalin and RCL2 in CD34 and Ki67 staining. Modifications in routine IHC protocols may be required for certain antibodies for tissues fixed in RCL 2.

DNA and RNA extraction:

Ghoddosi M et al demonstrated extraction of genomic DNA showed higher DNA extraction from specimens fixed in RCL 2 (6 to 7 times) than in formalin fixed tissues[40].

Cristina Zanini et al demonstrated nucleic acid extraction were superior to 10% NBF with regards to quality and amount of nucleic acid extracted from embedded blocks[1].

MATERIALS AND METHODS

The study was done after getting approval from Institutional Ethical Committee of Tirunelveli Medical College, Tirunelveli. The study was carried out in the Department of Pathology, Tirunelveli Medical College and Hospital, Tirunelveli.

Study design:

Cross sectional study.

Study location:

Tissue materials subjected to histopathological examination from Tirunelveli medical college were utilized for this study.

Sample size:

100 cases and 3 fixatives.

Duration of study:

October 2014 to June 2016.

Inclusion criteria:

Tissue materials collected from MOT (Main operation theatre) and GOT (Gynecology operation theatre) of Tirunelveli medical college.

Part of the tissue materials were utilized for this study.

Exclusion criteria:

- Autolysed specimens,
- Tissues fixed in other fixatives.

Materials required:

- Absolute ethyl alcohol
- Concentrated formalin
- Glycerin
- Methylene blue
- Sodium Dihydrogen Phosphate Monohydrate
- Sodium chloride
- Anhydrous disodium hydrogen phosphate
- Distilled water.

METHODOLOGY:

Minimal formalin containing fixatives were prepared with varying concentrations of formalin, ethanol, glycerin and hypotonic saline. The pH of the fixatives were maintained under 7.2 to 7.4. Ethanol as a dehydrant fixative, it will produce cell shrinkage. To overcome this, hypotonic saline was added. Glycerin was added to minimize evaporation. Methylene blue was added to monitor the colour of fixatives and subsequent dehydrants and to avoid the tendency to smell the solutions. The prepared solutions were light blue in colour(Fig. 3).

Fixation was done by three different combinations of proposed components at 3 different fixation times. Multiple human tissue materials of varying sites (skin, visceral organs,lymphnodes etc.,) and lesions (carcinoma, sarcoma and inflammatory lesions) were utilized. Tissue slices were immediately fixed in the prepared compound fixatives after collecting from MOT and GOT. Fixation hours were titrated between 7 to 10 hours for each of the 3 standard fixatives prepared .

Fixative 1:

It is composed of the following components.

- Formalin – 7%
- Ethanol - 20%
- Glycerin - 5%
- Methylene Blue- 0.05%
- 0.7% hypotonic saline
- Buffer

  Sodium dihydrogen phosphate monohydrate - 4g

  Anhydrous disodium hydrogen phosphate - 6g

Fixative 2:

Fixative 2 is prepared with the following chemicals.

- Formalin – 6%
- Ethanol - 30%
- Glycerin - 5%
- Methylene Blue- 0.05%
- 0.7% hypotonic saline
- Buffer

  Sodium dihydrogen phosphate monohydrate - 4g

  Anhydrous disodium hydrogen phosphate - 6g

Fixative 3:

Fixative 3 is prepared with the following ingredients.

- Formalin – 5%
- Ethanol - 40%
- Glycerin - 5%
- Methylene Blue- 0.05%
- 0.7% hypotonic saline
- Buffer

Sodium Dihydrogen Phosphate Monohydrate - 4g

Anhydrous disodium hydrogen phosphate - 6g

Fixation of tissues in above solutions were done at 10, 8 and 7 hours in all the 3 compound fixatives. Tissue processing was done in following steps;

70% ethanol - 1 hour

80% ethanol - 1 hour

90% ethanol - 1 hour

100% ethanol – 2 hours

Xylene I - 30 minutes

Xylene II - 30 minutes

Wax I - 1 hour

Wax II - 2 hours.

Processed tissues were embedded in paraffin wax. Then the sections were taken 4 micron thickness by using microtome and stained with routine hematoxylin and eosin staining.

Hematoxylin and eosin staining technique:

Preparation of Hematoxylin solution:

Hematoxylin 2.5 g

Potassium alum 50 g

Sodium iodate 0.5 g

Absolute ethanol 25 cc

Glacial acetic acid - 20 cc

Distilled water - 500cc

Hematoxylin is first dissolved in absolute alcohol, and then it is added to alum. The alum should be previously dissolved in warm distilled water. The mixture is made to boil and then sodium iodate is carefully added. The stain is rapidly cooled and when the solution becomes cold,acetic acid is added to it. The stain is now ready for immediate use.

Preparing Eosin:

Eosin Y - 1 g

95% ethanol - 80 cc

Glacial acetic acid - 0.2 cc

Distilled water - 20 cc

Dissolve 1g Eosin Y in 20 ml of distilled water and add 80 ml 95% ethanol and 0.2 ml glacial acetic acid.

Staining procedure:

1. Xylene 3 changes – 2 minutes each.
2. 90%, 80%, 70% alcohol – 10 dips each.
3. Bring sections to water.
4. Harris hematoxylin – 15 minutes.
5. Rinse in clean water.
6. Differentiate using 1% acid alcohol.
7. Rinse in clean water.
8. 0.5% lithium carbonate – until blue.
9. Wash in clean water.
10. Eosin – 15 seconds to 2 minutes depending on age of eosin.
11. Rinse in clean water.
12. Dehydrate in absolute alcohol - change twice, dip 10 times each.
13. Xylene – change twice and dip each 10 times.
14. Mount using DPX mountant.

Stained slides were studied under light microscope. Fixation artifacts, staining characteristics, architecture, nuclear and cytoplasmic details were analysed by two independent pathologists. Nuclear, cytoplasmic and architectural features were scored between 0- 3. Score 3 was given to nuclear, cytoplasmic and architectural features of all the tissues fixed in 24 hours conventional 10% NBF that is considered as absolute fixation.

Nuclear features were assessed based on following features, nuclear and nucleolar preservation, nucleus size, regularity of nuclear membrane, chromatin

pattern whether fine, coarse, granular/ reticular pattern and mitotic figures. Score 3 was given to tissues fixed in compound fixatives with similar nuclear features to tissues fixed in conventional 10% NBF. Score 2 was given to sections with 1 to 2 less defined nuclear features. Score 1 was given to sections with more than 2 less defined nuclear details. Score 0 was given to sections with poor preservation of details which was unsuitable for diagnosis.

Cytoplasmic features were assessed by colour of cytoplasm, abundance, cytoplasmic granules and mucin differentiation**.** Score 3 was given to tissues fixed in compound fixatives with similar cytoplasmic features to tissues fixed in conventional 10% NBF i.e. absolute fixation. Score 2 was given to sections with cytoplasmic shrinkage with less prominent cytoplasmic granules and considered as suboptimal fixation. Score 1 was given to sections with more than 2 less defined cytoplasmic details. Score 0 was given to sections with poor preservation of details which was unsuitable for diagnosis.

Architectural features were assessed based on shrinkage artifacts, distortion, cracking and formalin pigments. Score 3 was given to tissues fixed in compound fixatives with similar architectural features to tissues fixed in conventional 10% NBF i.e.optimal fixation. Score 2 was given to sections with 1 to 2 less defined architectural features. Score 1 was given to sections with more than 2 less defined nuclear details. Score 0 was given to sections with poor preservation of details which was unsuitable for diagnosis.

Scoring system:

3-Good fixation

2- Sub-optimal

1- poor

0- Unsuitable.

The fixation time and amount of reagents used in each fixation were evaluated and compared with conventional fixation procedures. The results were tabulated and analyzed with Mann-Whitney U test and Wilcoxon signed rank test. P value <0.05 is considered as statistically significant.

Schiff test:

The concentration of formaldehyde vapor in our compound fixatives was compared with conventional 10% NBF by using schiff's reagent. No .1 whatman filter paper was soaked in schiff's reagent and dried in air. Two glass beakers of 9 cm in length and 7.5 cm in diameter were taken and labeled as beaker A and beaker B. 10 ml of 10% NBF was poured into beaker A and 10 ml of fixative 2 was poured into beaker B. Both beakers were closed by whatman paper (schiff's reagent soakd) and allowed to stand. The time taken for the filter papers to change colour into pink/magenta was noted.

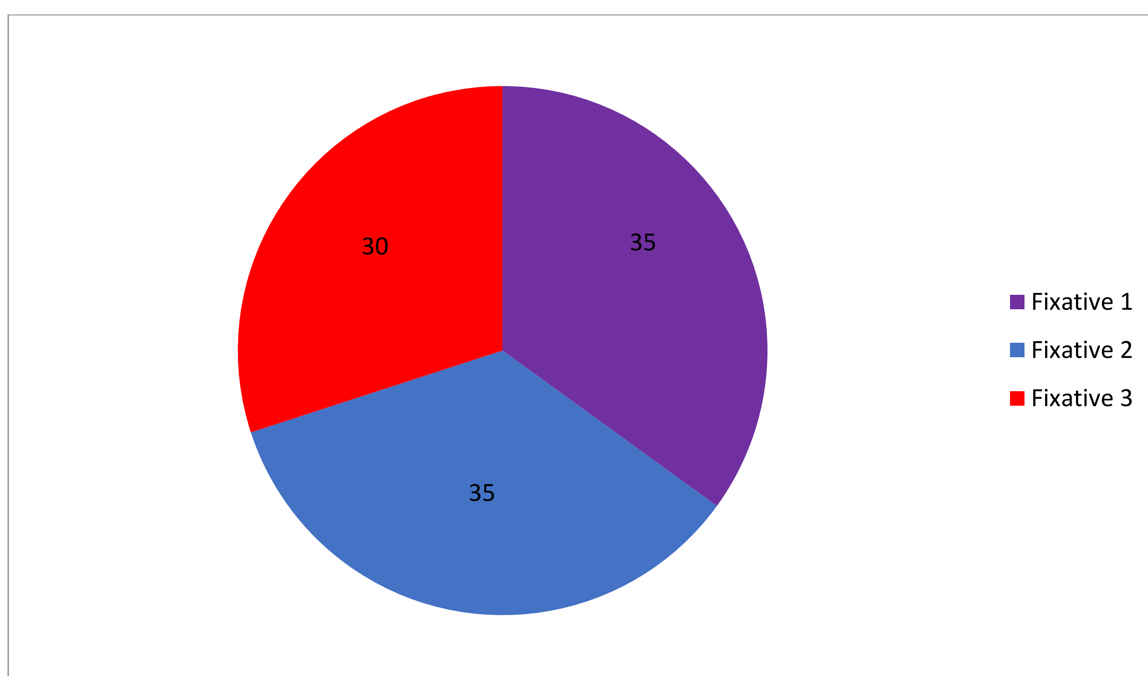
OBSERVATION AND RESULTS

A total of 100 specimens were included in this study. Among them 35 specimens were fixed in fixative 1, another 35 were fixed in fixative 2 and 30 specimens were fixed in fixative 3.

TABLE 1: Distribution of specimens

FIXATIVE	NO. OF SPECIMENS(n=100)	PERCENTAGE (%)
Fixative 1	35	35
Fixative 2	35	35
Fixative 3	30	30

CHART 1: Distribution of specimens



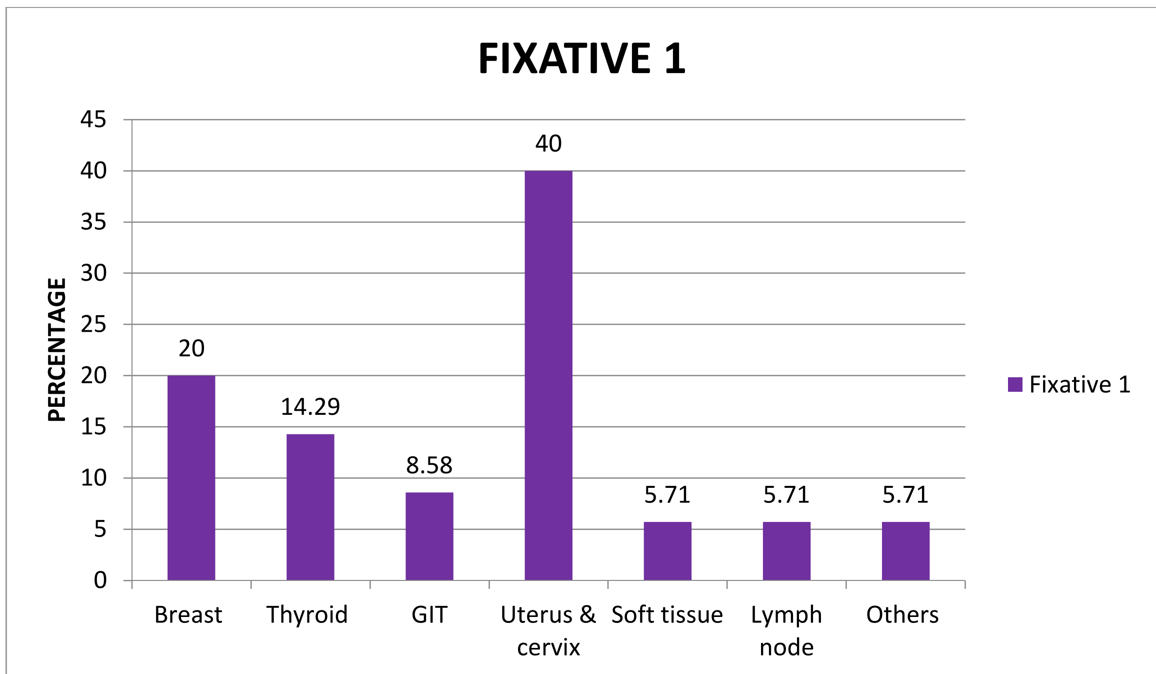
Distribution of specimens in fixative 1:

Totally 35 specimens were fixed in fixative 1. Among them 14 (40%) were uterus and cervix followed by breast 7 cases(20%), thyroid 5 (14.29%), gastrointestinal tract specimens 3 (8.58%), soft tissue 2 (5.71%), lymph node 2 (5.71%) and other ovary (1) and testis (1).

TABLE 2: Distribution of specimens in fixative 1

Specimen	No. of cases(n=35)	Percentage
Breast	7	20
Thyroid	5	14.29
Gastrointestinal tract	3	8.58
Uterus & cervix	14	40
Soft tissue	2	5.71
Lymph node	2	5.71
Others	2	5.71

CHART 2: Distribution of specimens in fixative 1



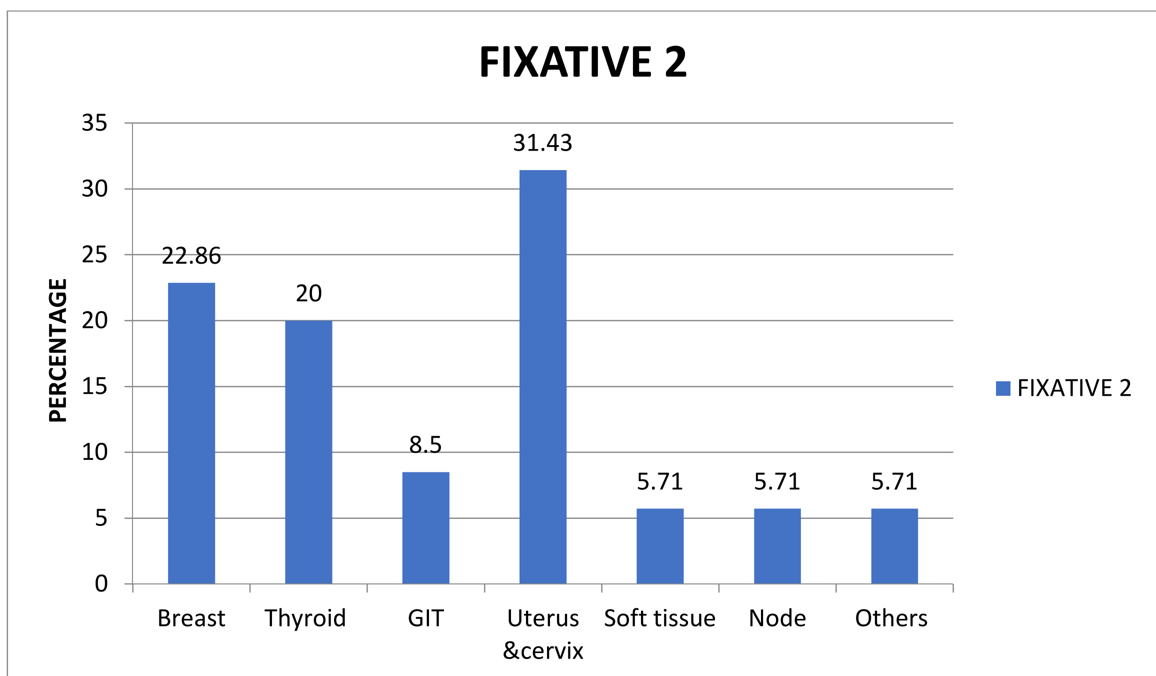
Distribution of specimens in fixative 2:

35 specimens were fixed in fixative 2. Out of 35, 11 (31.43%) were uterus and cervix, breast 8 (22.86%), thyroid 7 (20%), gastrointestinal tract 3 (8.5%), soft tissue 2 (5.71%), lymph nodes 2 (5.71%) and others were otongue (1) and ovary (1).

TABLE 3:Distribution of specimens in fixative 2

Specimen	No of cases	Percentage (%)
Breast	8	22.86
Thyroid	7	20
Gastrointestinal tract	3	8.5
Uterus & cervix	11	31.43
Soft tissue	2	5.71
Lymph node	2	5.71
Others	2	5.71

CHART 3: Distribution of specimens in fixative 2



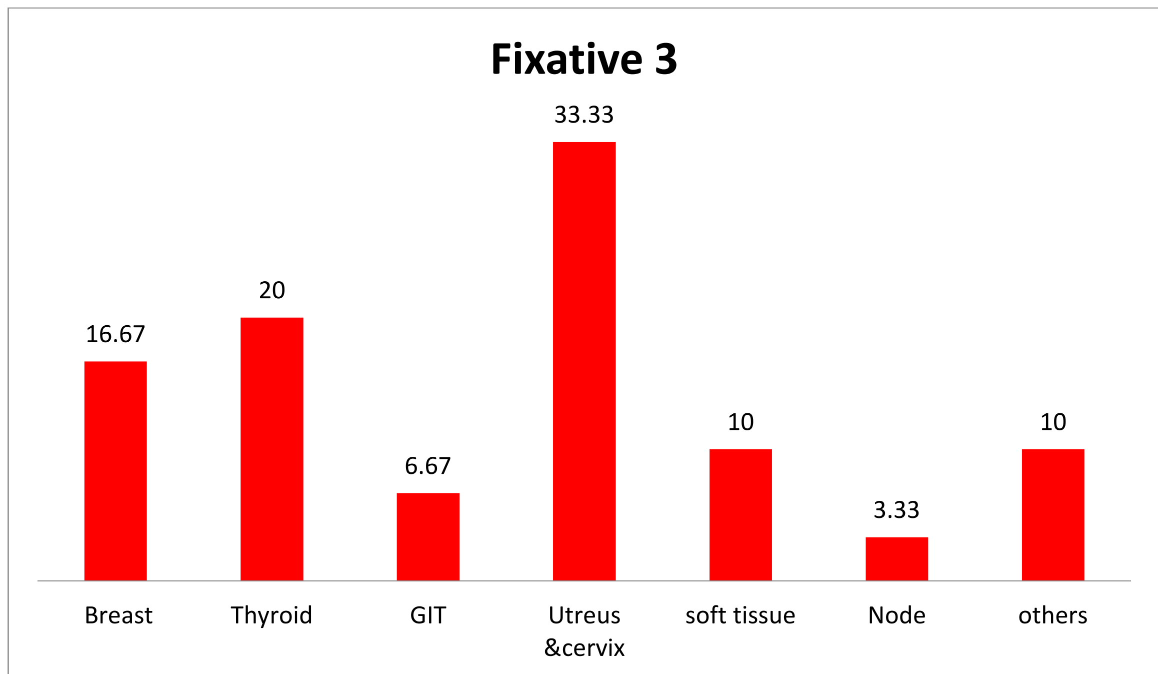
Distribution of specimens in fixative 3:

Out of 30 specimens fixed in fixative 3, uterus and cervix were 10 (33.33%) followed by thyroid 6 (20%), breast 5 (16.67%), soft tissue 3(10%), gastrointestinal tract 2 (6.67%), lymph node 1(3.33%) and others were testis(1), lung (1) and kidney (1) .

TABLE 4: Distribution of specimens in fixative 3

Specimen	No of cases (n= 30)	Percentage (%)
Breast	5	16.67
Thyroid	6	20
Gastrointestinal tract	2	6.67
Uterus & cervix	10	33.33
Soft tissue	3	10
Lymph node	1	3.33
Others	3	10

CHART 4: Distribution of specimens in fixative 3



MACROSCOPIC APPEARANCE:

- Tissues fixed in our newer compound fixatives were light blue in colour.
- Texture of tissues after fixation was same as tissues fixed in conventional 10% NBF.
- There was no difficulty in cutting and sectioning.

EVALUATION OF HISTOMORPHOLOGICAL FEATURES:

The stained slides were analyzed for fixation artifacts, staining characteristics, architecture, nuclear and cytoplasmic details.

COMPARISON OF NUCLEAR FEATURES:

Nuclear features were assessed based on following features, nuclear and nucleolar preservation, nucleus size, regularity of nuclear membrane,chromatin pattern whether fine, coarse, granular/ reticular pattern and mitotic figures .Score 3 was given to nuclear features of all the tissues(i.e.35 specimens) fixed in 24 hours conventional 10% NBF that is considered as absolute fixation. Score 2 and

1 were given to sections with nuclear shrinkage, less prominent nucleolus and mitotic figures and poorly defined chromatin pattern and it is considered as suboptimal fixation.

Comparison of fixative 1 and conventional 10% NBF:

Nuclear features of tissues fixed in fixative 1 was compared with conventional formalin fixed tissues. At 10 hours fixation in fixative 1, all the 35 specimens scored 3. At 8 hours fixation, only one specimen (lymph node) showed nuclear shrinkage compared to 10% NBF and got score 2. All the other 34 specimens received score 3. At 7 hours fixation more than half the cases showed nuclear shrinkage, less prominent nucleoli and mitotic figures and they got score 2.

TABLE 5: Comparison of Fixative 1 and conventional 10% NBF

	FIXATIVES	**SCORE**				**P value***
		3	**2**	**1**	**0**	
No. of Specimens	10% NBF	35	nil	nil	nil	**n/a**
	Fixative 1,10 Hrs	35	nil	nil	nil	**n/a**
	Fixative 1, 8Hrs	34	1	nil	nil	**0.325**
	Fixative1,7 Hrs	15	20	nil	nil	**<0.0001**

*Mann whitney U test

There is no significant difference between 10% NBF and fixative 1 at 10 and 8 hours fixation. So fixative 1 is as equal as 10% NBF in preserving nuclear features at 10 and 8 hours fixation. There is significant difference at 7 hours fixation on comparing to conventional formalin , so it is suboptimal in preserving nuclear details.

Comparison of fixative 2 and conventional 10% NBF:

Nuclear features of tissues fixed in fixative 2 was compared with conventional 10% NBF fixed tissues. At 10 and 8 hours fixation in fixative 2, two to three cases showed less defined chromatin pattern , less prominent nucleolus and nuclear shrinkage compared to NBF and received score 2. All other specimens scored 3 and comparable with NBF. More than half the tissues fixed at 7 hours fixation got score 2.

TABLE 6: Comparison of fixative 2 and conventional 10% NBF

	FIXATIVES	**SCORE**				**P value***
		3	**2**	**1**	**0**	
No. of Specimens	10% NBF	35	nil	nil	nil	**n/a**
	Fixative 2, 10 Hrs	33	2	nil	nil	**0.160**
	Fixative 2, 8Hrs	32	3	nil	nil	**0.083**
	Fixative 2, 7 Hrs	13	22	nil	nil	**<0.0001**

*Mann whitney U test

The above table shows there is no significant difference between 10% NBF and fixative 2 at 10 and 8 hours fixation. So fixative 2 is as equal as 10% NBF in preserving nuclear features at 10 and 8 hours fixation. There is significant difference at 7 hours fixation. So nuclear details are not well preserved in 7 hours fixation.

Comparison of fixative 3 and conventional 10% NBF:

Nuclear details of tissues fixed in fixative 3 was compared with conventional 10% NBF fixed tissues. Most of the specimens fixed in fixative 3 showed nuclear shrinkage, less defined nuclear membrane and chromatin pattern, less prominent nucleolus in all the 3 fixation hours. Few sections (2 to 6 cases) showed even more shrinkage and poorly preserved chromatin got score 1.

TABLE 7:Comparison of fixative 3 and conventional 10% NBF

	FIXATIVES	**SCORE**				**P value***
		3	**2**	**1**	**0**	
No. of Specimens	10% NBF	30	nil	nil	nil	**n/a**
	Fixative 3, 10 Hrs	6	22	2	nil	**<0.0001**
	Fixative 3, 8Hrs	6	23	1	nil	**<0.0001**
	Fixative 3, 7 Hrs	2	22	6	nil	**<0.0001**

*Mann whitney U test

The above table shows there is significant difference in all three different fixation times compared to conventional 10% NBF. So fixative 3 is inferior in preserving nuclear details compared to conventional formalin .

Comparison of fixative 1 and fixative 2:

Nuclear details were compared between fixative 1 and 2. Almost all of the cases fixed in both fixative 1 and 2 at 10 and 8 hours fixation received score 3 as they preserved well defined chromatin pattern , distinct nuclear membrane, nucleoli and mitotic figures. At 7 hours both fixatives received score 2 because they showed less prominent above nuclear details.

TABLE 8: Comparison of fixative 1 and fixative 2

	TIME	**FIXATIVES**	**SCORE**				**P value***
			3	**2**	**1**	**0**	
No. of Specimens	10 hours	Fixative 1	35	nil	nil	nil	**0.513**
		Fixative 2	33	2	nil	nil	
	8 hours	Fixative 1	34	1	nil	nil	**0.505**
		Fixative 2	32	3	nil	nil	
	7 hours	Fixative 1	15	20	nil	nil	**0.962**
		Fixative 2	13	22	nil	nil	

*Mann whitney U test

This table shows there is no significant difference between fixative 1 and 2 in all three fixation times. So both fixatives are equal in nuclear features preservation at 3 different fixation times.

Comparison of fixative 1 and fixative 3:

Nuclear details were compared between fixative 1 and 3 in the following table. At 10 and 8 hours fixation, almost all of the specimens fixed in fixative 1 scored 3 as they preserved nuclear details well whereas more than half of the tissues fixative fixative 3 showed nuclear shrinkage and less defined chromatin pattern and they got score 2. At 7 hours fixation , 20 cases fixed in fixative 1 got score 2 as they showed less prominent nucleoli and mitotic figures. 6 cases in fixative 3 showed even more shrinkage and got score1.

TABLE 9: Comparison of fixative 1 and fixative 3

	TIME	FIXATIVES	SCORE				P value*
			3	2	1	0	
No. of Specimens	10 hours	Fixative 1	35	nil	nil	nil	**<0.0001**
		Fixative 3	6	22	2	nil	
	8 hours	Fixative 1	34	1	nil	nil	**<0.0001**
		Fixative 3	6	23	1	nil	
	7 hours	Fixative 1	15	20	nil	nil	**<0.0001**
		Fixative 3	2	22	6	nil	

*Mann whitney U test

There is significant difference between fixative 1 and 3 in all three fixation times. So fixative 1 is superior to fixative 3 in preserving nuclear details.

Comparison of fixative 2 and fixative 3:

Nuclear details were compared between fixative 2 and 3. At 10 and 8 hours fixation, almost all of the specimens fixed in fixative 2 scored 3 as they preserved nuclear details well whereas more than half of the tissues fixative fixative 3 showed nuclear shrinkage and less defined chromatin pattern and they got score 2. At 7 hours fixation , 27 cases fixed in fixative 2 got score 2 as they showed less

prominent nucleoli and mitotic figures. 6 cases in fixative 3 showed even more shrinkage and got score1.

TABLE 10: Comparison of fixative 2 and fixative 3

	TIME	**FIXATIVES**	**SCORE**				**P value***
			3	**2**	**1**	**0**	
No. of Specimens	10 hours	Fixative 2	33	2	nil	nil	**<0.0001**
		Fixative 3	6	22	2	nil	
	8 hours	Fixative 2	32	3	nil	nil	**<0.0001**
		Fixative 3	6	23	1	nil	
	7 hours	Fixative 2	13	22	nil	nil	**<0.0001**
		Fixative 3	2	22	6	nil	

*Mann whitney U test

There is significant difference between fixative 2 and 3 in all three fixation times. So fixative 2 is superior to fixative 3 in preserving nuclear details.

COMPARISON OF CYTOPLASMIC FEATURES:

Cytoplasmic features were assessed by colour of cytoplasm, abundance, cytoplasmic granules and mucin differentiation**.** Score 3 was given to cytoplasmic

features of all the tissues(i.e.35 specimens) fixed in 24 hours conventional 10% NBF i.e. absolute fixation. Score 2 was given to sections with cytoplasmic shrinkage with less prominent cytoplasmic granules and considered as suboptimal fixation.

Comparison of fixative 1 and conventional 10% NBF:

In this table, cytoplasmic features were compared between fixative 1 and conventional formalin. At 10 and 8 hours fixation, 32 cases fixed in fixative 1 have received score 3 as they preserves cytoplasmic colour , granules and mucin differentiation well.3 cases got score 2 because of cytoplasmic shrinkage. At 7 hours fixation 25 cases received score 2 because of cytoplasmic shrinkage.

TABLE 11: Comparison of fixative 1 and conventional 10% NBF

	FIXATIVES	**SCORE**				**P value***
		3	**2**	**1**	**0**	
No. of Specimens	10% NBF	35	nil	nil	nil	**n/a**
	Fixative 1, 10 Hrs	32	3	nil	nil	**0.083**
	Fixative 1, 8Hrs	32	3	nil	nil	**0.083**
	Fixative1, 7 Hrs	10	25	nil	nil	**<0.0001**

*Mann whitney U test

There is no significant difference between 10% NBF and fixative 1 at 10 and 8 hours fixation. So fixative 1 is as equal as 10% NBF in preserving

cytoplasmic features at 10 and 8 hours fixation. There is significant difference at 7 hours fixation, so it is inferior than 10% NBF in preserving cytoplasmic details.

Comparison of fixative 2 and conventional 10%NBF:

In the following table, cytoplasmic features were compared between fixative 2 and conventional formalin. At 10 and 8 hours fixation, more than 30 specimens fixed in fixative 2 have received score 3 as they preserves cytoplasmic colour , granules and mucin differentiation well. At 7 hours fixation more than half of the cases got score 2 because of cytoplasmic shrinkage, less defined granules.

TABLE 12: Comparison fixative 2 and conventional 10%NBF

	FIXATIVES	**SCORE**				**P value***
		3	**2**	**1**	**0**	
No. of Specimens	10% NBF	35	nil	nil	Nil	**n/a**
	Fixative 2, 10 Hrs	33	2	nil	Nil	**0.160**
	Fixative 2, 8Hrs	32	3	nil	Nil	**0.083**
	Fixative 2, 7 Hrs	13	22	nil	Nil	**<0.0001**

*Mann whitney U test

The above table shows there is no significant difference between 10% NBF and fixative 2 at 10 and 8 hours fixation. So fixative 2 is as equal as 10% NBF in preserving cytoplasmic details at 10 and 8 hours fixation. There is significant

difference at 7 hours fixation. So 7 hours fixation in fixative 2 is inferior to conventional formalin.

Comparison of fixative 3 and conventional 10% NBF:

Cytoplasmic features between fixative 3 and conventional formalin were compared in this table. All three fixation hours, more than half of the tissues got score 2 because of cytoplasmic shrinkage. 4 to 7 cases showed even more shrinkage and received score 1.

TABLE 13: Comparison of fixative 3 and conventional 10% NBF

	FIXATIVES	**SCORE**				**P value***
		3	**2**	**1**	**0**	
No. of Specimens	10% NBF	30	nil	nil	nil	**n/a**
	Fixative 3, 10 Hrs	5	18	7	nil	**<0.0001**
	Fixative 3, 8Hrs	5	21	4	nil	**<0.0001**
	Fixative 3, 7 Hrs	5	18	7	nil	**<0.0001**

*Mann whitney U test

The above table shows there is significant difference in all three different fixation times compared to conventional 10% NBF. So fixative 3 is inferior to conventional 10% NBF in preserving cytoplasmic details.

Comparison of Fixative 1 and Fixative 2:

This table shows cytoplasmic features comparison between fixative 1 and 2. At 10 8 hours fixation, more than 30 cases fixed in fixative 2 received score 3. At 7 hours fixation, more than half of the specimens fixed in both fixatives got score 2 because of cytoplasmic shrinkage.

TABLE 14:Comparison of fixative 1 and fixative 2

	TIME	FIXATIVES	SCORE				P value*
			3	2	1	0	
No. of Specimens	10 hours	Fixative 1	32	3	nil	nil	**1.000**
		Fixative 2	33	2	nil	nil	
	8 hours	Fixative 1	32	3	nil	nil	**n/a**
		Fixative 2	32	3	nil	nil	
	7 hours	Fixative 1	10	25	nil	nil	**0.003**
		Fixative 2	13	22	nil	nil	

*Mann whitney U test

This table shows there is no significant difference between fixative 1 and 2 at 10 hours fixation times. So both fixatives are equal in cytoplasmic features preservation at 10 and 8 hours. There is significant difference at 7 hours fixation. At 7 hours fixative 1 is superior than 2 in the preservation of cytoplasmic details.

Comparison of Fixative 1 and Fixative 3:

Fixative 1 and 3 were compared in this table. Both 10 and 8 hours fixation, fixative 1 got good score. More than half of the cases fixed in fixative 3 showed cytoplasmic shrinkage and got score 2. At 7 hours fixation both fixatives resulted in suboptimal score.

TABLE 15: comparison of fixative 1and fixative 3

	TIME	FIXATIVES	SCORE				P value*
			3	**2**	**1**	**0**	
No. of Specimens	10 hours	Fixative 1	32	3	nil	nil	**<0.0001**
		Fixative 3	5	18	7	nil	
	8 hours	Fixative 1	32	3	nil	nil	**<0.0001**
		Fixative 3	5	21	4	nil	
	7 hours	Fixative 1	10	25	nil	nil	**<0.0001**
		Fixative 3	5	18	7	nil	

*Mann whitney U test

There is significant difference between fixative 1and 3 in all three fixation times. So fixative 1 is superior to fixative 3 in preserving cytoplasmic details.

Comparison of Fixative 2 and Fixative 3:

Cytoplasmic features of fixative 2 and 3 were compared in this table. Both 10 and 8 hours fixation , fixative 2 got optimal score. More than half of the cases fixed in fixative 3 showed cytoplasmic shrinkage and got score 2. At 7 hours fixation, more than half of the tissues fixed in both fixatives resulted in suboptimal score.

TABLE 16:Comparison of fixative 2 and fixative 3

	TIME	**FIXATIVES**	**SCORE**				**P value***
			3	**2**	**1**	**0**	
No. of Specimens	10 hours	Fixative 2	33	2	nil	nil	**<0.0001**
		Fixative 3	5	18	7	nil	
	8 hours	Fixative 2	32	3	nil	nil	**<0.0001**
		Fixative 3	5	21	4	nil	
	7 hours	Fixative 2	13	22	nil	nil	**<0.0001**
		Fixative 3	5	18	7	nil	

*Mann whitney U test

There is significant difference between fixative 2 and 3 in all three fixation times. So fixative 2 is superior to fixative 3 in preserving cytoplasmic details.

COMPARISON OF ARCHITECTURAL FEATURES:

Architectural features were assessed based on shrinkage artifacts, distortion, cracking and formalin pigments. Score 3 was given to architecture feature of all the tissues(i.e.35 specimen) fixed in 24 hours conventional 10% NBF i.e. absolute fixation. Score 2 was given to sections with shrinkage artefacts , distortion and cracking.

Comparison of Fixative 1 and Conventional 10% NBF:

Architectural features were compared between fixative 1 and 10% NBF. 33 specimens fixed in both 10 and 8 hours, got optimal score 3. 2 cases received score 2 because of shrinkage artefact and distortion. At 7 hours more than half of the tissues received suboptimal score.

TABLE 17: Comparison of Fixative 1 and Conventional 10% NBF

	FIXATIVES	**SCORE**				**P value***
		3	**2**	**1**	**0**	
No. of Specimens	10% NBF	35	nil	nil	nil	**n/a**
	Fixative 1, 10 Hrs	33	2	nil	nil	**0.160**
	Fixative 1, 8Hrs	33	2	nil	nil	**0.160**
	Fixative1, 7 Hrs	12	23	nil	nil	**<0.0001**

*Mann whitney U test

There is no significant difference between 10% NBF and fixative 1 at 10 and 8 hours fixation. So fixative 1 is as equal as 10% NBF in preserving architectural features at 10 and 8 hours fixation. There is significant difference at 7 hours fixation, so it is poor in preserving architecture than conventional fixation.

Comparison of Fixative 2 and Conventional 10% NBF:

In this table architecture features were compared between fixative 2 and 10%NBF. 32 specimens fixed in both 10 and 8 hours, got optimal score 3. 3 cases received score 2 because of shrinkage artefact and distortion. At 7 hours more than half of the tissues received suboptimal score because of shrinkage artefact .

TABLE 18: Comparison of Fixative 2 and Conventional 10% NBF

	FIXATIVES	**SCORE**				**P value***
		3	**2**	**1**	**0**	
No. of Specimens	10% NBF	35	nil	nil	nil	**n/a**
	Fixative 2, 10 Hrs	32	3	nil	nil	**0.083**
	Fixative 2, 8Hrs	32	3	nil	nil	**0.083**
	Fixative 2, 7 Hrs	3	20	12	nil	**<0.001**

*Mann whitney U test

The above table shows there is no significant difference between 10% NBF and fixative 2 at 10 and 8 hours fixation. So fixative 2 is as equal as 10% NBF in preserving architectural features at 10 and 8 hours fixation. There is significant difference at 7 hours fixation , so it is poor in preserving architecture compared to conventional formalin.

Comparison of fixative 3 and conventional 10% NBF:

Architecture features were compared between fixative 3 and conventional formalin. In all the three fixation hours fixative 3 received score 2 and 3 , because of more shrinkage artefacts , distortion and cracking.

TABLE 19:Comparison of fixative 3 and conventional 10% NBF

	FIXATIVES	**SCORE**				**P value***
		3	**2**	**1**	**0**	
No. of Specimens	10% NBF	30	nil	nil	nil	**n/a**
	Fixative 3, 10 Hrs	nil	17	13	nil	**<0.001**
	Fixative 3, 8Hrs	nil	19	11	nil	**<0.001**
	Fixative 3, 7 Hrs	nil	10	20	nil	**<0.001**

*Mann whitney U test

The above table shows there is significant difference in all three different fixation times compared to conventional 10% NBF. So fixative 3 is inferior compared to conventional 10% NBF in preserving architecture.

Comparison of Fixative 1 and Fixative 2:

Architecture was compared between fixative 1 and 2. More than 30 cases fixed in both fixative 1 and 2 got optimal score with minimal distortion. At 7 hours fixation, both fixatives got suboptimal and poor score because of shrinkage artifact and cracking especially thyroid and nodal tissues.

TABLE 20:Comparison of fixative 1 and fixative 2

	TIME	**FIXATIVES**	**SCORE**				**P value***
			3	**2**	**1**	**0**	
No. of Specimens	10hours	Fixative 1	33	2	nil	nil	**0.812**
		Fixative 2	32	3	nil	nil	
	8 hours	Fixative 1	33	2	nil	nil	**0.812**
		Fixative 2	32	3	nil	nil	
	7 hours	Fixative 1	12	23	nil	nil	**<0.0001**
		Fixative 2	3	20	12	nil	

*Mann whitney U test

The above table shows there is no significant difference between fixative 1 and 2 at both 10 and 8 hours fixation. So both fixatives are equal in architectural detail preservation at 10 and 8 hours. There is significant difference at 7 hours fixation. At hours fixative 1 is superior than 2.

Comparison of Fixative 1 and Fixative 3:

Architecture features were compared between fixative 1 and 3 in this table.. More than 30 cases fixed in both fixative 1 at both 10 and 8 hours fixation got optimal score with minimal distortion. Fixative 3 in all three fixation hours got suboptimal and poor score because of shrinkage artifact and cracking.

TABLE 21: Comparison of fixative 1 and fixative 3

	TIME	**FIXATIVES**	**SCORE**				**P value***
			3	**2**	**1**	**0**	
No. of Specimens	10 hours	Fixative 1	33	2	nil	nil	**<0.0001**
		Fixative 3	nil	17	13	nil	
	8 hours	Fixative 1	33	2	nil	nil	**<0.0001**
		Fixative 3	nil	19	11	nil	
	7 hours	Fixative 1	12	23	nil	nil	**<0.0001**
		Fixative 3	nil	10	20	nil	

*Mann whitney U test

There is significant difference between fixative 1 and 3 in all three fixation times. So fixative 1 is superior compared to fixative 3 in preserving tissue architecture.

Comparison of fixative 2 and fixative 3:

Architecture features were compared between fixative 2 and 3 in this table.. More than 30 cases fixed in both fixative 2 at both 10 and 8 hours fixation got optimal score with minimal distortion. Fixative 3 in all three fixation hours got suboptimal and poor score because of shrinkage artifact and cracking **TABLE 23:Comparison of fixative 2 and fixative 3**

	TIME	FIXATIVES	SCORE				P value*
			3	2	1	0	
No. of Specimens	10 hours	Fixative 2	32	3	nil	nil	**<0.0001**
		Fixative 3	nil	17	13	nil	
	8 hours	Fixative 2	32	3	nil	nil	**<0.0001**
		Fixative 3	nil	19	11	nil	
	7 hours	Fixative 2	3	20	12	nil	**<0.0001**
		Fixative 3	nil	10	20	nil	

*Mann whitney U test

There is significant difference between fixative 2 and 3 in all three fixation times. So fixative 2 is superior compared to fixative 3 in preserving architectural details.

COMPARISON OF FIXATION TIME:

Comparison of fixation time in 3 different fixatives was carried out by Wilcoxon signed rank test.

Fixation time and nuclear details:

Nuclear features were analyzed based on size, regularity of nuclear membrane, presence or absence of nucleolus, chromatin pattern and mitotic figures.

Comparison of fixation time and nuclear details in fixative 1:

On comparing between 10 and 8 hours almost all the tissues fixed in fixative 1 received score 3. At 7 hours fixation more than half of the cases (20) got score 2 because of nuclear shrinkage and less defined chromatin pattern.

TABLE 23: Fixation time and nuclear details in fixative 1

	TIME	**SCORE**				**P value***
		3	**2**	**1**	**0**	
No. of Specimens	10 hours	35	nil	nil	nil	**0.317**
	8 hours	34	1	nil	nil	
	10 hours	35	nil	nil	nil	**<0.0001**
	7 hours	15	20	nil	nil	

*Wilcoxon signed rank test

The above table shows there is no significant difference between tissues fixed in 10 and 8 hours. So fixative 1 gives optimal fixation at 10 and 8 hours. There is significant difference between 10 and 7 hours fixation. This indicates 7 hours fixation results in suboptimal fixation.

Comparison of fixation time and nuclear details in fixative 2:

On comparing between 10 and 8 hours more than 30 cases fixed in fixative 2 received score 3. At 7 hours fixation more than half of the cases (27) got score 2 because of nuclear shrinkage and less defined chromatin pattern, less prominent nucleoli.

TABLE 24: Fixation time and nuclear details in fixative 2

	TIME	**SCORE**				**P value***
		3	**2**	**1**	**0**	
No. of Specimens	10 hours	33	2	nil	nil	**0.324**
	8 hours	32	3	nil	nil	
	10 hours	33	2	nil	nil	**<0.0001**
	7 hours	13	22	nil	nil	

* Wilcoxon signed rank test

This table also shows there is no significant difference between tissues fixed in 10 and 8 hours. So fixative 2 also gives optimal fixation at 10 and 8 hours.

There is significant difference between 10 and 7 hours fixation. This indicates 7 hours fixation results in suboptimal fixation.

Comparison of fixation time and nuclear details in fixative 3:

On comparing nuclear features, more than 20 cases received score 2 in all the three fixation hours because of nuclear shrinkage and poorly defined chromatin pattern. Few cases got score 1, as they showed even more nuclear shrinkage and poorly defined chromatin and mitotic figure details.

TABLE 25: Fixation time and nuclear details in fixative 3

	TIME	SCORE				P value*
		3	2	1	0	
No. of Specimens	10 hours	6	22	2	nil	1.000
	8 hours	6	23	1	nil	
	10 hours	6	22	2	nil	0.008
	7 hours	2	22	6	nil	

* Wilcoxon signed rank test

The above table shows there is no significant difference between 10 and 8 hours fixation and 10 and 7 hours fixation. This indicates fixative 3 gives nearly equal results in all three different fixation times.

FIXATION TIME AND CYTOPLASMIC FEATURES:

Cytoplasmic features were assessed by colour of cytoplasm, abundance, cytoplasmic granules and mucin differentiation.

Comparison of fixation time and cytoplasmic details in fixative 1:

On comparing cytoplasmic details between 10 and 8 hours 32 cases fixed in fixative 1 received score 3. At 7 hours fixation more than half of the cases (25) got score 2 because of cytoplasmic shrinkage.

TABLE 26: Fixation time and cytoplasmic details in fixative 1

	TIME	**SCORE**				**P value***
		3	**2**	**1**	**0**	
No. of Specimens	10 hours	**32**	**3**	**Nil**	**nil**	**n/a**
	8 hours	**32**	**3**	**Nil**	**nil**	
	10 hours	**32**	**3**	**Nil**	**nil**	**<0.0001**
	7 hours	**10**	**25**	**Nil**	**nil**	

* Wilcoxon signed rank test

This table shows fixative 1 scored equal number of maximum score at 10 and 8 hours fixation. So p value is not available, both fixation hours produce similar results. There is significant difference between 10 and 7 hours. Tissues fixed in 10 hours fixation gives better cytoplasmic details than 7 hours fixation.

Comparison of fixation time and cytoplasmic details in fixative 2:

On comparing cytoplasmic details between 10 and 8 hours more than 30 cases fixed in fixative 2 received score 3. At 7 hours fixation more than half of the cases (27) got score 2 because of cytoplasmic shrinkage.

TABLE 27:Fixation time and cytoplasmic details in fixative 2

	TIME	**SCORE**				**P value***
		3	**2**	**1**	**0**	
No. of Specimens	10 hours	33	2	Nil	nil	**0.324**
	8 hours	32	3	Nil	nil	
	10 hours	33	2	Nil	nil	**<0.0001**
	7 hours	13	22	Nil	nil	

* Wilcoxon signed rank test

There is no significant difference between 10 and 8 hours fixation. Between 10 and 7 hours, there is significant difference. This indicates optimal fixation achieved at 8 hours fixation in fixative 2.

Comparison of fixation time and cytoplasmic details in fixative 3:

On comparing cytoplasmic features, more than 20 cases received score 2 in all the three fixation hours because of cytoplasmic shrinkage. Few cases got score 1, as they showed even more cytoplasmic shrinkage and poorly defined cytoplasmic granules.

TABLE 28: Fixation time and cytoplasmic details in fixative 3

	TIME	SCORE				P value*
		3	2	1	0	
No. of Specimens	10 hours	5	18	7	nil	**0.414**
	8 hours	5	21	4	nil	
	10 hours	5	18	7	nil	**n/a**
	7 hours	5	18	7	nil	

* Wilcoxon signed rank test

The above shows there is no significant difference between 10 and 8 hours fixation. And received similar scores in both 10 and 7 hours fixation. This indicates fixative 3 gives nearly equal results in all three different fixation times.

FIXATION TIME AND ARCHITECTURAL FEATURES:

Shrinkage artifacts, distortion, cracking and formalin pigments were included to assess architecture**.**

Comparison of fixation time and architectural features in fixative 1:

Architecture features of tissues fixed in fixative 1 were compared between 10 and 8 hours and between 10 and 7 hours. Both 10 and 8 hours fixation, 33 specimens got score 3. At 7 hours , 22 cases got score 2 because they showed shrinkage artifact, distortion and cracking.

TABLE 29: Fixation time and architectural features in fixative 1

	TIME	**SCORE**				**P value***
		3	**2**	**1**	**0**	
No. of Specimens	10 hours	33	2	nil	nil	**1.000**
	8 hours	33	2	nil	nil	
	10 hours	33	2	nil	nil	**0.000**
	7 hours	13	22	nil	nil	

* Wilcoxon signed rank test

The above table shows there is no significant difference between tissues fixed in 10 and 8 hours. So fixative 1 gives optimal fixation at 10 and 8 hours. There is significant difference between 10 and 7 hours fixation. This indicates 7 hours fixation results in suboptimal fixation in preserving architecture.

Comparison of fixation time and architectural features in fixative 2:

Architecture features of tissues fixed in fixative 2 were compared between 10 and 8 hours and between 10 and 7 hours. Both 10 and 8 hours fixation, 32 specimens got score 3. At 7 hours , 20 cases got score 2 because they showed shrinkage artifact, distortion and cracking.

TABLE 30: Fixation time and architectural features in fixative 2

	TIME	SCORE				P value*
		3	2	1	0	
No. of Specimens	10 hours	32	3	nil	nil	n/a
	8 hours	32	3	nil	nil	
	10 hours	32	3	nil	nil	<0.0001
	7 hours	3	20	12	nil	

*Wilcoxon signed rank test

The above table shows fixative 2 scored equal number of maximum score at 10 and 8 hours. So p value is not available , fixative 2 produces similar results in both fixation hours. There is significant difference between 10 and 7 hours. Tissues fixed in 10 hours fixation gives better cytoplasmic details than 7 hours fixation.

Comparison of fixation time and architectural features in fixative 3:

More than half of the tissues fixed in fixative 3 showed shrinkage artifacts and cracking and they got score 2 in 10 and 8 hours fixation. At 7 hours fixation 20 case received score 1 because they showed more shrinkage, craking and distortion.

TABLE 31: Fixation time and architectural features in fixative 3

	TIME	SCORE				P value*
		3	2	1	0	
No. of Specimens	10 hours	nil	17	13	nil	**0.739**
	8 hours	nil	19	11	nil	
	10 hours	nil	17	13	nil	**<0.0001**
	7 hours	nil	10	20	nil	

*Wilcoxon signed rank test

The above table shows there is no significant difference between 10 and 8 hours fixation. So fixative 3 gives equal results in both 10 and 8 hours fixation. There is significant difference between 10 and 7 hours fixation. This indicates tissues fixed at 10 and 8 hours give better architectural features than7 hours.

FORMALDEHYDE VAPOR IN COMPOUND FIXATIVES:

Formaldehyde vapor from the compound fixatives were qualitatively measured and compared with conventional 10% NBF by Schiff test. In this test, filter paper over beaker A (10 % NBF)started to change colour in 10 minutes and completely changed colour in 25 minutes. Whereas filter paper over beaker B (fixative 2) started to change colour in 55 minutes and completely changed in 90 minutes.

SCHIFF TEST(FIG: 15)

	EARLY CHANGE	**COMPLETE CHANGE**
Beaker A	10 minutes	25 minutes
Beaker B	55 minutes	90 minutes

This clearly documents the fact that formaldehyde vapor from fixative 2 is comparatively minimal in contrast to conventional 10% formalin.

FIG 3: COMPOUND FIXATIVE

FIXATIVE 1, 10 HOURS

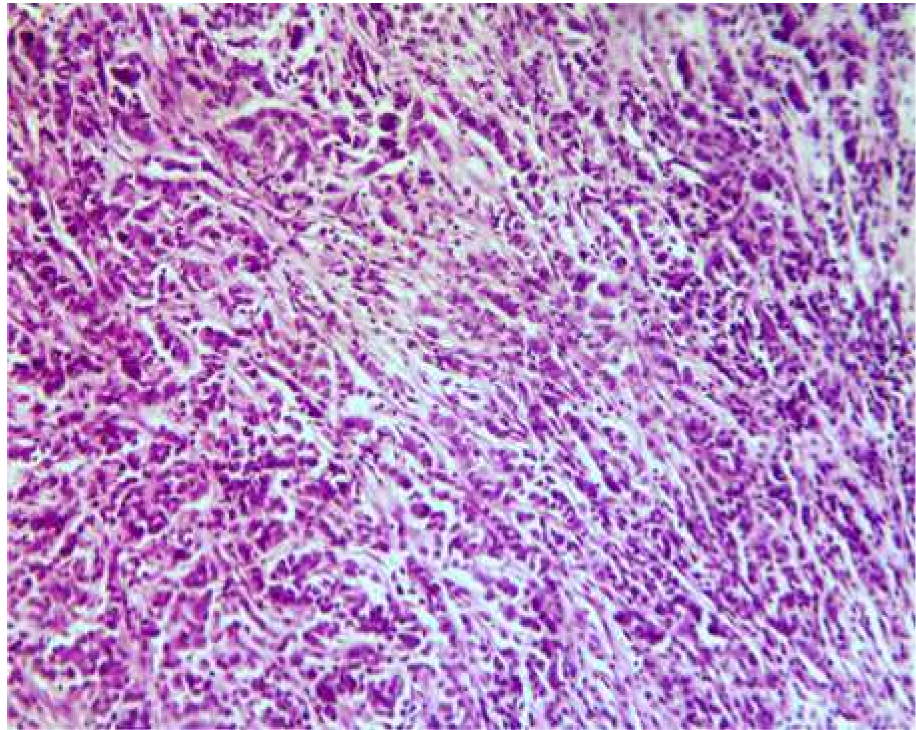


FIG 4a: Invasive ductal carcinoma breast, H&E, (10x)

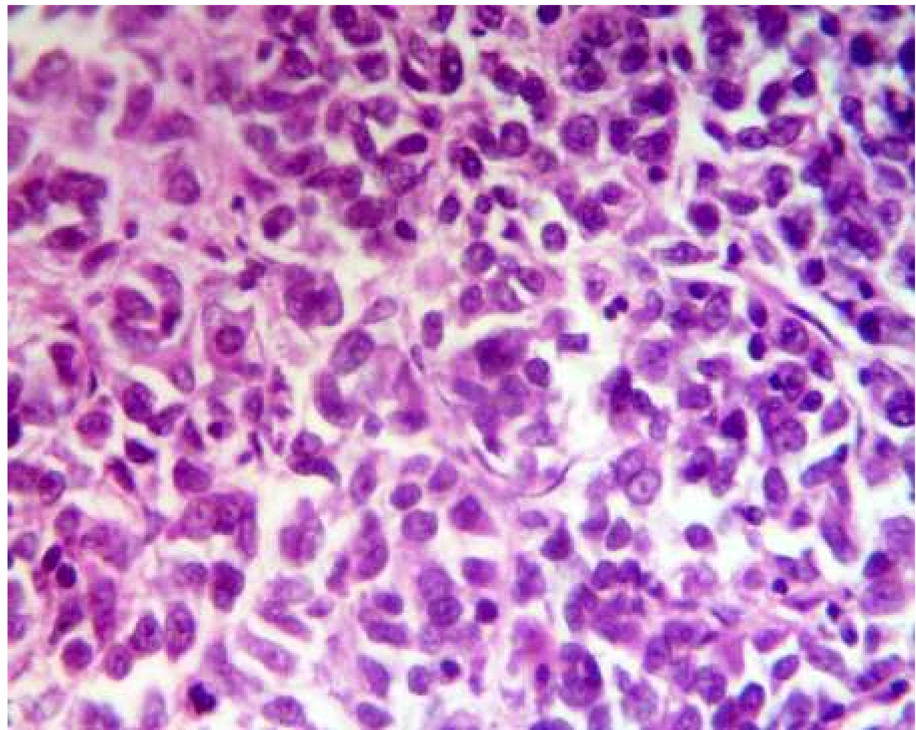


FIG 4b: Invaive ductal carcinoma breast, H&E (40x)

FIXATIVE 1, 8 HOURS

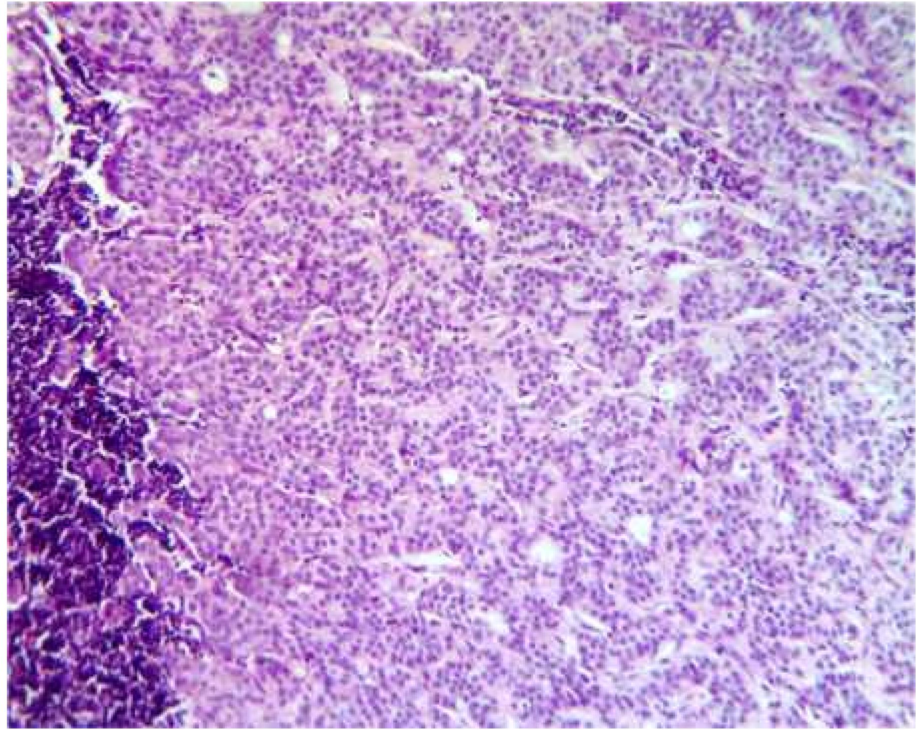


FIG 5a: Metastatic carcinomatous deposits lymph node, H&E , (10x)

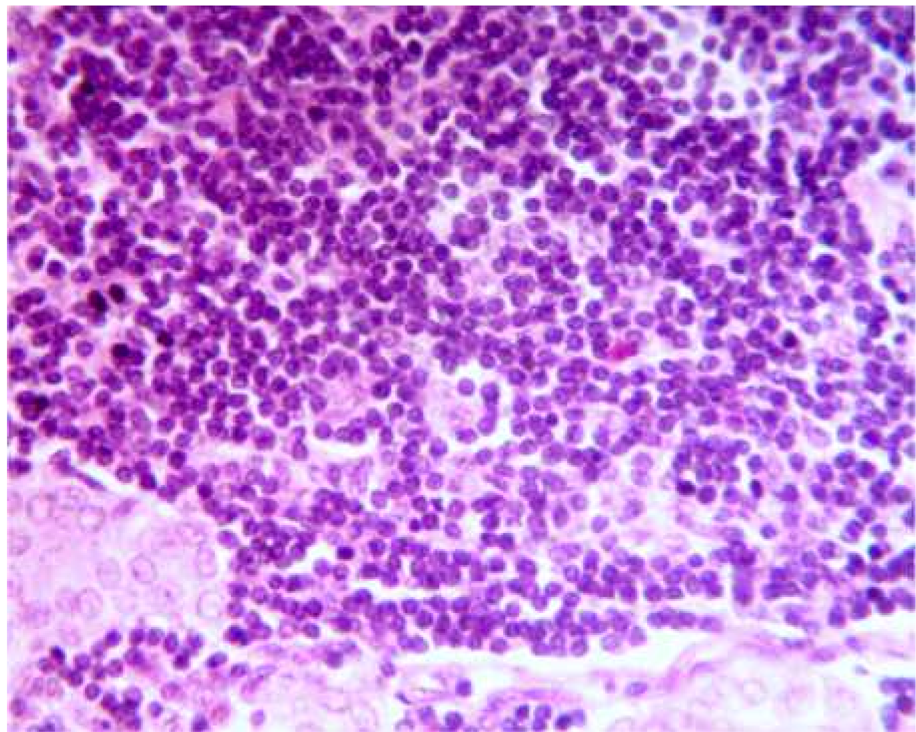


FIG 5b: Metastatic carcinomatous deposits node, H&E, (40x)

FIXATIVE 2, 10 HOURS

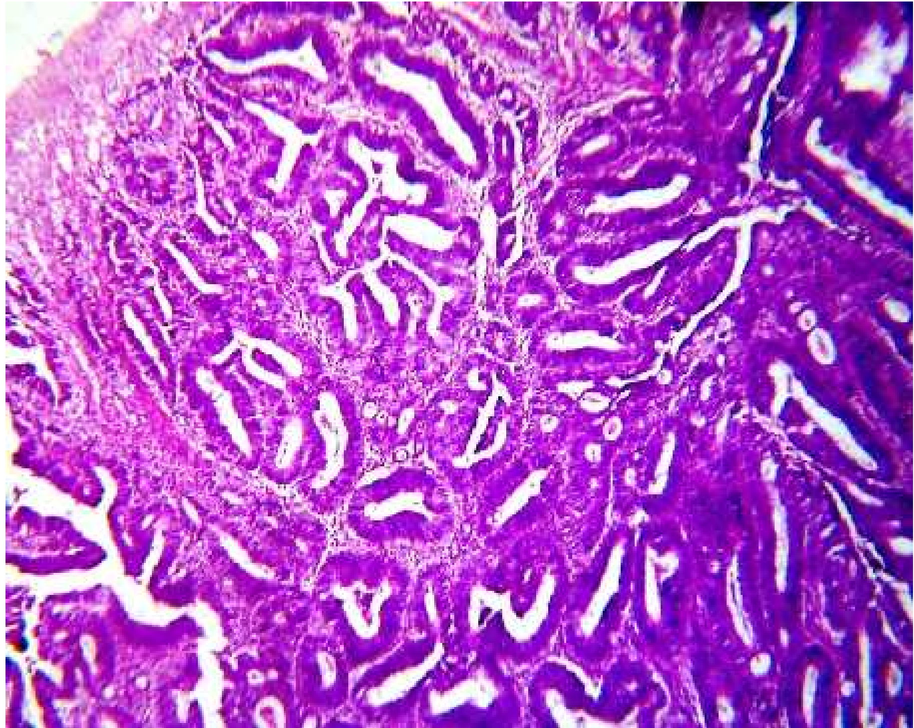


FIG 6a: Well differentiated adenocarcinoma colon, H&E, (10x)

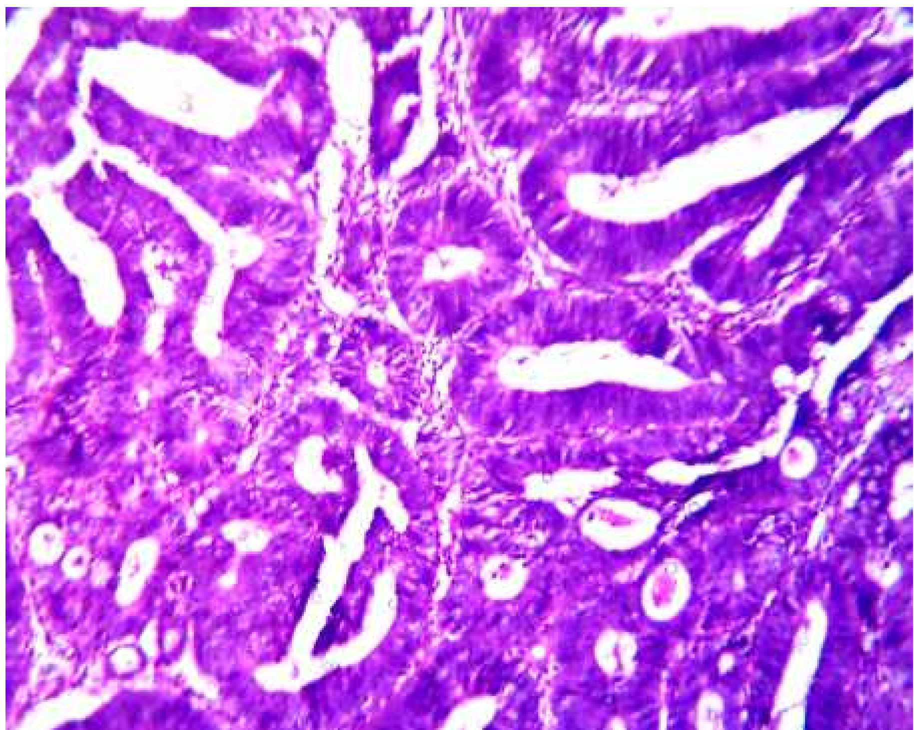


FIG 6b: Well differentiated adenocarcinoma colon, H&E (40x)

FIXATIVE 2, 10 HOURS

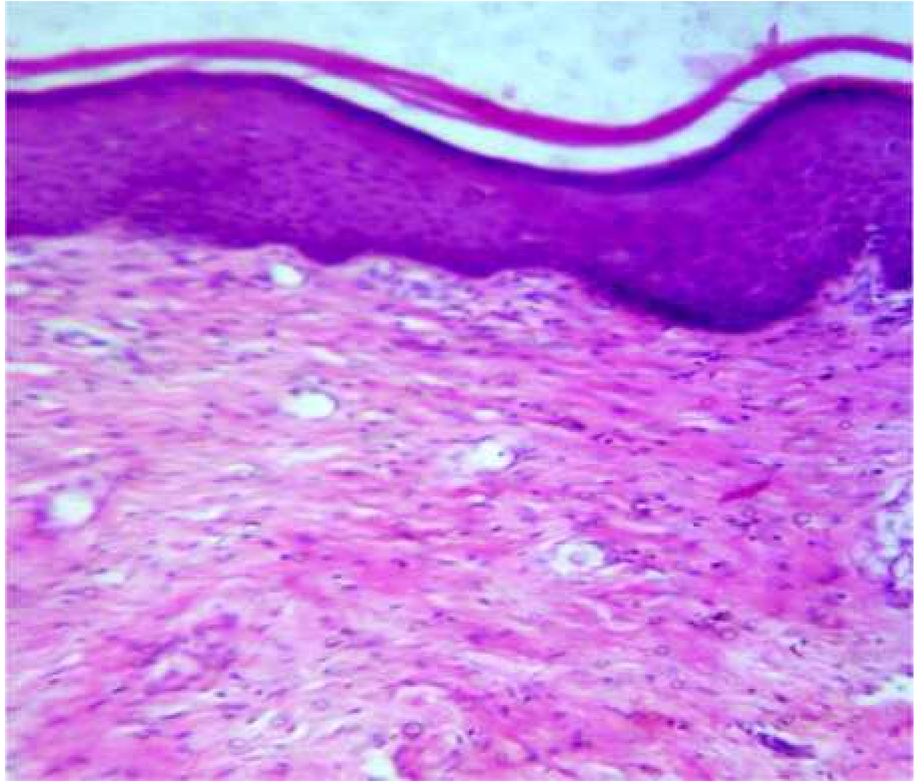


FIG 7: Ectocervix, H&E, (10x)

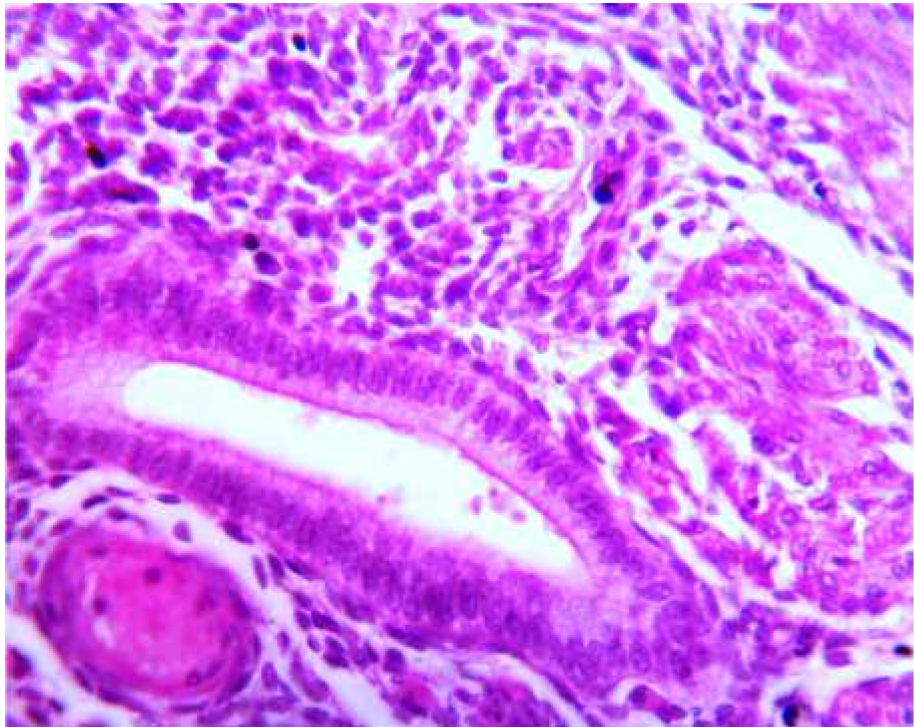


FIG 8: Proliferative endometrium, H&E (40x)

FIXATIVE 2, 8 HOURS

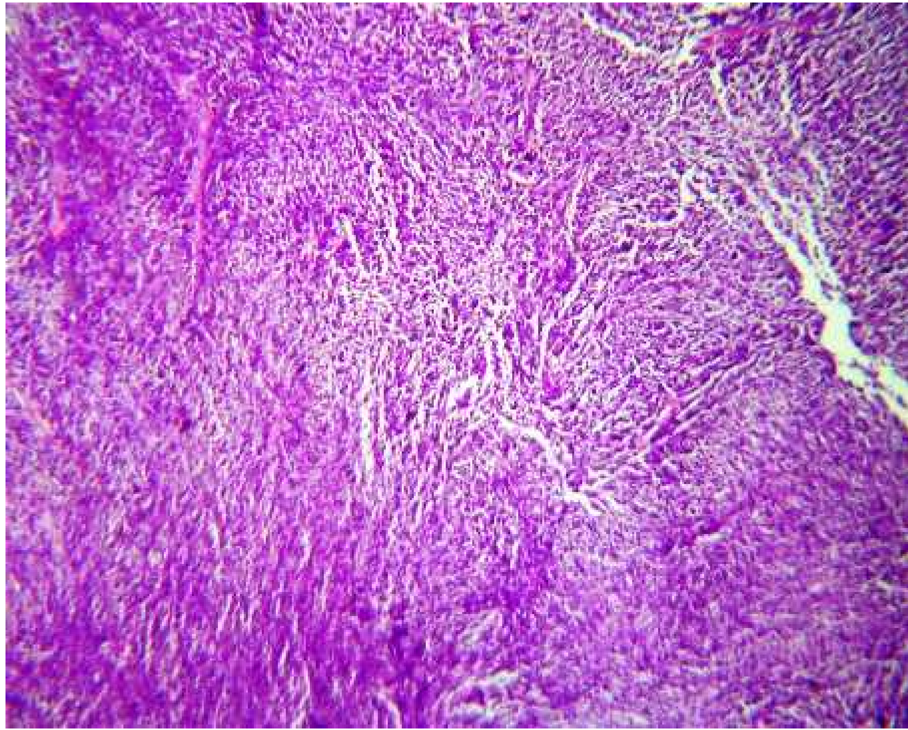


FIG 9a:Undifferentiated sarcoma, H&E ,(10x)

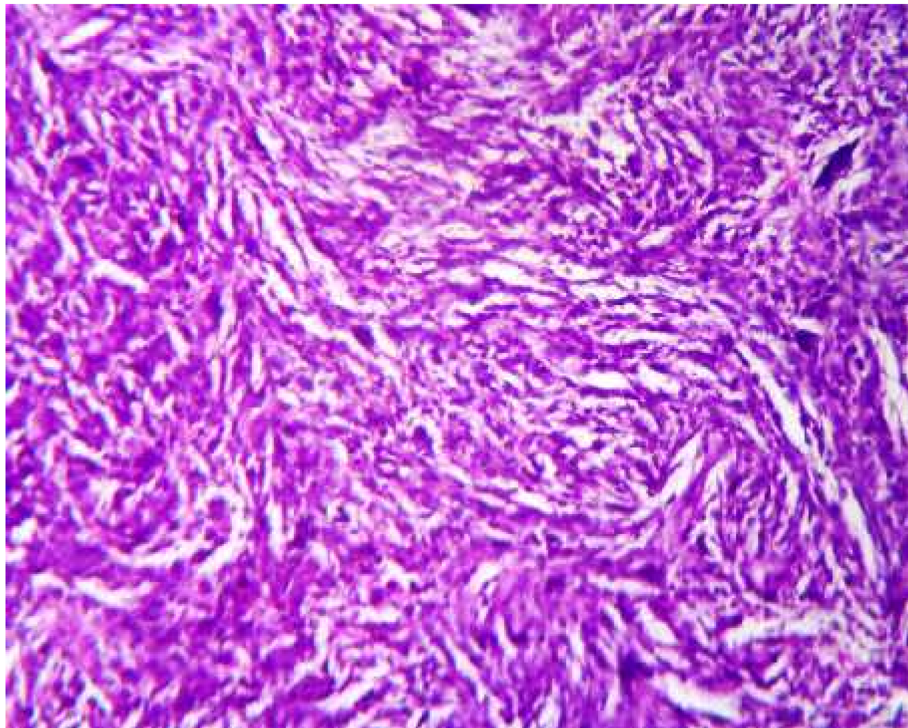


FIG 9b: Undifferentiated sarcoma , H&E (40x)

FIXATIVE 2, 8 HOURS

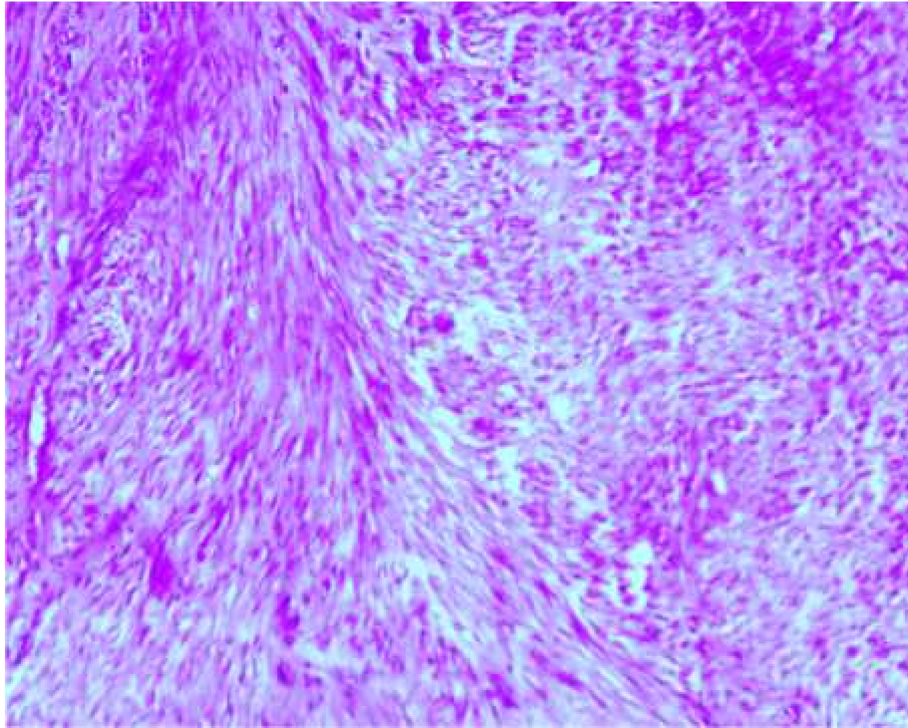


Fig 10a :Leiomyoma uterus, H&E, (10x)

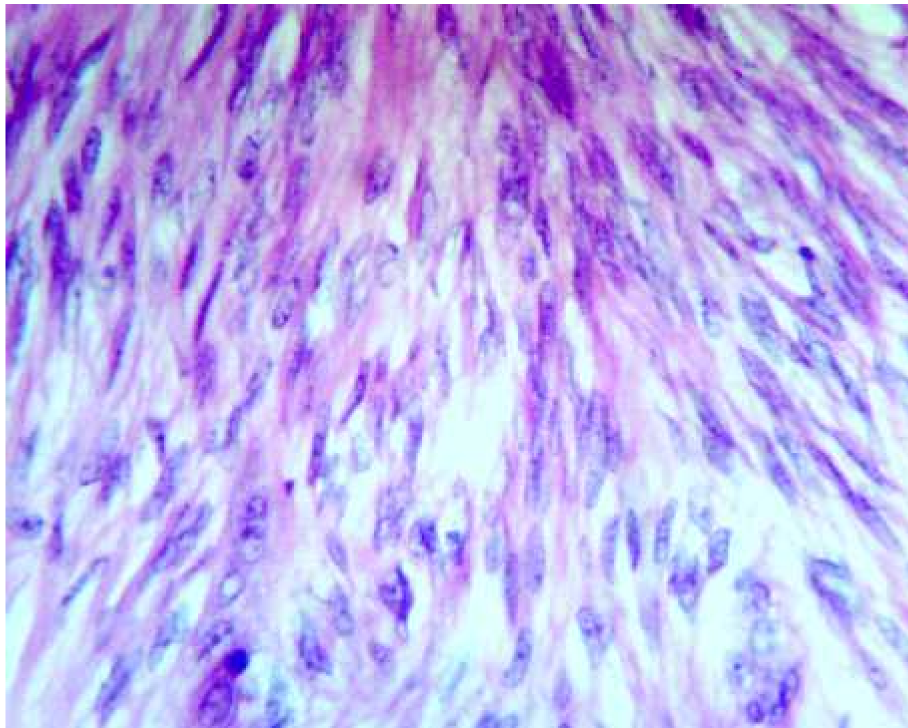


Fig 10b :Leiomyoma uterus, H&E, (40x)

FIXATIVE 2, 7 HOURS

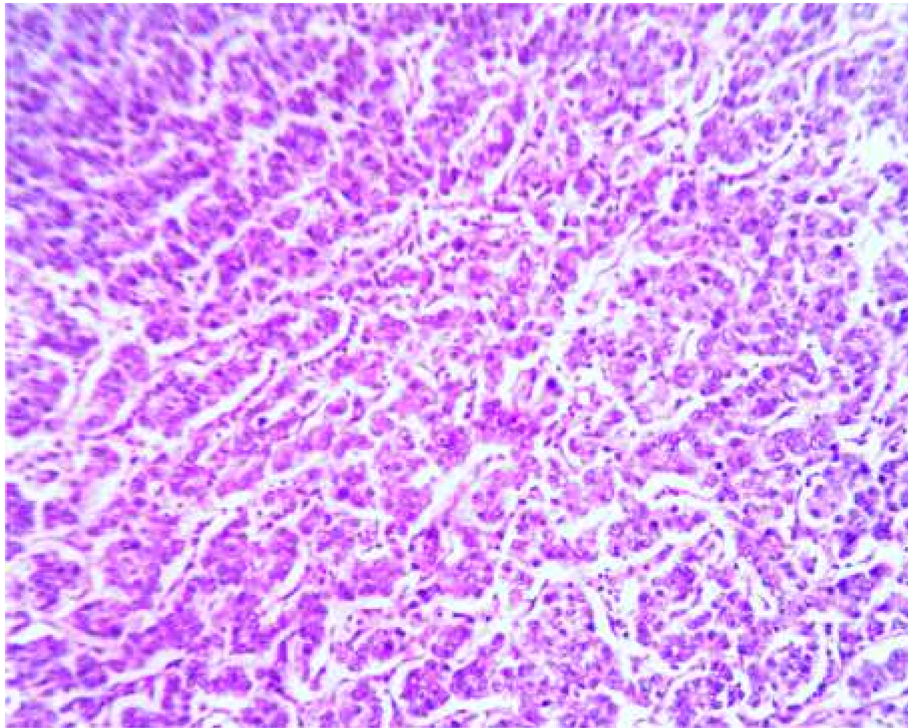


Fig 11a :Dysgerminoma ovary H&E (10x)

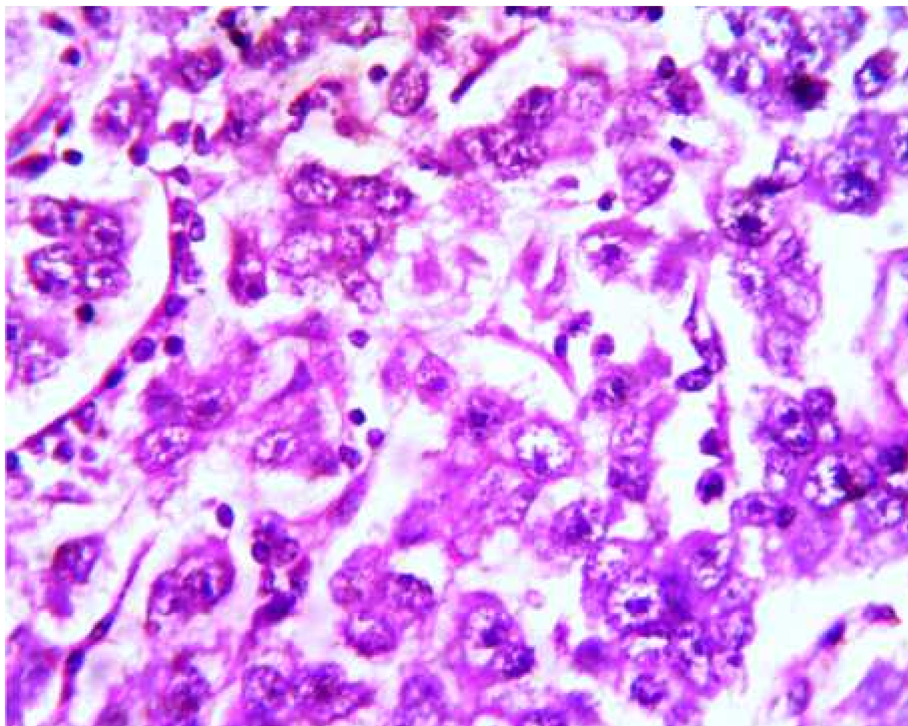


Fig 11b:Dysgerminoma ovary H&E (40x)

FIXATIVE 2, 7 HOURS

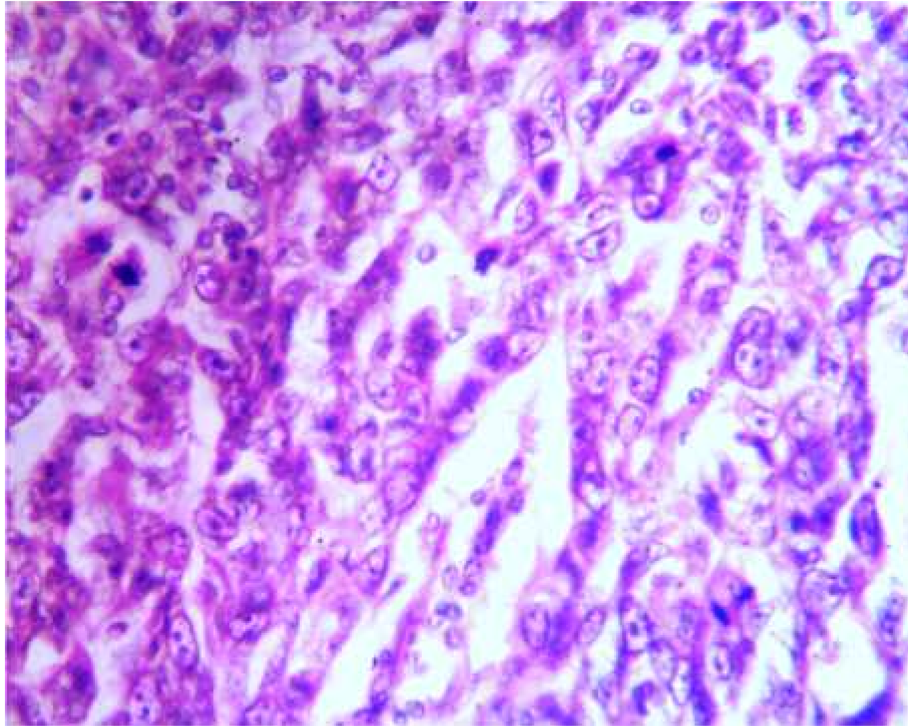


FIG12: Metastatic carcinomatous deposits node, H&E (10x)

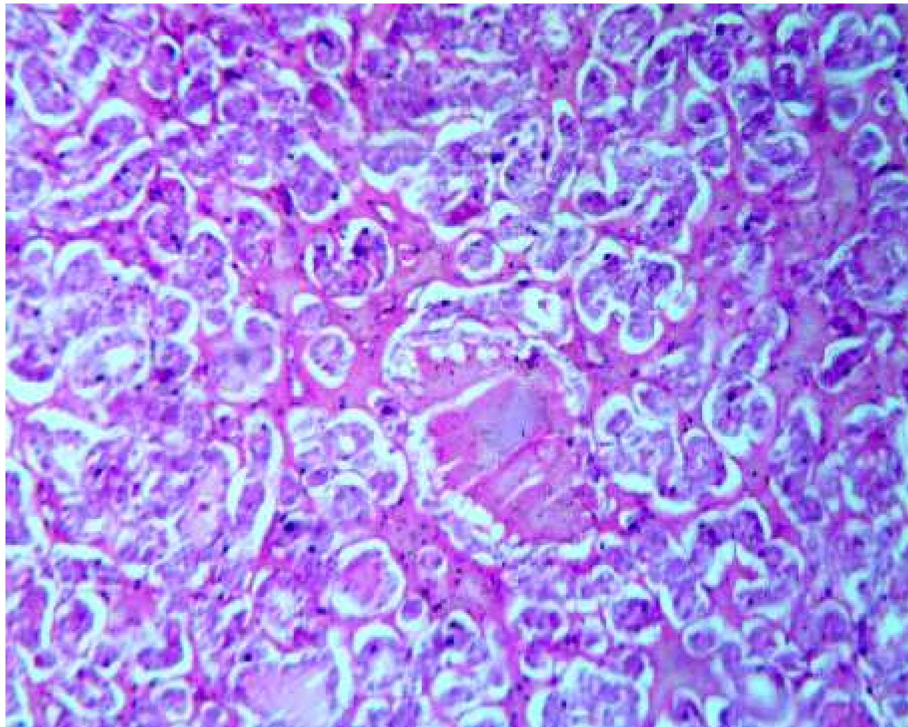


FIG 13:Nodular colloid goiter, H&E (10x)

FIXATIVE 3, 8 HOURS

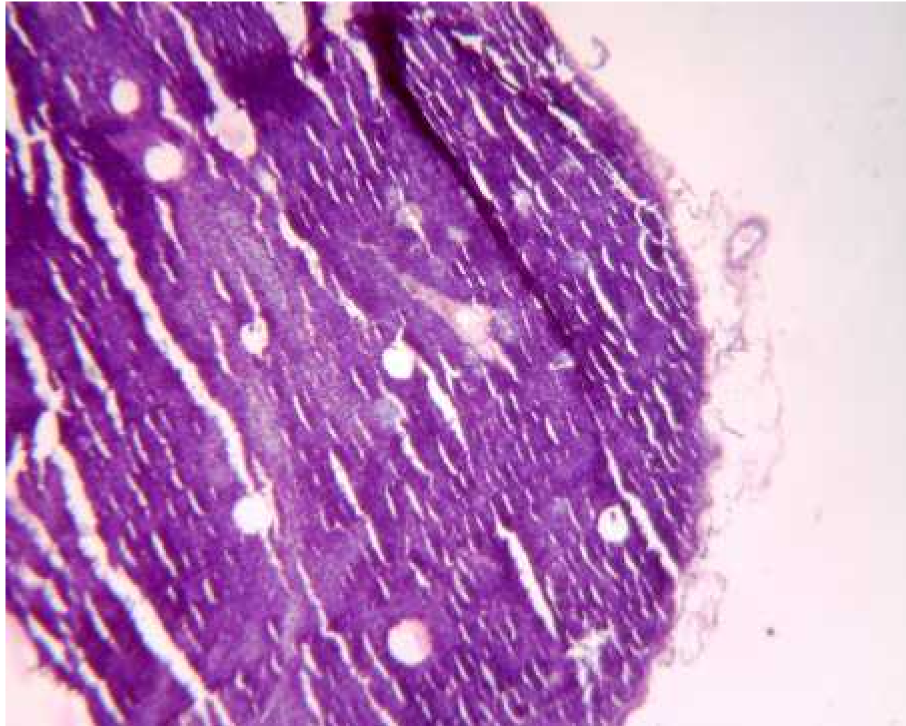


FIG 14 a: Reactive hyperplasia node, H&E,(10x)

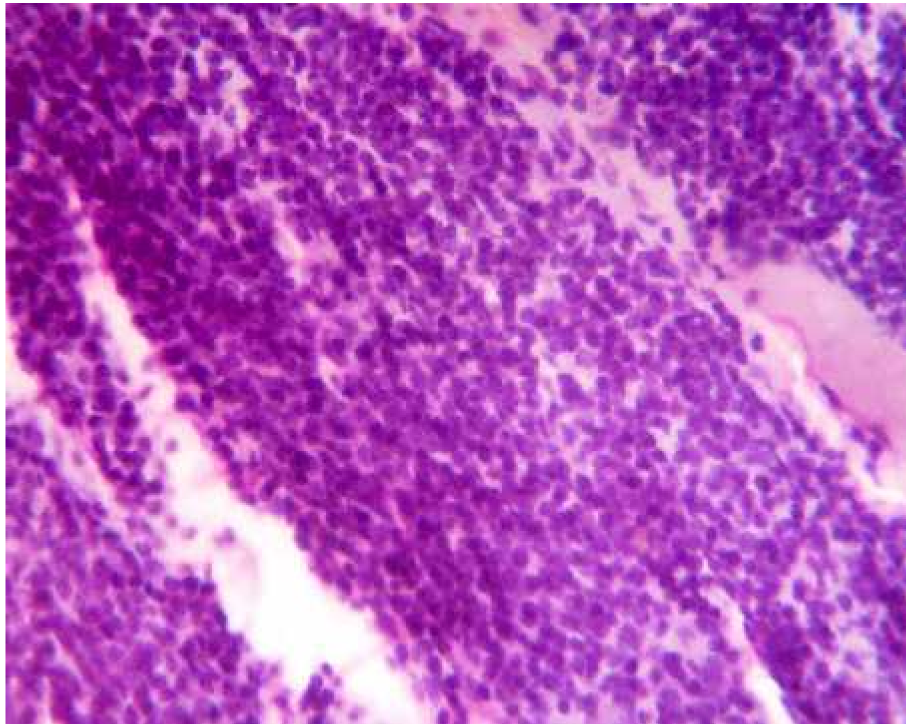


FIG 14 b: Reactive hyperplasia node , H&E,(40x)

FIG 15: Schiff aldehyde detection test -at 55 minutes

Beaker A- 10% Neutral buffered formalin

Beaker B- Fixative 2

DISCUSSION

Fixation is a very important step in histopathological analysis as it preserves tissues in a life like manner. Formalin is considered the gold standard fixative and has been used for over 100 years. It is widely used for preserving the structure, antigenicity and molecular characteristics of most types of tissues, and is accepted by most pathologists following standardization of protocols. But the role of FFPE (formalin fixed paraffin embedded) tissues in molecular diagnostics is controversial. Many studies demonstrated that FFPE tissues gave low yield of DNA and RNA than other alternative fixatives.

Another fact under consideration is that pathology laboratories use huge quantities of formalin and often do not give due importance to its toxic hazards. Technicians and pathologists are constantly exposed to a dilute solution of formaldehyde and its vapor. As the exposure occurs every day, the role of formaldehyde as a chemical carcinogen must be given due consideration [63,65,66].Over the past 20 years, many pathology laboratories have tried to replace formalin with other less toxic alternatives, but the results obtained have not been satisfactory, due to factors like alterations in cellular structure and antigenicity[67]. Hence it is necessary to search for an alternative to formalin fixation. This alternative fixative should offer better performance and has to be safer for health workers [68].

In present study we have tried to minimize formalin exposure in histopathology laboratory by reducing formalin concentration.We have included 100 specimens and three different fixatives. 35 specimens were fixed in fixative

1, another 35 were fixed in fixative 2 and 30 specimens were fixed in fixative 3 at 3 different fixation times. In fixative 1 formalin concentration was reduced from 10 to 7%. Alcohol concentration was 20%. To minimize the evaporation of absolute ethanol, we have added glycerol. Ethanol is a dehydrant coagulative fixative, it removes water molecules from tissues leads to shrinkage of cells. To overcome this defect, we have added 0.7% hypotonic saline. Methylene blue was added to monitor spillage and contamination of subsequent dehydrants in processing. The pH of the solution was maintained under 7 to 7.2 by adding sodium dihydrogen phosphate monohydrate and anhydrous disodium hydrogen phosphate. In fixative 2 formalin concentration was further reduced to 6%. Ethanol concentration was increased from 20 to 30%. Fixative 3, ethanol concentration was further increased to 40%. Formalin concentration was reduced to 5 %. Glycerin and methylene blue concentration was similar in all the three fixatives. In our study, we have analyzed the fixation characteristics and cytomorphological features of minimal formalin containing compound fixatives.

The study by Cathy.B.Moelans et al found that tissues fixed in Finefix and RCL2 were to be paler when compared to specimens fixed with NBF [3]. The study by Cristina Zanini et al showed that tissues fixed in PAGA, ZBF, Z7, RCL2 and CellBlock (alternative fixatives) do not change the color in in a similar manner as formalin[1]. Tissues fixed in our fixatives were light blue in colour and it does not interfere with macroscopic analysis.. Another factor is that the odour associated with compound fixatives is less irritant than formalin.

Regarding texture of tissues, Cathy.B.Moelans et.al reported that tissues fixed using F-solv and Finefix were found to be more rigid, and tissue fixed in RCL2 was much softer and comparatively more slippery [3]. Tissues fixed in our compound fixative is same as formalin fixed tissues.

Cristina Zanini et al found that tissues fixed in alternative fixatives were suitable for microtomy[1,40]. Cathy.B.Moelans et al reported that tissue fixed using RCL2 were softer and slippery, making cutting difficult[3]. Tissues fixed by using compound fixatives are suitable for microtomy. There is no difficulty in cutting.

Rate of fixation time depends on the rate at which diffusion of fixative into the tissue occurs and the rate at which chemical reactions with various components occurs [70]. The study by Cathy.B.Moleans et al, penetration speed of alcohol based fixatives was found to be faster than 10% NBF[1]. In the present study, fixative 1 shows there is no significant difference between tissues fixed at 10 and 8 hours fixation in all three morphological features (nuclear, cytoplasm and architectural details). But there is significant difference between tissues fixed at 10 and 7 hours fixation in all three parameters. Fixative 2 also shows there is no significant difference between tissues fixed at 10 and 8 hours fixation. But there is significant difference between tissues fixed at 10 and 7 hours fixation in all three parameters. This indicates 7 hours fixation is inadequate. 8 hours fixation in both fixative 1 and 2 is optimum for histopathological evaluation.

In the study by Cristina Zanini et.al, nuclear features were better preserved in alcohol based fixatives[1]. L. Benerini Gatta et al – Bouin fixative showed higher resolution in the nucleus[4]. In the study by Cathy.B.Moelans et al demonstrated

highest score for nuclear and cytoarchitectural features tissues fixed in NBF and lowest for FineFIX[3]. On comparing nuclear features of fixative 1 and 10% NBF, the present study indicates that there is no significant difference between them at 10 and 8 hours fixation. So both are equal in preserving nuclear features. Tissues fixed with fixative 2 also compared with conventional formalin fixed tissues. And there is no significant difference between them at 10 and 8 hours. So fixative 2 is comparable with conventional formalin in nuclear details preservation. On comparison between fixative 1 and 2, there is also no significant difference. Both fixatives 1and 2 are comparable with conventional formalin fixation. Nucleolus, chromatin and mitotic figures are better demonstrated in these compound fixatives (Fig. 4b,11b, 12). Table 7 shows there is significant difference between 10% NBF fixed tissues and fixative 3. Comparison between fixative 1 and 3, and fixative 2 and 3 also shows significant difference. So fixative 3 is poor in nuclear details preservation. Tissues fixed in fixative 3 nuclear shrinkage, especially lymphoreticular system (Fig. 14a and 14b).

The study by L. Benerini Gatta et al found that there was no differences in cytoplasmic and nuclear morphology between alternative fixatives and formalin [4]. In the study by Mahdiieh Ghoddosi et al, RCL2 fixed tissues got slightly better score for cytoplasmic features [40]. Regarding cytoplasmic features, the present study demonstrates that there is no significant difference between fixative 1 and 10% NBF. And also there is no significant difference between fixative 2 and 10% NBF. Cytoplasmic features also compared between fixative 1 and 2 that results no significant difference. So both fixatives are comparable with conventional

formalin in cytoplasmic features preservation. There is significant difference between fixative 3 and 10% NBF. So fixative 3 is inferior to conventional formalin and our compound fixatives 1 and 2 in preserving cytoplasmic features.

In the study Cristina Zainini et al, alcohol based fixatives showed shrinkage artifacts especially when concentration of alcohol is more than 50%. Fixatives containing zinc also had shrinkage artifacts[1]. In the study by Mahdiieh Ghoddosi et al, more than 90% cases in their study receive good score morphological features. On comparing architectural features, our study found that there is no significant difference between fixative 1 and conventional formalin at 10 and 8 hours fixation. Fixative 2 also shows that there is no significant difference at 10 and 8 hours fixation when compared with conventional formalin. There is significant difference between fixative 3 and NBF. From these findings we have found that fixative 1 and 2 are as good as conventional formalin in architecture preservation. Fixative 3 is poor in preservation of architecture. Tissues fixed in fixative 3 especially lymphoreticular system show shrinkage artifacts (Fig.14a and 14b). Thyroid specimens also showed significant retraction artifacts (Fig 13).This study also shows formalin pigments when the prepared solutions were stored more than 10 days (Fig.13).The present study also demonstrates more than 90% specimens of fixative 1 and 2 have been received maximum score for cytoarchitectural features.

Even though the present study demonstrates that both fixatives 1 and 2 result in good morphological features comparable with conventional 24 hours formalin fixation, fixative 2 is better than 1 because it gives good results with

minimal formalin concentration (6%). Fixative 2 is found to evaporate less formaldehyde vapor than 10% NBF and fixation time is reduced upto 8 hours.

SUMMARY AND CONCLUSION

This study was conducted in Department of Pathology, Tirunelveli Medical College as a pilot study in order to minimize carcinogenic formalin exposure in histopathology laboratory. It includes 100 specimens fixed in three minimal formalin containing compound fixatives and 3 different fixation times. Compound fixatives are composed of ethanol, formalin, hypotonic saline and glycerin. Hypotonic saline was added to overcome cell shrinkage.Tissues fixed in compound fixatives are light blue in colour and it do not interfere with histopathology diagnosis. There is no difficulty in cutting.

Nucleus and cytoplasmic features are well preserved and compared with conventional 10% NBF both in fixative 1and 2. Most of the tissues fixed in both fixative 1and 2 [>90%] scored maximum score of 3 for cytoarchitectural features. Architectural features show very minimal structural distortion in selective group of tissues compared to conventional formalin. Tissues fixed in fixative 3 especially lymphoreticular system show marked cell shrinkage. So fixative 3 is not suitable routine histopathology evaluation. Fixation time is reduced upto 8 hours.

With regard to cytoarchitectural features preservation, both fixatives 1 and 2 gives comparably similar results as a conventional formalin fixation. However Fixative 2 gives an optimal fixation at a minimized formalin concentration (6%) than fixative 1 (7%) and at a reduced fixation time of 8 hours . Qualitative measurement of formaldehyde vapor in fixative 2 is considerably less compared to conventional 10% NBF. This enables reduced formalin exposure in histopathology laboratory.

As formaldehyde is a group 1 human carcinogen[2] , it should be replaced by less toxic fixatives in histopathology laboratory. The present study demonstrates that minimal formalin containing fixatives can be easily prepared in the laboratory and they are suitable for histopathological examination of routine surgical specimens. In this study we have taken into account only the histomorphological features of H & E stained sections. Tissue characteristics in special histochemical and immunohistochemistry reactions were not taken into account. However for a routine diagnostic histopathology using H & E stain, the effectiveness of these compound fixatives are comparable to conventional formalin fixation with an improved air quality of the working laboratory and considerably reduced formalin vapor density.

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S. No	HPE NO	SPECIMEN	DIAGNOSIS	FIXATIVE 3									CONVENTIONAL 10% NBF		
				NUCLEUS			CYTOPLASM			ARCHITECTURE			Nucleus	Cytoplasm	Architecture
				10 Hrs	8 Hrs	7 Hrs	10 Hrs	8 Hrs	7 Hrs	10 Hrs	8 Hrs	7 Hrs			
1	808/15	Breast	Invasive ductal carcinoma	3	3	3	3	3	3	2	2	2	3	3	3
2	2508/15	Thyroid	hashimoto's thyroiditis	2	2	2	2	2	2	1	1	2	3	3	3
3	2515/15	Thyroid	microfollicular adenoma	2	2	2	1	2	2	1	1	1	3	3	3
4	2545/15	Thyroid	colloid goitre	2	2	2	2	1	1	1	1	1	3	3	3
5	2547/15	Breast	invasive ductal carcinoma	3	3	2	3	3	3	2	2	2	3	3	3
6	2626/15	GIT	Mucinous adenocarcinoma	2	2	3	2	2	2	2	1	1	3	3	3
7	2483/15	Thyroid	follicular adenoma	2	2	2	2	2	2	2	1	2	3	3	3
8	1495/16	Soft tissue	lipoma	2	2	2	2	2	2	2	2	2	3	3	3
9	2721/15	Breast	Invasive ductal carcinoma	2	2	2	2	2	2	2	2	2	3	3	3
10	2895/15	Testis	Teratocarcinoma	2	2	1	2	2	1	1	2	1	3	3	3
11	3092/15	Breast	Invasive ductal carcinoma	3	3	2	3	3	3	2	2	1	3	3	3
12	3093/15	GIT	Adenocarcinoma	2	2	2	2	2	2	1	2	1	3	3	3
13	3206/15	Thyroid	Nodular colloid goitre	2	2	2	1	1	1	1	1	1	3	3	3
14	3213/15	Soft tissue	Low grade fibrosarcoma	2	2	1	1	1	1	2	1	1	3	3	3
15	3238/15	Kidney	Renal cell carcinoma	2	2	2	2	2	2	2	2	1	3	3	3
16	3275/15	Node	Reactive hyperplasia	1	1	1	1	1	1	1	1	1	3	3	3
17	238/16	Breast	Invasive ductal carcinoma	3	3	2	3	2	2	1	1	1	3	3	3
18	1006/16	Soft tissue	Liposarcoma	2	2	2	2	2	1	1	2	1	3	3	3
19	1134/16	Lung	Aspergilloma	2	2	2	1	2	2	1	2	2	3	3	3
20	1534/16	Uterus	Proliferative endometrium	2	2	2	2	2	1	2	2	1	3	3	3
21	1821/16	Thyroid	Trabecular adenoma	3	3	2	3	3	3	2	2	1	3	3	3
22	gy1634/15	Uterus	Leiomyoma	2	2	2	2	2	2	2	2	2	3	3	3
23	gy2042/15	Uterus	Proliferative endometrium	2	2	2	1	2	2	1	1	1	3	3	3
24	gy941/16	Cervix	Chronic cervicitis	3	3	2	3	3	3	2	2	1	3	3	3
25	gy1039/16	Uterus	Leiomyoma	2	2	2	2	2	2	2	2	2	3	3	3

S. No	HPE NO	SPECIMEN	DIAGNOSIS	FIXATIVE 3									CONVENTIONAL 10% NBF		
				NUCLEUS			CYTOPLASM			ARCHITECTURE			Nucleus	Cytoplasm	Architecture
				10 Hrs	8 Hrs	7 Hrs	10 Hrs	8 Hrs	7 Hrs	10 Hrs	8 Hrs	7 Hrs			
26	gy1148/16	Uterus	Proliferative endometrium with leiomyoma	2	2	1	2	2	2	1	2	1	3	3	3
27	gy1189/16	Uterus	Proliferative endometrium	2	2	1	1	2	1	1	1	1	3	3	3
28	gy1190/16	Uterus	Leiomyoma	2	2	2	2	2	2	2	2	2	3	3	3
29	gy1323/15	Uterus	Secretory endometrium	2	2	1	2	2	2	2	2	1	3	3	3
30	gy478/16	Uterus	Leiomyoma	2	2	1	2	2	2	2	2	1	3	3	3