A COMPARATIVE ASSESSMENT OF TISSUE FIXATION PROPERTIES OF SELECTED EXPERIMENTAL FORMALIN BASED FIXATIVES IN AHMADU BELLO UNIVERSITY TEACHING HOSPITAL, ZARIA.

BY

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BY

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DECLARATION

I declare that the work in this dissertation entitled: **A Comparative Assessment of Tissue Fixation Properties of Selected Experimental Formalin Based Fixatives in Ahmadu Bello University Teaching Hospital, Zaria** has been carried out by me in the Department of Pathology ABUTH Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

Name of Student	**Signature**	**Date**

CERTIFICATION

This dissertation entitled 'A Comparative Assessment of Tissue Fixation Properties of Selected Experimental Formalin Based Fixatives in ABUTH Zaria' by Fatimah Yalaraba ABDULQADIR meets the regulation governing the award of the Degree of M.Sc. Pathology (Clinical Laboratory Management) of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my parents Abdulqadir Iye Madihu (Mi Zawangi) and Aishatu Ope Zubairu (Aayi Mi Zawangi).

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ABSTRACT

This study on formalin based combination fixatives was carried out in the Department of Pathology ABUTH Zaria Nigeria a training institution located in the North West region of Nigeria that offers specialist medical services and processes about 3000 to 4000 samples yearly in its pathology laboratory. The study was aimed at identifying a potentially less toxic formalin based combination fixative as compared to the local (10% formol saline-FS) and international (10% Neutral Buffered Formalin-NBF) standards by virtue of reduction of the concentration of formaldehyde in the combinations to a range within the known functional limits of formaldehyde. The combination fixatives were prepared with less concentration of formaldehyde (Group A – A1: 8% Formol saline, A2: 6% Formol saline and A3: 4% Formol saline) and some with phenol as additive inclusive (Group B– B1: 8% Formol saline with 20g phenol crystals, B2: 6% Formol saline with 20g phenol crystals and B3: 4% Formol saline with 20g phenol crystals). Fresh tissues from *Sus domestica* were procured based on organ system, passed into freshly prepared fixatives and allowed to fix for 24hours. The tissues were then grossed and processed in the laboratory. The slides were stained with the routine stain (Haematoxyline and Eosin - H&E), special stains (PAS & MT) and immunohistochemistry-IHC (EMA & CD23). The screened slides were presented to five pathologists in a single blinded manner for microscopic histomorphologic assessment using modified Allred scoring system. The results showed that the fixative with the best performance was NBF with a percentage overall performance grade of 13.2%, followed by FS with a performance grade of 12.6%, A3 with 12.5%, the phenol containing groups i.e. B1, B2 and B3 with percentage of 12.4% each, then A2 with 12.3% and A1 the lowest grade percentage of 12.1%. These grades were a fair reflection of tissue preservation properties and staining properties of the fixatives. Individual performances based on tissue type, some of the combination fixatives performed better than the standards. The IHC performances in a

decreasing order are; EMA- B3(6.7%), B2(6.3%), B1(6.3%), NBF(5.9%), FS(5.9%), A1(5.5%), A2(5.1%). CD23- A3(6.7%), B1(6.7%), B3(6.7%), B2(6.3%), NBF(6.3%), FS(6.3%), A1(6.3%), A2(6.3%). For special stains performances in a decreasing order are; PAS- B2(7.5%), FS(6.3%), NBF(5.9%), A1(5.9%), A2(5.9%), B1(5.9%), A3(5.5%), B3(5.5%). MT- FS(7.1%), NBF(6.7%), A1(6.7%), B1(6.7%), A2(6.3%), A3(6.3%), B3(5.9%), B2(5.5%). The overall difference between the best grade and the lowest grade percentage was 1.1% i.e. less than 10% of the responses. The overall result of responses showed 3.67% (less than 10% of responses) graded the performances as suboptimal and poor while 75.63% graded the performances good and 20.70% graded it very good. The results from microscopic assessments done by the pathologists, showed virtually an unremarkable difference in the performances of the combination formalin based fixatives when compared to the local and international standards as evidenced by the hypothesis test (Chi-square and ANOVA) results and p values greater than 0.05, all supported the maintenance of the null hypothesis. Thus, it is recommended that any one of the experimental formalin based combination fixatives can be used as a potentially less toxic suitable substitution to 10% formol saline.

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LIST OF ABBREVIATIONS

ABUTH – Ahmadu Bello University Teaching Hospital
ANOVA – Analysis of variance
A1 – 8% formol saline
A2 – 6% formol saline
A3 – 4% formol saline
BRF – Bundesinstitut für Risikobewertung
B1 – 8% formol saline with phenol
B2 – 6% formol saline with phenol
B3 – 4% formol saline with phenol
CD – Cluster of differentiation
DNA – Deoxyribonucleic acid
EMA – Epithelial membrane antigen
EPA – Environmental Protection Agency
Fig. – Figure
FEF – Forced expiratory flow
FEV_1 – Forced expiratory volume
FFPET – Formalin fixed paraffin embedded
FVC – Forced vital capacity
FS – 10% Formol saline
GMS – Gomori methanamine silver
H&E – Haematoxyline and eosin
HIER – Heat induced epitope retrieval
IARC – International Agency for Research on Cancer
IBF – Trade name
IDLH – Immediately Dangerous to life and health
IHC – Immunohistochemistry
IgE – Immunoglobulin E
IgG – Immunoglobulin G
MT – Massons trichrome
NBF – 10% Neutral buffered formalin
OSHA – Occupational safety and health administration
PAS – Periodic Acid Schiff
PBS – Phosphate buffered solution
Pen Fix – Trade name
ppm – Parts per million = g/m^3
RNA – Ribonucleic acid
TWA – Time weighted average
UK – United Kingdom
US – United States
Statfix – Trade name

CHAPTER ONE

1.0. INTRODUCTION

Fixation is a process in which tissues are preserved as life state form as possible using various agents, a process that requires time to complete (David, 1996; Rosai 2011 , Fixation 2014). It should be of note that over or under fixation can be detrimental and may be the cause of inappropriate results for some assays especially under fixation (David, 1996; Fixation, 2014).

Fixation is the first stage in the process to prepare a surgical specimen sample for diagnostic microscopy. Tissue fixation must demonstrate preservation of the integrity and morphology of cells and tissues so that they can withstand the harsh conditions of further processing i.e. dehydration, clearing, embedding and staining that are performed during routine histological processes. In addition, fixation also helps prevent decomposition, putrefaction, autolysis of tissues and optimizes tissue morphology for proper microscopic evaluation (David, 1996 ; Rosai, 2011).

Once tissues are removed from the body, they begin to undergo a process of self-destruction or autolysis which is initiated soon after cell death by the action of intracellular enzymes causing the breakdown of protein and eventual liquefaction of the cell (David, 1996; Anthony, 2000). Autolysis is independent of any bacterial action, retarded by cold and greatly accelerated with increase temperature (Anthony, 2000).

Autolysis is more severe in tissues which are rich in enzymes, such as the brain and is less rapid in tissues such as elastic fibre and collagen (Anthony, 2000). By light microscopy, autolysed tissue presents a `washed-out' appearance with swelling of cytoplasm, eventually

converting to a granular, homogeneous mass which fails to take up stains. The nuclei of autolytic cells may show some of the changes of necrosis including condensation (pyknosis), fragmentation (karyorrhexis) and lysis (karyolysis) but these are not accompanied by an inflammatory or cellular response (David, 1996; Rosai, 2011; Anthony, 2000).

Bacterial decomposition can also produce changes in tissues that are similar to autolysis but it is as a result of bacterial proliferation/action in the dead tissue (Anthony, 2000). Such bacteria may be normal flora, or may be present in diseased tissues at the time of death such as in septicaemia (Anthony, 2000).

It is relevant to point out that fixation in itself can constitute a major artefact. The living cell is in fluid or in a semi-fluid state, fixation produces coagulation of tissue proteins and constituents, a necessary event to prevent their loss or diffusion during tissue processing. The passage of tissue through hypertonic and hypotonic solutions during tissue processing would actually disrupt the cells if not fixed (Anthony, 2000).

Over the years, various classifications of fixatives have been proposed, according to function (coagulants and non-coagulants), or according to their chemical nature into three general categories which include alcohol, aldehyde and heavy metal fixatives (David, 1996; Anthony, 2000; Rosai, 2011).

The current standard for proper fixation of surgical specimen, is achieved when tissue sample is immersed in a 10% neutral buffered formalin fixative at a minimum volume of 10-20 times greater than the volume of the tissue to be fixed. 10 % neutral buffered formalin has proven, over several years, to successfully diffuse through the tissue to render appropriate fixation processes (David,1996; Anthony, 2000; Rosai, 2011; Fixation, 2014; Virginia, 2014).

Formalin contains formaldehyde. Formaldehyde is a colorless, pungent gas which polymerizes slowly at room temperature. The four basic uses for formaldehyde are as a

bactericide or fungicide, in the production of resins, as intermediates in the production of chemicals, and as a component of end-use consumer items. Formaldehyde is also used as embalming fluid and in textile treating to impart wrinkle-resistance to clothing. Facilities reporting the highest exposures (i.e. 0.1 to above 1.0 ppm) to formaldehyde include those producing hardwood plywood, particle board, fiberboard and resins, foundries, laboratories and funeral services (Toxicity of formaldehyde, 2014).

Formalin (aqueous formaldehyde) is highly acutely toxic, it can cause death secondary to ingestion of as little as 30 ml. The inhalation of high concentrations of formaldehyde is also extremely dangerous, with the IDLH (Immediately Dangerous to Life and Health) concentration being 100 ppm. Accidental splash exposures into the eyes may cause blindness especially in cases where immediate flushing of the eyes with water does not occur (Toxicity of formaldehyde, 2014).

The United State Occupational Safety and Health Administration (OSHA) have established limits for the amount of formaldehyde that workers can be exposed to at their place of work. At present, the limit is at 0.75 ppm on average over an 8 hour workday (Toxicity of formaldehyde, 2014). The highest concentration that a worker can be exposed to is 2 ppm, and that can only occur over 15 minutes (Toxicity of formaldehyde, 2014). Employers must monitor formaldehyde levels and provide respirators and protective clothing as needed to limit exposure. This includes workers in any workplace where formaldehyde exposure is likely, including hair salons that use commercial hair smoothing products that release formaldehyde (Toxicity of formaldehyde, 2014).

Epidemiological studies have evaluated the relationship between formaldehyde exposure and carcinogenicity in humans. It showed that there is an increased risk of lymphohaematopoietic, nasopharyngeal, sinonosal and brain cancers in humans due to formaldehyde exposure (Carcinogenic effects of Formaldehyde, 2012) Some studies have confirmed the link of

maternal exposure to high amounts of formaldehyde during pregnancy and increased incidences of benign and malignant tumors of the gastrointestinal tract (Carcinogenic effects of Formaldehyde, 2012).

In a study by Kilburn *et al.*, 1989 done on histology technicians (280 subjects) showed reduced pulmonary function, as measured by FVC, FEV1, FEF25-75, and FEF75-85, as compared with 486 controls. The range of formaldehyde concentrations was 0.2 - 1.9 ppm, volatilized from formalin preservative solution (Tatjana and Jana, 1997).

1.1. PROBLEM STATEMENT

Formaldehyde is a potentially hazardous substance that affects personnel that work with it e.g. staff and students in the laboratory, plywood industries, end consumer products industries, e.t.c. Formaldehyde was classified as "carcinogenic to humans" by the International Agency for Research on Cancer. The cancers that have been scientifically proven to be association to exposure to formalin in man are nasopharyngeal cancers, sinonasal cancers and myeloid leukemia. In animals many other cancers are implicated. Exposure to formaldehyde causes a myriad of diseases ranging from injury to the eyes, nasal and skin irritation, to the emergence of malignancy in individuals exposed to formaldehyde and their off-springs. Formalin (aqueous formaldehyde) is highly acutely toxic, it can cause death secondary to ingestion of as little as 30ml. Accidental splash exposures into the eyes may cause blindness especially in cases where immediate flushing of the eyes with water does not occur. The therapeutic responses of cancers are limited, they are difficult to treat and pose a morbidity and mortality burden to us. Chronic and recurrent exposure to formaldehyde fumes at a concentration greater than 0.75ppm poses a significant hazard for these malignancies hence the need to develop alternative fixatives that combine the tissue fixation

properties without the attendant malignancies. This prompted studies to either eliminate, reduce or prevent exposure to formaldehyde.

1.2. JUSTIFICATION FOR THE STUDY

An optimal fixative should be non-toxic and allow for detailed morphologic analysis, high-quality special histochemical and immunohistochemical staining, and good preservation of DNA and RNA at a reasonable price (David, 1996; Rosai, 2011). Unfortunately, such a universal fixative does not exist, but it is important to still assess the advantages and drawbacks of existing fixatives (David, 1996; Cathy *et al*, 2011). With the advent of safety of laboratory personnel, formalin has been found to not only be toxic but carcinogenic to personnel in the recent time (Cathy *et al*, 2011). An exposure greater than 0.75ppm over 8hrs is prone to develop malignancies and levels greater than 1ppm causes acute diseases. In surgical pathology laboratory, neutral buffered formalin (NBF; aqueous solution of 4% buffered formaldehyde) has been the "gold standard" fixative for decades (Rosai, 2011). It is cheap, enables long-term storage of surgical material, preserves morphologic features well, allows for special histologic stains in combination with antigen retrieval and for reliable immunohistochemical analysis (David, 1996; Anthony 2000; Rosai, 2011). Increased cost of health care delivery on a background of dwindling insurance support for occupational hazards especially among laboratory workers exposed to formaldehyde has prompted a global quest for alternative less toxic fixatives. The significance of formaldehyde in the emergence of occupational malignancies varies from "probable human carcinogen" through a "definite carcinogen" to being" risk factors for nasopharyngeal cancer and leukemia" by the U.S. Environmental Protection Agency and the International Agency for Research on Cancer respectively (Anthony, 2000; Cathy *et al*, 2011). The overwhelming consensus is that Formaldehyde is a carcinogenic mutagen in high doses and therefore, represents a risk to

anyone handling the solution(Cathy *et al*, 2011; National Toxicology Program, 2011; Carcinogenic effects of Formaldehyde, 2012).

Health challenges remain a limitation to formaldehyde sustainable use in the future, prompting the need for this research which entails a comparative study between formol saline and modified formalin based combination fixatives to help find a more suitable alternative fixative which will be affordable, less toxic, very effective, easy to handle, and compatible with the routine procedures.

1.3. AIM

The aim of the study is to determine the suitability of potentially less toxic alternative experimental combination formalin based fixatives here named A and B (appendix 3) over the formol saline for routine surgical pathology procedures.

1.4. OBJECTIVES

i. To determine an alternative fixative from the experimental combination formalin fixatives A and B to formol saline.

ii. To determine the fixing abilities of the alternative combination fixatives A and B as compared to formol saline (local laboratory standard) and 10% neutral buffered formalin (international standard).

iii. To determine the most compatible of A and B fixatives with Haematoxylin and Eosin (H & E) stain.

iv. To determine the most compatible of A and B fixatives with Periodic acid schiff (PAS) and Massons Trichrome (MT).

v. To determine the most compatible of A and B fixatives with Epithelial membrane antigen (EMA) and Cluster of differentiation 23 (CD23).

1.5. RESEARCH QUESTIONS

i. Which of the experimental combination formalin fixatives A and B (in appendix 3) can be an alternative fixative to formol saline.

ii. Which of the experimental combination fixatives have fixing abilities as comparable to formol saline (local laboratory standard) and 10% neutral buffered formalin (international standard).

iii. Which of the experimental combination fixatives is the most compatible with Haematoxylin and Eosin (H & E) stain.

iv. Which of the experimental combination fixatives is the most compatible with Periodic Acid Schiff (PAS) and Massons Trichrome (MT).

v. Which of the experimental combination fixatives is the most compatible with Epithelial membrane antigen (EMA) and Cluster of differentiation 23 (CD23).

1.6. HYPOTHESIS

NULL HYPOTHESIS:

There is no significant difference between the experimental combination formalin fixatives to the formol saline used for routine surgical pathology procedures.

CHAPTER TWO

2.0. LITERATURE REVIEW

Fixation is a critical step in the preparation of histological sections by which surgical tissue samples are preserved and prevented from autolysis or putrefaction (David, 1996; Rosai, 2011; Fixation, 2014). The structure of a tissue is determined by the shapes and sizes of macromolecules (proteins and nucleic acids) in the tissue. Fixation terminates any ongoing biochemical reactions and may also increase the mechanical strength or stability of the treated tissues. The broad objective of tissue fixation is to preserve cells and tissue components as life state form as possible and to allow for the preparation of thin sections (Rosai, 2011; Nietner *et al*, 2012; Fixation, 2014). Fixatives denature proteins by coagulation, by forming additive compounds, or by a combination of coagulation and additive processes. Fixation of tissue is done for several reasons. To achieve this, several conditions usually must be met (Rosai, 2011; Nietner *et al*, 2012; Fixation, 2014).

There are generally three types of fixation process: Heat fixation, Perfusion (Fixation via blood flow) and Immersion. The latter is basically the most common method used in the laboratory, in which the tissue is immersed in fixative of volume at a minimum of 10 - 20 times greater than the volume of the tissue to be fixed. The fixative must diffuse through the tissue to fix, so tissue size and density, as well as type of fixative to be used must be considered (Rosai, 2011; Fixation, 2014).

In surgical pathology, neutral buffered formalin (NBF; aqueous solution of 4% buffered formaldehyde) has been the “gold standard” fixative for decades (Rosai, 2011). It is cheap, enables long-term storage of surgical material, preserves morphologic features well, allows for special histologic stains in combination with antigen retrieval and for reliable immunohistochemical analysis (Bancroft and Stevens, 1996; Rosai, 2011; Anthony, 2000). It

contains formaldehyde which has been found to be carcinogenic and placed under Class I substance which makes surveillance of exposed personal mandatory, and may involve Directors of Surgical Pathology Departments as well as Hospital Administrators responsible for the health of workers (Zanini *et al*, 2012).

Over the past 20 years, several laboratories tried to replace formalin with other less toxic fixatives, but the results have been unsatisfactory, owing to alterations in cellular morphology and antigenicity. The search for an alternative to formalin fixation, which offers better technical performance and greater protection for health workers, is unavoidably needed (Gatta *et al*, 2012).

Formaldehyde was discovered in 1859 by the Russian chemist Alexander M. Butlerov, since then formalin became the standard fixative for routine work, and even today, it is the fixative of choice in 81% of US histology laboratories, in almost all laboratories in the UK and in close to two thirds in the rest of the world (René, 2008). Formaldehyde came into the practice of histology during the last decade of the 19th century, it was first used as an antiseptic in a 1:10 dilution of the formaldehyde concentrate (37%-40%) aqueous solution manufactured in Germany 1891 under the trade names of Formalin or Formol (René, 2008) Neutral buffered formalin (NBF), which is a 10% solution of the concentrated formalin, buffered at pH 7 with phosphate salts to prevent its acidification when the aldehyde (methanal) oxidizes into acid (methanoic or formic acid) that can lead to the formation of "formalin pigment" in tissues or to a slower fixation rate. The German trade name Formalin was the one selected by the American chemical companies producing the concentrated solution, and the name remains in common use (René, 2008).

The quest for formaldehyde substitutes has been motivated by: the OSHA regulation standard declaring it hazardous and advocating its substitution with less dangerous chemicals. As such the large potential market involved for any successful substitute, many chemical companies

and private individuals alike have developed many new fixatives all aimed at substituting formalin (René, 2008).

The alternate fixatives are alcoholic fixatives, fixatives for nucleic acids, nonalcoholic substitutes, and fixatives with less than 10% of formalin (René, 2008). Their lower formalin contents could qualify them as "less toxic" than the regular NBF example of such studies is René, 2008 where Pen Fix and Stat Fix, which have undisclosed amounts of formalin but less than 10% was used and outcome were good, although reports of drying small biopsies, because of the alcoholic components of the fixative (René, 2008). IBF, is alcoholic with less than 3% formalin, has also been reported as producing excellent morphology in prostate biopsies, and STF has been evaluated as producing from better to comparable, to worse morphology than formalin, With undisclosed amounts of formalin (<10%) and other components, its unknown mode of action, and the associated quality problems it presented, it is no longer available.

Evidently, the four (4) commercial fixatives with less than 10% formalin are not good alternatives to NBF. It now becomes necessary to take a hard look at formalin and what would it mean to find a substitute (René, 2008).

In a blind study, formalin fixation provided the highest histomorphologic quality for tissue stained with hematoxylin-eosin and examined for diagnostic surgical pathology (Titford *et al*, 1987).

2.1. ADVANTAGES OF FORMALIN

One of the advantages of formalin is its continuous and universal use for at least 100 years and all the accumulated scientific knowledge on it. The formalin-fixed paraffin-embedded tissue (FFPET) stained with H&E is the "gold standard" and it has been said that there is no other histopathology technique that provides so much information so quickly and for such

little cost (René, 2008). Formalin preserves the secondary structure of proteins, insolubilizing them in more than 90% and even its cross linking is reversible using hypotonic buffers at 37°C for 2 days, as was done before the advent of the more recent HIER techniques using different pH buffers (René, 2008).

All blocks and slides archives, all the diagnostic images learnt during pathology training, and most of the scientific articles on histopathology are based on formalin fixed paraffin embedded tissue (FFPET) (René, 2008).

Also, formalin is readily available, cheap, fairly convenient to store, allows long-term storage, preserves lipids well, and has been accepted as the closest thing there is to the perfect fixative, with no clear "all-purpose" alternative found to date(René, 2008).

Should the use of formalin be discontinued, pathologists will have to familiarize themselves with a different set of microscopic details associated with the replacement fixatives (Titford +*et al.*, 1987)**.**

In a study by Sandra et al in 2009, all the formalin substitute fixatives used in the study provided good histomorphologic quality for the different stained thyroid tissues, but individually, some fixatives performed better for immunohistochemical and molecular biological procedures for different thyroid pathologies (Sandra *et al*, 2009).

In another study by Ozkhan et al in 2011, results suggested that even honey can be used as a safe alternative to formalin in histopathology (Ozkan *et al*, 2011).

In a study by Cathy et al in 2011, alcohol-based fixatives had positive and negative attributes and environmental drawbacks, and none was overall comparable to NBF with regard to microscopy, morphologic evaluation, and immunohistochemical studies (Cathy *et al*, 2011).

2.2. DISADVANTAGES OF FORMALIN

Formaldehyde is a carcinogen, is a slow fixative requiring from 24 to 48 hours to completely bind (René, 2008). The production for dangerous formaldehyde fumes requiring additional precautions to limit the exposure and evidence that this procedure causes thermal coagulation, rather than an increment in the formaldehyde diffusion or reaction rates. Its less effectiveness as a fixative for the molecular tests using FFPET (René, 2008).

2.3. USING FORMALIN SAFELY

The advantages of formalin, added to the intrinsic problems to be created by its substitution, outweigh its disadvantages. Formalin can be used safely, the contact between personnel and formalin should be kept to a minimum (René, 2008). Laboratory safety can be improved to allow an operation at a TWA level of 0.4 ppm or less, which will require additional personnel training. The formaldehyde contents in the NBF should be lowered to 8%, and if recycling is practiced, it should be by filtration methods, with all neutralization procedures discouraged (René, 2008).

A total of 81% of US laboratories use NBF, 15% do not monitor for its presence in the work areas, 15% recycles it. The airflow is not tested in 13% of the laboratories, and 6% do not have formalin spills neutralizing substances, and all these deficiencies have to be eliminated (René, 2008).

Recycling is another way but can be a cost-effective practice, distillation not only is less efficient (80%-90% recuperation), but also requires adding the buffer salts to the distillate increasing the exposure (René, 2008). Another step that can be taken is to reduce the formaldehyde contents in the NBF from 10% to 8% as is practiced in some European laboratories (René, 2008). Recent reports comparing a group of pathologists and industrial workers, and using individual devices to monitor exposure to formaldehyde demonstrated

that environmental exposure in pathology departments is trivial in most areas while the sampling activity in the gross room may result in exposure to toxic levels that exceed the recommended values (Zanini *et al.*, 2012).

Individual exposure to formaldehyde was monitored by measuring Malondialdehyde-deoxyguanosine adducts on peripheral leukocytes and the alkylation of hemoglobin to form a terminal N-methylene valine residue. These data show that formaldehyde exposure in the gross room is comparable to that of workers in plastic factories (Zanini *et al.*, 2012).

2.4. EXPOSURE TO FORMALDEHYDE

Based on data obtained from animals and man, Bundesinstitut für Risikobewertung (BfR) established a "safe level" on the basis of the two mechanisms of action, that is 0.124 milligram per cubic metre indoor air. But, repeated, clear exceeding of this level may entail health risks (Carcinogenic effect of inhaled formaldehyde, 2006). Formaldehyde is normally present in both indoor and outdoor air at low levels, usually less than 0.03 parts of formaldehyde per million parts of air (ppm) (Formaldehyde and Cancer Risk - National Cancer Institute, 2011). When formaldehyde is present in the air at levels exceeding 0.1 ppm, some individuals may experience adverse effects such as watery eyes; burning sensations in the eyes, nose, and throat; coughing; wheezing; nausea; and skin irritation (Formaldehyde and Cancer Risk - National Cancer Institute, 2011). Some people are very sensitive to formaldehyde, whereas others have no reaction to the same level of exposure.

In 1987, Occupational Safety And Health Administration (OSHA) established a Federal standard that reduced the amount of formaldehyde to which workers can be exposed over an 8-hour workday from 3 ppm to 1 ppm (Formaldehyde and Cancer Risk - National Cancer Institute, 2011). In May 1992, the standard was amended, and the formaldehyde exposure limit was further reduced to 0.75 ppm. Some presswood products emit less formaldehyde

because they contain phenol resins (Formaldehyde and Cancer Risk - National Cancer Institute, 2011).

2.5. FORMALDEHYDE CARCINOGENIC TO HUMANS

Formaldehyde is a clear substance which is present in gaseous form at room temperature and has a distinct pungent smell which is noticeable even at low concentrations. It has germicidal, preserving and disinfectant properties hence its produced on a large scale around the world (Carcinogenic effect of inhaled formaldehyde, 2006). It is present in many consumer products used daily such as disinfectants, household cleaning agents, cosmetics, paints, varnishes and building materials, pressed-wood products, cigarette smoke, and fuel-burning appliances (Anthony, 2000; Carcinogenic effect of inhaled formaldehyde, 2006). Small amounts of formaldehyde are also formed during cell metabolism in man and animals. The Federal Institute for Risk Assessment to the general public has revealed that formaldehyde is harmful, it irritates the mucous membranes and can trigger cancer in the nasopharynx when it is inhaled (Carcinogenic effect of inhaled formaldehyde, 2006). The Institute believes that there is sufficient evidence that this substance can trigger tumours in the nasopharynx when inhaled. The harmful action of formaldehyde depends on the concentration. Carcinogenic effect can occur at indoor air levels of or below 124 micrograms formaldehyde per cubic metre, repeated clear exceeding of this value may entail risks to health (Carcinogenic effect of inhaled formaldehyde, 2006).

Highly comprehensive studies on workers in the USA have confirmed an elevated, exposure-related mortality rate caused by tumours in the nasopharynx. Also cases of spontaneous tumours are rare in human beings with the exception of some occupational exposure situations. Following its reevaluation the International Agency for Research on Cancer (IARC), proposed a reclassification of formaldehyde as a human carcinogen

(Carcinogeniceffect of inhaled formaldehyde, 2006). More comfirmation by the German Cancer Research Centre in Heidelberg was made, they came to the conclusion that formaldehyde can trigger cancer in man if inhale (Carcinogenic effect of inhaled formaldehyde, 2006).

Epidemiological studies have evaluated the relationship between formaldehyde exposure and carcinogenicity in humans. It showed that there is an increased risk of lymphohematopoietic, nasopharyngeal, sinonosal and brain cancers in humans due to formaldehyde exposure (Carcinogenic effects of Formaldehyd., 2011). Some studies have confirmed the link of maternal exposure to high amounts of formaldehyde during pregnancy and increased incidences of benign and malignant tumors of the gastrointestinal tract (Carcinogenic effects of Formaldehyde, 2011).

2.6. CARCINOGENIC PATHWAY OF FORMALDEHYDE

Individual reactions to formaldehyde exposure might have occurred due to the binding of formaldehyde to endogenous proteins creating haptens that can elicit an immune response (Tatjana and Jana 1997). Chronic exposure to formaldehyde has been associated with immunological hypersensitivity as evidenced by elevated circulating IgG and IgE autoantibodies to human serum albumin, immune activation, and formaldehyde-albumin adducts in patients occupationally exposed, or residents of mobile homes or of homes containing particleboard sub-flooring (Tatjana and Jana 1997). There is also a decrease of T-cells which indicates an altered immunity (Tatjana and Jana 1997). Hypersensitivity induced by formaldehyde may be the mechanism for asthma and other health complaints associated with formaldehyde exposure (Tatjana and Jana 1997).

The carcinogenic action of formaldehyde leads to a change in genetic information. The carcinogenic action of formaldehyde is based on two biological mechanisms:

- The cytotoxic effect to which the body responds with cell proliferation
- A change in genetic information.

Both mechanisms develop joint action from a specific level upwards (Carcinogenic effect of inhaled formaldehyde, 2006). Formaldehyde is a direct-acting genotoxic compound that affects multiple gene expression pathways, including those involved in DNA synthesis and repair and regulation of cell proliferation and produces mutations in a number of diverse ways (Carcinogenic effects of Formaldehyde., 2011). In-vitro studies with mammalian and human cells were positive for DNA adducts, DNA-protein cross links, DNA-DNA cross links, unscheduled DNA synthesis, single-strand breaks, mutations, and cytogenetic effects (chromosomal aberrations, sister chromatid exchange, and micronucleus induction). In in-vivo studies in rats, formaldehyde caused DNA-protein cross links (in the nasal mucosa and fetal liver but not bone marrow), DNA strand breaks (lymphocytes and liver), dominant lethal mutations, chromosomal aberrations (pulmonary lavage cells and bone marrow in one of two studies), and micronucleus induction in the gastrointestinal tract (Carcinogenic effects of Formaldehyde., 2011).

2.7. AIMS OF FIXING A TISSUE

The aims are:

i. To prevent autolysis and putrefaction.
ii. Tissue should not change shape or volume.
iii. No small molecule should be lost.
iv. Tissue should remain in a condition which subsequently allow for other processing.

Unfortunately no single fixative is able to achieve these aims hence there is no ideal fixative (David, 1996).

So the objective of fixation is to preserve cells and tissue constituents in as close as life-like state as possible and to allow them to undergo further preparative procedures without change. Fixation arrests autolysis and bacterial decomposition and stabilises the cellular and tissue constituents so that they withstand the subsequent stages of tissue processing. Aside from these requirements for the production of tissue sections, increasing interest in cell constituents and the extensive use of immunohistochemistry to augment histological diagnosis has imposed additional requirements. Fixation should also provide for the preservation of tissue substances and proteins. Fixation is, therefore, the first step and the foundation in a sequence of events that culminates in the final examination of a tissue section under the microscope (David, 1996; Anthony, 2000; Rosai, 2011; Fixation, 2014).

2.8. QUALITIES OF A GOOD FIXATIVE

The good fixative is expected to be able to prevent autolysis, putrefaction, penetrate rapidly into tissue and kill the tissue, prevent osmosis and leaching of cell and tissue constituents, harden tissue for easy handling, allow accurate histochemistry of tissue constituents, give good optical differentiation to unstained tissue constituents, non-toxic, safe to use by staff, safe and easy to dispose and cheap. Again unfortunately no single fixative is able to achieve these (David, 1996).

2.9. CLASSIFICATION OF FIXATIVES

Hopwood's classification (David, 1996).

- Aldehydes - formaldehyde, gluteraldehyde, acrolein, glyoxal.
- Oxidizing agents – osmium tetroxide, potassium permanganate, potassium dichromate.

- Protein denaturing agents – acetic acid, methyl alchohol, ethyl alchohol.
- Other cross linking agents – carbodiimides.
- Physical – heat, microwaves.
- Miscellaneous – mercuric chloride, picric acid, non-aldehyde containing fixatives.

2.10. ALDEHYDES

Aldehydes is a cross linking fixatives that act by creating covalent chemical bonds between proteins in tissue. This anchors soluble proteins to the cytoskeleton, and gives additional rigidity to the tissue (David, 1996). The most commonly used aldehyde fixative in histology is formaldehyde. It is usually used as a 10% Neutral Buffered Formalin (NBF), which is approximately 3.7%-4.0% formaldehyde in phosphate buffered saline (Rosai, 2011; Fixation, 2014). Formaldehyde is a gas at room temperature, formalin-formaldehyde gas dissolved in water (~37% w/v)-is used when making the former fixative (Fixation, 2014). Formalin occurs purely as a concentrated (40%) solution of the gas formaldehyde in water. Thus a 10% formalin solution represents a 4% solution of the gas, which is 1.3 molar (Rosai, 2011). If the final dilution is maintained in a range between 8% and 12%, no noticeable differences will be noted (Rosai, 2011; Fixation, 2014). However, once the concentration of formalin drops below 5%, the quality of the preparation will suffer. Paraformaldehyde is a polymerised form of formaldehyde, usually obtained as a fine white powder, which depolymerises back to formalin when heated (Rosai, 2011; Fixation, 2014). Formaldehyde fixes by cross-linking the proteins, primarily the residues of the basic amino acid lysine (Rosai, 2011). Formaldehyde is generally used in a 4% aqueous solution (10% formalin) and buffered at pH 7 by acetate or phosphate. In this form, it is named NBF, and fixation is achieved through cross-linking of amines, amides, aromatic rings, hydroxyls, guanidine groups, sulfhydryl groups, and reactive hydrogen atoms (Cathy *et al*., 2011)**.**

Formaldehyde fixation is typically performed at room temperature. Using a low and wide specimen container to allow for adequate penetration and ease of retrieval by a technician is the best choice for adequate volume ratio. In addition, 1:20 volume ratio of fluid to tissue and 3–4 mm specimen thickness are recommended for good penetration. To prevent swelling or shrinking of the cells, an isotonic solution buffered to pH 7.2–7.4 is recommended, to maintain ultrastructure and minimize cell distortion. The shorter the time elapsed between removing the sample from the body and immersing it in fixative, the better. The duration of exposure to the fixative must be optimized for each specimen type (Nowacek *et al*, 2010).

Formaldehyde has been found to:

- Slow formation of covalent bonds in aqueous solution but rapid diffusion in tissue.
- When formaldehyde is used as a fixative in aqueous solution at least 24 hr at room temperature is required for the reaction to reach equilibrium.
- While 1.3 M aqueous formaldehyde solutions are standard, this concentration is not critical to fixation, since most of the formaldehyde is present as methylene glycol.
- Formic acid, a spontaneous oxidation product in formaldehyde solutions, seems to have little effect other than as an acid causing formation of "formalin pigment" in blood rich tissues.
- Aqueous solutions of formaldehyde in the usual concentrations produce marked alterations in cellular membranes and in mitochondrial organization. Whether these changes are a result of the methylene glycol present or are due to some other mechanism is not clear.
- Shrinkage of tissues is minimal in formaldehyde fixation, but becomes manifest in later steps of tissue processing (Fox *et al*, 1985).

Gluteraldehyde operates in a similar way to formaldehyde by causing deformation of the alpha-helix structures in proteins. But it is a larger molecule so its rate of diffusion across

membranes is slower than formaldehyde. One of the advantages of glutaraldehyde fixation is that it may offer a more rigid or tightly linked fixed product. It causes rapid and irreversible changes, fixes quickly and is well suited for electron microscopy. However it is not ideal for immunohistochemistry staining (Rosai, 2011).

Acrolein (acrylic aldehyde) is mainly used in the tanning industry. It produces more cross-links than formaldehyde under optimal conditions but is unpleasant to use and unstable at alkaline pH levels. Acrolein has a tendency to polymerise into disacryl, a solid plastic when exposed to light. It has been employed as a fixative for enzyme cytochemistry as labile enzymes like glucose-6-phosphatase are retained in tissue fixed in 4% acrolein (Anthony, 2000).

Glyoxal (ethanedial, diformyl), malonaldehyde (malonic dialdehyde), diacetyl (2,3-butanedione) and the polyaldehydes are other aldehydes which have been infrequently employed for fixation, mostly for special situations, to retain specific enzymes or proteins for histochemistry. In terms of effectiveness as cross-linking agents glutaraldehyde is the most efficient although acrolein, when present in excess, is nearly as efficient and succinic dialdehyde is also comparable (Anthony, 2000).

2.11. PROTEIN DENATURING AGENTS

Denaturing (Precipitating) fixatives act by reducing the solubility of protein molecules and sometimes by disrupting the hydrophobic interactions that give many proteins their tertiary structure. The precipitation and aggregation of proteins is a different process from the crosslinking in the aldehyde fixatives (David, 1996; Fixation, 2014).

The most common precipitating fixatives are ethanol, methanol and acetone. They are rarely used alone for fixing blocks unless studying nucleic acids. They are commonly used to fix

frozen sections and smears (Fixation, 2014). Acetone has been shown to produce better histological preservation than frozen sections. Alcohol-based non–cross-linking fixatives advantages include faster fixation, elimination of carcinogenic vapours, better preservation of glycogen, DNA, and RNA, greater staining avidity and no need for enzyme predigestion for immunohistochemical analysis. Disadvantages are variability of tissue staining, tissue shrinkage and hardening, artifactual pigment deposition in bloody specimens, partial or complete lysis of erythrocytes, and increased flammability (Cathy *et al*, 2011; Fixation, 2014).

Another component is acetic acid, it complements the action of other ingredients such as alcohol, makes collagen fibers swell, precipitates nucleoprotein, and may have a solvent action on cytoplasmic granules (Cathy *et al*, 2011)**.**

The alcohols, by themselves, are known to cause considerable shrinkage and hardening of tissue during fixation while acetic acid alone is associated with tissue swelling; combining the two may result in better preservation of tissue morphology (Fixation, 2014).

2.12. OXIDISING AGENTS

These fixatives react with various side chains of proteins and other biomolecules causing the formation of crosslinks that stabilize tissue structure. They cause extensive denaturation and are used mainly as secondary fixatives (David, 1996; Fixation, 2014). Osmium tetroxide is often used as a secondary fixative when samples are prepared for electron microscopy. It is not used for light microscopy because it penetrates thick tissue sections very poorly (Fixation, 2014).

Potassium dichromate, chromic acid, and potassium permanganate all find use in certain specific histological preparations (Fixation, 2014).

2.13. PHYSICAL AGENTS

Fixation can also be achieved with microwaves, which are defined as electromagnetic waves with a frequency between 300 MHz - 300 GHz, temperature of 60º and penetrates at 10-15mm (David, 1996; Rosai, 2011). They can also be used in combination with conventional chemical fixation. Most laboratories use for this purpose household ovens, which have obvious limitations in terms of reproducibility. Ovens specifically designed for histology use should offer the standardization and calibration that these kitchen instruments sorely lack, and would presumably render the procedure even more satisfactory. Microwaves are also used in the pathology laboratory for decalcification, processing for electron microscopy and immunohistochemical staining, including antigen retrieval (Rosai, 2011).

2.14. MISCELLANEOUS

2.14.1. MERCURIALS

Mercurials such as Zenker's fixative have an unknown mechanism of action but they are fast, mercurials penetrate poorly and produce tissue shrinkage. They increases staining brightness and give excellent nuclear detail. They are used for fixation of hematopoietic and reticuloendothelial tissues. When it comes to disposal of mercury care must be taken (Fixation, 2014).

2.14.2. PICRATES

Picrates react with histones and basic proteins to form crystalline picrates with amino acids and precipitate proteins. It is a good fixative for connective tissue and preserves glycogen well but it causes loss of basophilia except if the specimen is thoroughly washed following fixation (Fixation, 2014).

2.15.0. FACTORS AFFECTING FIXATION (David, 1996)

2.15.1. pH (HYDROGEN ION CONCENTRATION)

pH value for each fixative varies. It is normally adjusted using buffers to fall within the physiological range i.e. pH 4-9. Satisfactory fixation occurs between pH of 6 – 8, the pH for the ultrastructure preservation should be buffered between 7.2 - 7.4. Buffer systems most commonly used are phosphate, s-collidine, veronal acetate, bicarbonate, Tris and cacodylate. Buffers should not react with fixatives in order not to reduce the buffering power and fixation ability. (David, 1996)

2.15.2. OSMOLARITY

Hypertonic solutions give rise to cell shrinkage. Isotonic and hypotonic solutions result in cell swelling and poor fixation. Best results are obtained using slightly hypertonic solutions i.e. 400 – 450mOsm. 10% neutral buffer formalin is 4% formaldehyde (1.33 osmolar) in PBS buffer (0.3 osmolar) sums to 1.63 osmolar. This is a very hypertonic solution yet it has worked well as a general tissue fixation condition for many years in pathology laboratories. (David, 1996)

2.15.3. TEMPERATURE

Increasing the temperature increases speed of fixation. However, care is required to avoid cooking the specimen. Fixation is routinely carried out at room temperature, for electron microscopy 0-4ºC. (David, 1996)

2.15.4. PENETRATION OF FIXATIVES

Penetration is relatively slow process for most fixatives, either the tissue should be small or thin in order to obtain satisfactory fixation. Large tissues could be opened or sliced or kept in fixatives for weeks before grossing of tissue. Medawar (1941) investigated the law of tissue penetrance by fixatives which states that the depth of penetrance (d) is directly proportional to the square root of time (t) (David, 1996).

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

K is the coefficient of diffusibility of the fixative which is specific to each fixative. K in 1hour is the distance in millimeters that the fixative has diffuse into the tissue. The rate of reaction with tissue is also important in fixation, a slow rate of diffusion and reaction will give rise to various zones in tissue. (David, 1996)

2.15.5. CONCENTRATION OF FIXATIVES

Concentration of fixatives are fixative specific but is also influenced by many factors e.g. cost, solubility, customs etc. gluteraldehyde is normally used as a 3% solution but had been found to be effective down to 0.25% provided the pH is correct. (David, 1996)

2.15.6. DURATION OF FIXATION

It is fixative and other factors discussed below dependant. A general rule of 1hr per 1mm of tissue, especially formalin. So if we have a three-dimensional tissue block, it is the shortest dimension that determines fixation time. (David, 1996)

2.16. METHODS OF ENHANCING FIXATION(David, 1996)

Fixation can be enhanced by various manipulations ranging from:

- Immersion of tissue into ample amount of fixatives.
- Opening up or slicing up of tissue to enhance fixation.
- Agitation.
- Increase pressure.
- Mixing of fixative to enhance each order.
- Addition of additives or buffers.
- Increasing the temperature of fixing.
- The use of microwaves.

2.17. PHENOL

Phenol is formally known as carbolic acid, it is an aromatic organic compound with the molecular formula C6H5OH. It is a white crystalline solid that is volatile. The molecule consists of a phenyl group (−C6H5) bonded to a hydroxyl group (−OH). It is mildly acidic and requires careful handling due to its propensity to cause chemical burns. Phenol was first extracted from coal tar, but today is produced on a large scale (about 7 billion kg/year) from petroleum. It is an important industrial commodity as a precursor to many materials and useful compounds. Phenol is appreciably soluble in water, with about 84.2 g dissolving in 1000 mL (0.88 M). Homogeneous mixtures of phenol and water at phenol to water mass ratios of ~2.6 and higher are also possible. The sodium salt of phenol, sodium phenoxide, is far more water-soluble. Its uses involve its conversion to plastics or related materials, its chemical derivatives are key for building polycarbonates, epoxies, Bakelite, nylon, detergents, herbicides such as phenoxy herbicides, and numerous pharmaceutical drugs.(Phenol, 2016)

Phenol is also a versatile precursor to a large collection of drugs, most notably aspirin but also many herbicides and pharmaceutical drugs. Phenol is also used as an oral

anesthetic/analgesic in products such as Chloraseptic or other brand name and generic equivalents, commonly used to temporarily treat pharyngitis. Phenol is also component in liquid/liquid phenol–chloroform extraction technique used in molecular biology for obtaining nucleic acids from tissues or cell culture samples. Depending on the pH of the solution either DNA or RNA can be extracted.(Phenol, 2016) It has also been used to enhance fixing abilities of formaldehyde, the addition of 2% phenol had a marked accelerating effect on neutral buffered 4% formaldehyde as a fixative.(Hopwood, 1989)

CHAPTER THREE

3.0. METHODOLOGY

3.1. STUDY AREA:

This study was carried out in the Department of Pathology, Ahmadu Bello University Teaching Hospital (ABUTH), Zaria. The hospital is a training institution located in the North West region of Nigeria and offers specialist medical services to the population of Kaduna state and neighbouring states of Zamfara, Jigawa, Katsina and Niger. About 3000 to 4000 samples are processed yearly in this laboratory.

3.2. STUDY DESIGN:

The study was conducted on following fixatives i.e. formol saline (local laboratory standard), 10% neutral buffered formalin (international standard), 8% formol saline (A1), 6% formol saline (A2), 4% formol saline (A3), 8% formol saline (B1), 6% formol saline (B2) and 4% formol saline (B3) (Appendix 3). All fixatives were stored and used at room temperature.

3.3. SAMPLE POPULATION/ SAMPLE SIZE DETERMINATION:

Purposive sampling method was used. The average number of a Consultant and four Residents to a team in ABU Zaria laboratory per day.

The total number of 104 slides were analyzed by five (5) pathologists from ABUTH, Zaria for various parameters.

The sample size was determined as follows:

Nine (9) pieces of tissues based on organ systems namely; brain, heart, lungs, skin, liver, kidney, spleen, lymphnode and skeletal muscle (Appendix 7), was passed into each of the eight (8) containers of freshly prepared fixatives,

Hence for H&E number of slides = 8 X 9 = 72.

For PAS number of slides = 8 (liver only)

For MT number of slides = 8 (muscles only)

For EMA number of slides = 8 (skin only)

For CD23 number of slides = 8 (lymph node only)

:. Total number of slides = 72 + 8 + 8 + 8 + 8 = 104

i.e. 104 slides for evaluation.

3.4. SAMPLING TECHNIQUE

The fixatives were prepared at three varying concentrations each and placed in well labeled containers. Fresh tissue samples from Sus domestica which was sacrificed and the following tissues based on organ system in appendix 7 were harvested; skin, brain, heart, lungs, skeletal muscle, liver, lymph nodes, spleen and kidneys. Tissues obtained were, divided into equal sizes (1x1x1cm=1cm^3) and passed into the containers of fixatives (x10 volume of tissues). The tissues were grossed after 24hours and processed using an Automated Rapid Tissue Processor (Leica®) which dehydrates using graded alcohol, clears using xylene and impregnates using paraffin. After paraffin embedding, blocks were stored at room temperature. Paraffin sections were cut at 3-4μm, mounted on glass slides, dried for at least 10 minutes on a hot plate, and processed for haematoxyline & eosin (H&E), special stains (PAS & MT) and Immunohistochemistry (IHC)(EMA & CD23).

3.5. STUDY INSTRUMENTS

They include:

Transparent 200cm^3 plastic containers (x8), labels, measuring cylinders, funnel, weighing scale, spatula, filter paper, cassettes, slides, scapel, blade, forceps, knife, grossing board cover slips, moulds, pen, pencils and brushes.

Animal: Domestic pig (*Sus domestica*) was sacrificed at the Department of Veterinary Pathology of ABU Zaria, brain, heart, lungs, skin, liver, kidney, spleen, lymphnode and skeletal muscle (Appendix 7) were then obtained and what was left of it was incinerated.

Chemicals: Concentrated formalin, Sodium chloride, Sodium dihydrogen orthophosphate, Phenol crystals and Disodium hydrogen orthophosphate (anhydrous), were used for compounding the fixatives. Alcohol for dehydrating f tissue, xylene for clearing of tissue and paraffin wax for impregnating the tissue by the processing machine. Parafin wax was used for embedding. H&E, PAS, MT, EMA and CD23 were used for staining. Distrene pthalal diphenyl xylene (DPX) was used for mounting the cover slip on slides. And water distilled water was used at various stages of the work.

Machines: An automated Rapid Tissue Processor (Leica®) was used to process the tissues.

An embedding machine was used to embed the tissues into blocks.

Microtome was used for sectioning tissues at 3-4μm.

Warm (40-50^{o}C) water bath was used to straighten sections from microtome.

Hot plate (40-50^{o}C) was used to dry sections and melt wax from slides.

An automated / manual staining machines was used for staining the slides.

Light microscopes was used to screen and analyze the slides.

3.6. DATA COLLECTION METHODS

The slides were analyzed by the pathologists, each set was scored by the pathologists in a single blind manner for evaluation of the physical quality of the sections (artifacts associated with fixatives), the quality of tissue preservation (nucleus, cytoplasm, extracellular components) and the quality of staining (uniformity, nuclear, cytoplasmic, and extracellular components). The overall evaluation of morphological details and staining quality on the stained slides were graded on a scale of 4-1 (4- very good, 3- good, 2- suboptimal and 1-

poor). For each fixative, a total percentage was calculated based on the ratio of the sum of all scores. (Modified Allred score in Appendix 5)

3.7. DATA MANAGEMENT

For each fixative, a total percentage was calculated based on the ratio of the sum of all scores. Results was made in frequencies, charts and photomicrographs while data analysis in descriptive statistics was analyzed using SPSS 21.

3.8. ETHICAL CONSIDERATIONS:

All ethical guide lines on the use of animals for experimental research were complied with. Ethical clearance was obtained from the Ethics and Scientific Committee of the ABUTH Zaria for the study.

3.9. LIMITATIONS

i. Inter-observer variation could occur.

ii. Inter-observer error could occur.

CHAPTER FOUR

4.0. RESULTS

4.1. PHYSICAL QUALITIES ATTRIBUTED TO FIXATIVES

On passing the tissues into freshly prepared fixatives the phenolic groups show clearer solution compared to the others.(**Plate 4.1**) On gross examination after fixation, all the tissues were similar, gray white with soft to firm consistency. Microscopic examination was further used to compare the overall effectiveness of each fixative. In the whole study no artifact associated with fixation was observed.

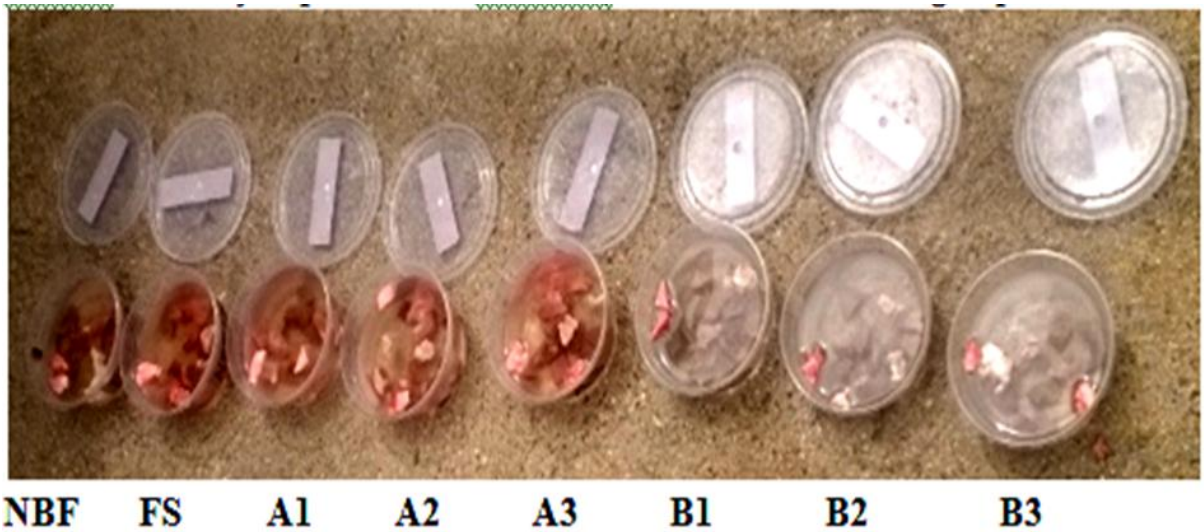


Plate 4.1. Tissues just passed into freshly prepared fixatives. Note how clear the B group solutions are.

4.2. Overall cumulative performances of fixatives

The quality of tissue preservation, based on total score of tissue types: the skin; formol saline was the highest with score 18, the Brain; B1 was the highest with score 17, the lungs; formol saline was the highest with score 17, the heart; NBF was the highest with score 18, the Liver; B3 was the highest with score 16, the spleen; NBF and B2 are the highest with score 16, the kidney; B2 was the highest with score 18, the lymph nodes formol saline and A3 are the highest with score 17, skeletal muscles; NBF was the highest with score 18. **(Table. 4.1), (Fig.4.29-4.31)**

NBF has the highest percentage i.e. 13.2%, followed by FS with 12.8%, then B2 with 12.5%, the lowest was A1 with 12.1%. **(Fig 4.1)**

The quality of staining of tissues total score and percentages, based on total score of tissue types: the skin; B1 was highest with score 17, the Brain; A3, B1 and B3 were the highest with score 16, the lungs; NBF was the highest with score 19, the heart; NBF was the highest with score 16, the Liver; NBF and B1 was the highest with score 16, the spleen; FS was the highest with score 16, the kidney; NBF, A2, A3 and B2 are the highest with score 17, the lymph nodes A2 was the highest with score 18, skeletal muscles; A3 was the highest with score 17**.(Table 4.2), (Fig.4.32-4.34)**

In general NBF has the highest percentage i.e. 13.1%, followed by A3 with 12.7%, then B1 and B3 with 12.5%, the lowest was A1 with 12.0%.**(Fig 4.2)(Table 4.31-4.37)**

The overall average score, mean and median of averages, variance, standard deviation and p values of all fixative showed a good score based on averages, the mean and median of averages were equal i.e. 3.1 hence the distribution was normal**.(Table 4.3)**

The p value for each of the fixative were greater than 0.05 hence null hypothesis should be retained**.(Table 4.4)** Using alpha=0.05, confidence interval level=95, ANOVA and chi-square tests were performed and the results supports the acceptance of the null hypothesis.

i.e. there is no significant difference between the experimental combination formalin fixatives to the formol saline**.(Table 4.5-4.8)**

4.3. Overall percentage grade responses

Less than 10% (3.67%) of responses graded the performances suboptimal and poor. 75.63% graded the performances good and 20.70% graded it very good.(**Fig.4.35, 4.36, 4.37**)

4.4. H&E Performances based on tissue types

The skin; for tissue preservation properties formol saline was the best with a percentage grade of 14%, second best A2 and A3 13.2% each, third best NBF and B3 12.4% each then others 11.6% each.(**Fig.4.3, Table 4.9**) For quality of staining of the skin, B1 was the best with a percentage grade of 13.4%, second best were NBF, FS, A1, A2 and B3 12.6% each, third best A3 and B2 11.8% each.(**Fig.4.3, Table 4.10**)

The brain; for tissue preservation properties B1 was the best with a percentage grade of 13.7%, second best NBF and B3 12.9% each, third best a were others with 11.6% each.(**Fig.4.5, Table 4.11**) . For quality of staining of the brain NBF was the best with a percentage grade of 14.7%, second best was B2 with 13.2%, the third best were FS, B1 and B3 with 12.4% each. The others were 11.6% each. (**Fig.4.6, Table 4.12**)

The lungs; for tissue preservation properties formol saline was the best with a percentage grade of 14.2%, second best B1 13.3%, third best A1 and B2 12.5% each then the others 11.7% each. (**Fig.4.7, Table 4.13**) For tissue quality of staining of the lungs, NBF was the best with a percentage grade of 14.7% , second best was B2 with 13.8%, third best were FS, B1 and B3 with 12.4% each then others with 11.6% each.(**Fig.4.8, Table 4.14**)

The liver; for tissue preservation properties NBF was the best with a percentage grade of 14.4%, second best were A1 and B2 with 12.7%, third best were FS, A2 and B2 with 11.9% each then A3 11%.(**Fig.4.9, Table 4.15**) For tissue quality of staining of the liver, NBF and B1 were the best with a percentage grade of 13.6% each, second best were FS, A3 and B3 with 12.7%, third best were A2 and B2 with 11.9% each then A1 11%.(**Fig.4.10, Table 4.16**)

The spleen; for tissue preservation properties FS, A1 and B2 are the best with a percentage grade of 13.2%, second best were NBF, A3 and B3 with 12.4%, third best were A2 and B1 with 11.6% each.(**Fig.4.11, , Table 4.17**) For tissue quality of staining of the spleen, FS was the best with a percentage grade of 13.2%, second best were the others with 12.4% each.(**Fig.4.12, , Table 4.18**)

The heart; for tissue preservation properties NBF was the best with a percentage grade of 15.3%, second best were A2 and B1 with 13.6%, third best were FS, A3, B2 and B3 with 11.9% each then A1 10.2%.(**Fig.4.13, Table 4.19**) For tissue quality of staining of the heart, NBF was the best with a percentage grade of 13.8%, second best were B1, B2 and B3 with 12.9%, third best were FS, A2 and A3 with 12.1% each then A1 11.2%.(**Fig.4.14, Table 4.20**)

The lymph nodes; for tissue preservation properties FS and A3 were the best with a percentage grade of 13.2%, second best were the others with 12.4%, except for B1 with 11.6%.(**Fig.4.15, Table 4.21**)

For tissue quality of staining of the lymph node, A2 was the best with a percentage grade of 13.7% each, second best were FS and A3 with 13%, third best were the others with 12.2% except B1 11.5%. (**Fig.4.16, Table 4.22**)

The skeletal muscles; for tissue preservation properties NBF was the best with a percentage grade of 14.8%, second best was A1 with 13.1%, third best were FS, A3, B1 and B2 with 12.3% each then A2 and B3 with 11.5%.(**Fig.4.17, Table 4.23**) For tissue quality of staining of the skeletal muscle, A3 was the best with a percentage grade of 13.8%, second best were NBF, FS and A1 with 13%, third best wrre B1, B2 and B3 with 12.2% each then A2 with 10.6%.(**Fig.4.18, Table 4.24**)

The kidney; for tissue preservation properties B2 was the best with a percentage grade of 14.2%, second best were NBF, FS, A2,A3 and B3 with 12.6%, third best A1 with 11.8% then B1 11%.(**Fig.4.19, Table 4.25**) For tissue quality of staining of the kidney, NBF, A2, A3 and B2 were the best with a percentage grade of 13.4%, second best was A1 with 12.6%, third best B3 with 11.8% then FS and B1 with 11.0% each. (**Fig.4.20, Table 4.26**)

4.5. Special stain Performances

The PAS done on liver tissue result; for quality of staining B2 was the best with a percentage grade of 7.5%, second best were FS with 6.3%, third best were NBF, A1, A2 and B1 with 5.9% each then A3 and B3 5.5% each.

The MT done on skeletal muscle tissue result; for quality of staining FS was the best with a percentage grade of 7.1%, second best were NBF, A1 and B1 with 6.7%, third best were A2 and A3 with 6.3% each then B3 5.9% and B2 5.5%.(**Fig.4.21, 4.22, 4.23, Table 4.27, 4.28**)

4.6. IHC Performances

The EMA done on skin tissue result; for quality of staining B3 was the best with a percentage grade of 6.7%, second best were A3, B1 and B2 with 6.3%, third best were NBF and FS with 5.9% each then A1 with 5.5% and A2 5.1%.(**Fig.4.25, Table 4.29, 4.30**)

The CD23 done on lymph nodes result; for quality of staining A3, B1 and B3 were the best with a percentage grade of 6.7%, second best were the others with 6.3% each.(**Fig.4.26, 4.27, 4.28**)

4.7. GENERAL CUMMULATIVE H&E RESULTS OF PERFORMANCES OF EACH FIXATIVE.

Table 4.1: Tissue preservation cummulative grades of fixatives based on tissue type

TISSUES	NBF	FS	A1	A2	A3	B1	B2	B3	TOTAL
SKIN	16	18	15	17	17	15	15	16	129
BRAIN	16	15	15	15	15	17	15	16	124
LUNGS	14	17	15	14	15	16	15	14	120
HEART	18	14	12	16	14	16	14	14	118
LIVER	17	14	15	14	13	15	14	16	118
SPLEEN	15	16	16	14	15	14	16	15	121
KIDNEY	16	16	15	16	16	14	18	16	127
LYMPHNODES	16	17	16	16	17	15	16	16	129
SKELETAL MUSCLES	18	15	16	14	15	15	15	14	122
TOTAL	**146**	**142**	**135**	**136**	**137**	**137**	**138**	**137**	**1108**
TOTAL %	**13.2**	**12.8**	**12.2**	**12.3**	**12.4**	**12.4**	**12.5**	**12.4**	**100.0**

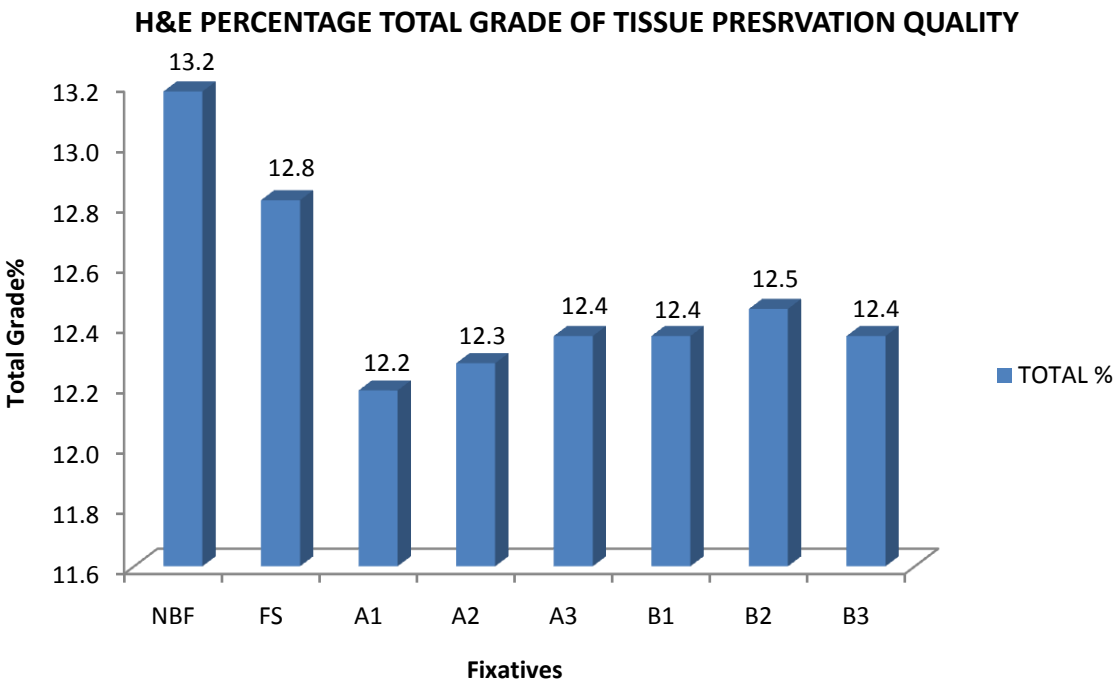


Fig. 4.1 showing the percentage total grade for tissue preservation quality by each fixatives.

TABLE 4.2: Quality of staining cumulative grades of fixatives based on tissue type

TISSUES	NBF	FS	A1	A2	A3	B1	B2	B3	TOTAL
SKIN	16	16	16	16	15	17	15	16	127
BRAIN	15	14	13	15	16	16	14	16	119
LUNGS	19	16	15	15	15	16	17	16	129
HEART	16	14	13	14	14	15	15	15	116
LIVER	16	15	13	14	15	16	14	15	118
SPLEEN	15	16	15	15	15	15	15	15	121
KIDNEY	17	14	16	17	17	14	17	15	127
LYMPHNODES	16	17	16	18	17	15	16	16	131
SKELETAL MUSCLES	16	16	16	13	17	15	15	15	123
TOTAL	**146**	**138**	**133**	**137**	**141**	**139**	**138**	**139**	**1111**
TOTAL %	**13.1**	**12.4**	**12.0**	**12.3**	**12.7**	**12.5**	**12.4**	**12.5**	**100.0**

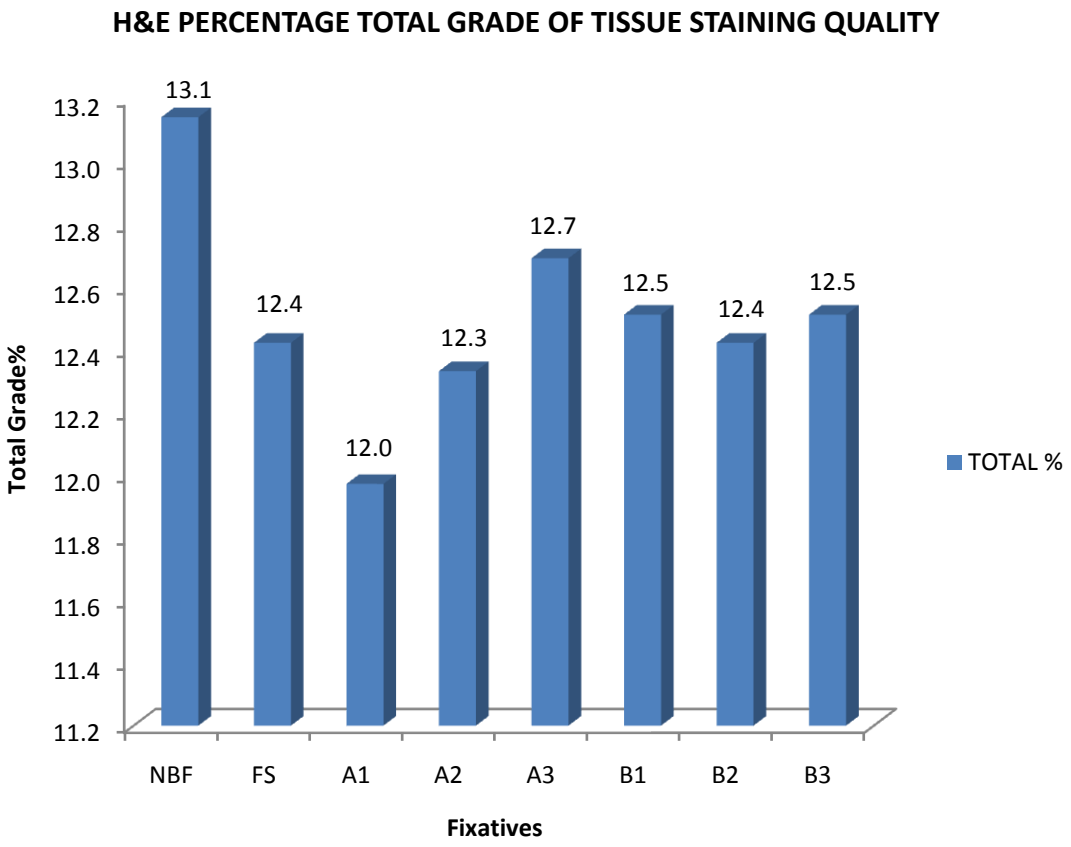
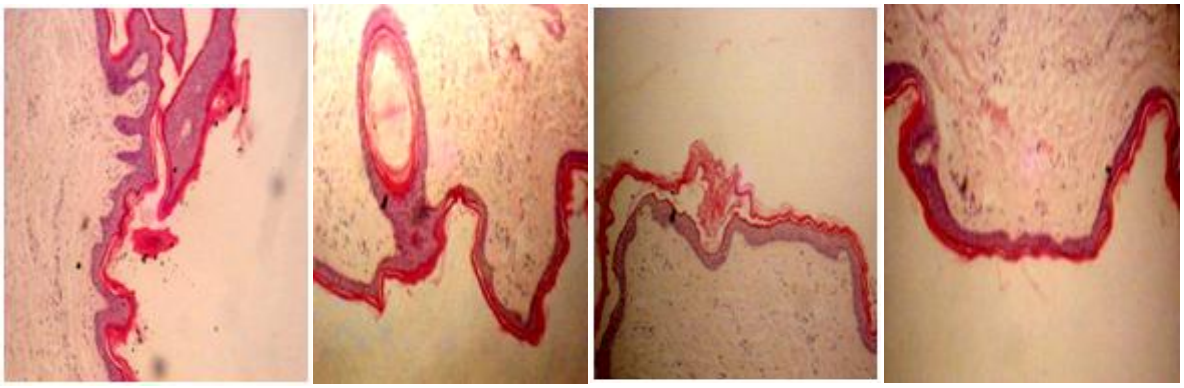
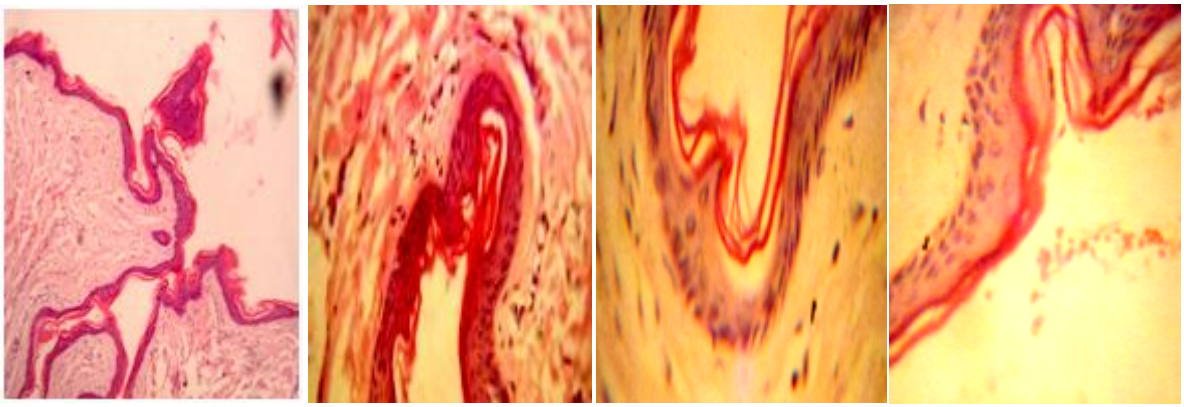


Fig4.2 showing the percentage total grade for tissue staining quality by each fixatives.



NBF(x200) **FS(x200)** **A1(x200)** **A2(x200)**



A3(x200) **B1(x400)** **B2(x400)** **B3(x400)**

Plate 4.2: Photomicrographs of skin for all fixatives (H&E at X200 and X400)

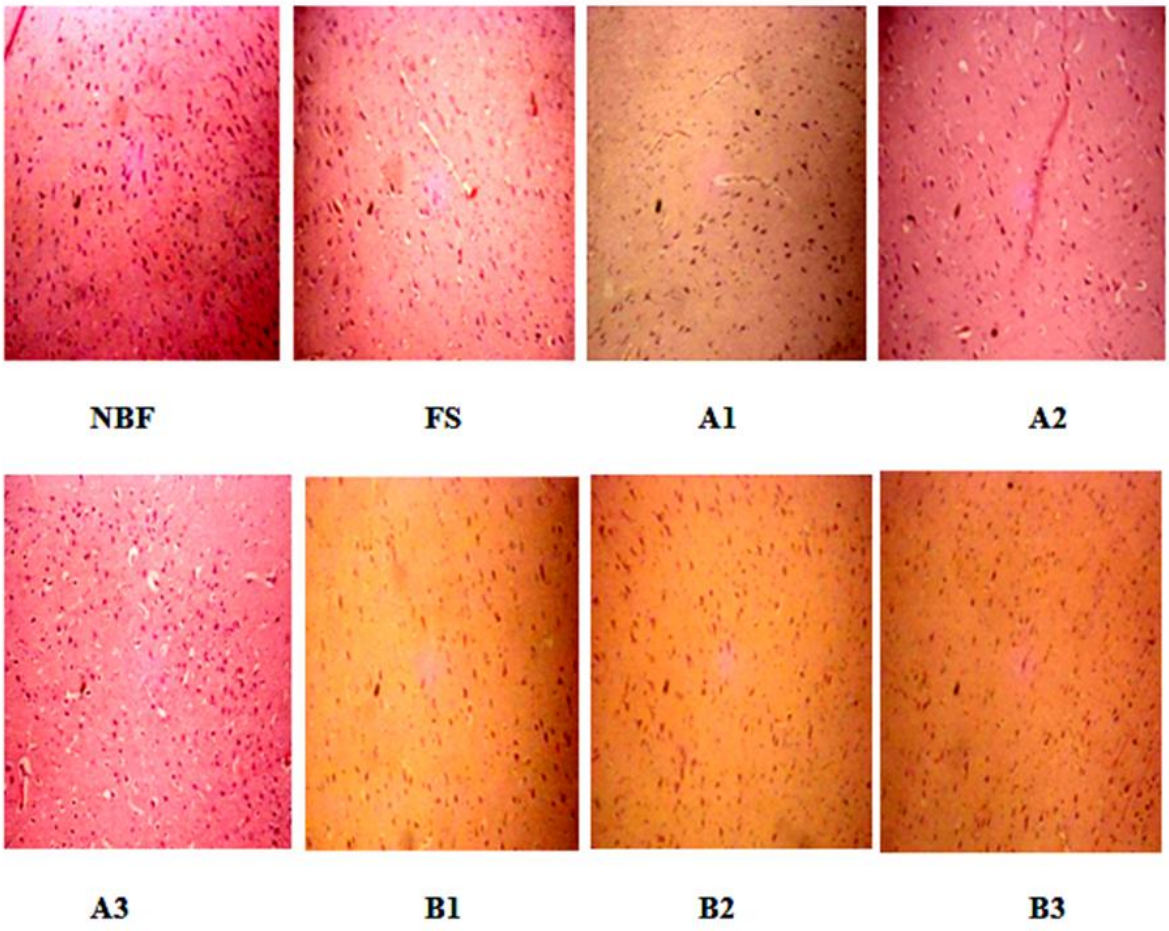


Plate 4.3: Photomicrographs of brain for all fixatives (H&E X 400)

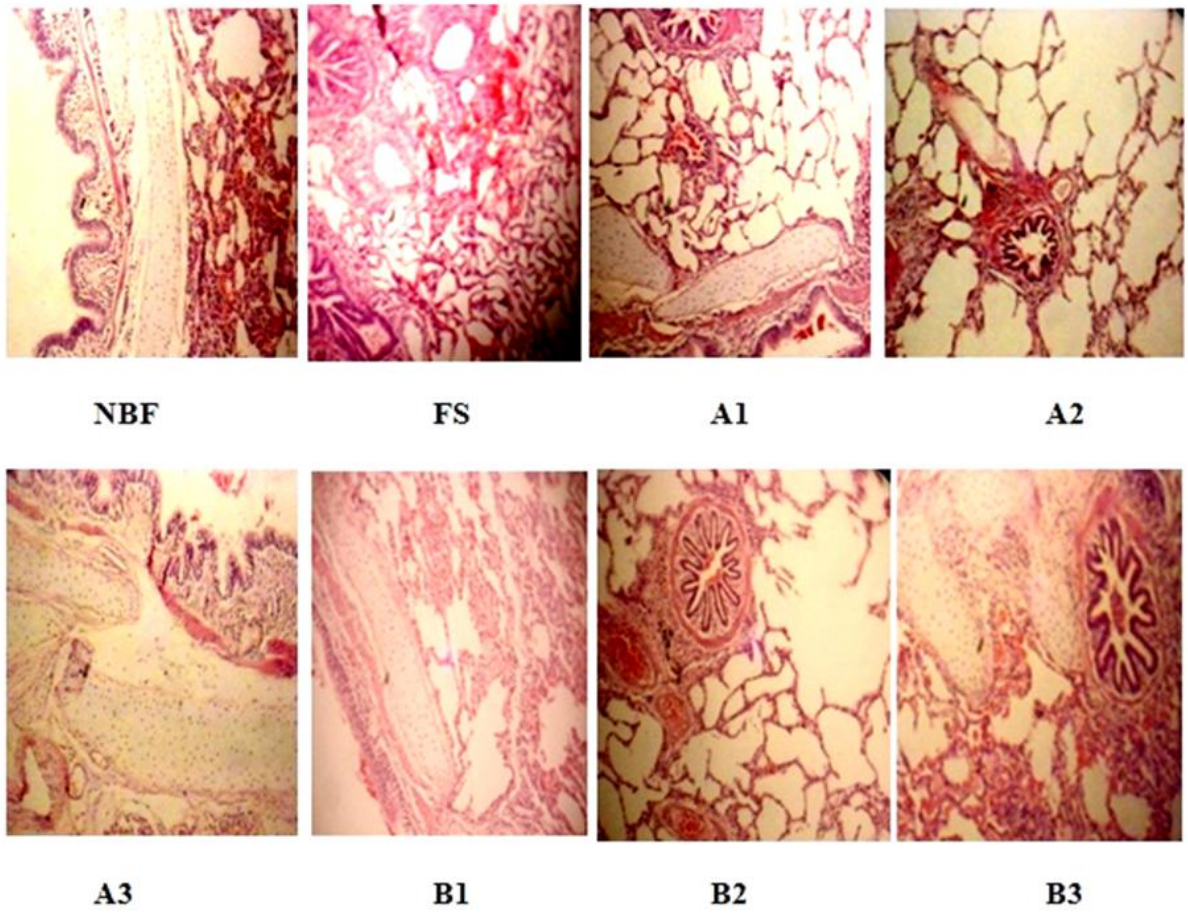


Plate 4.4: Photomicrographs of lungs for all fixatives (H&E X 200)

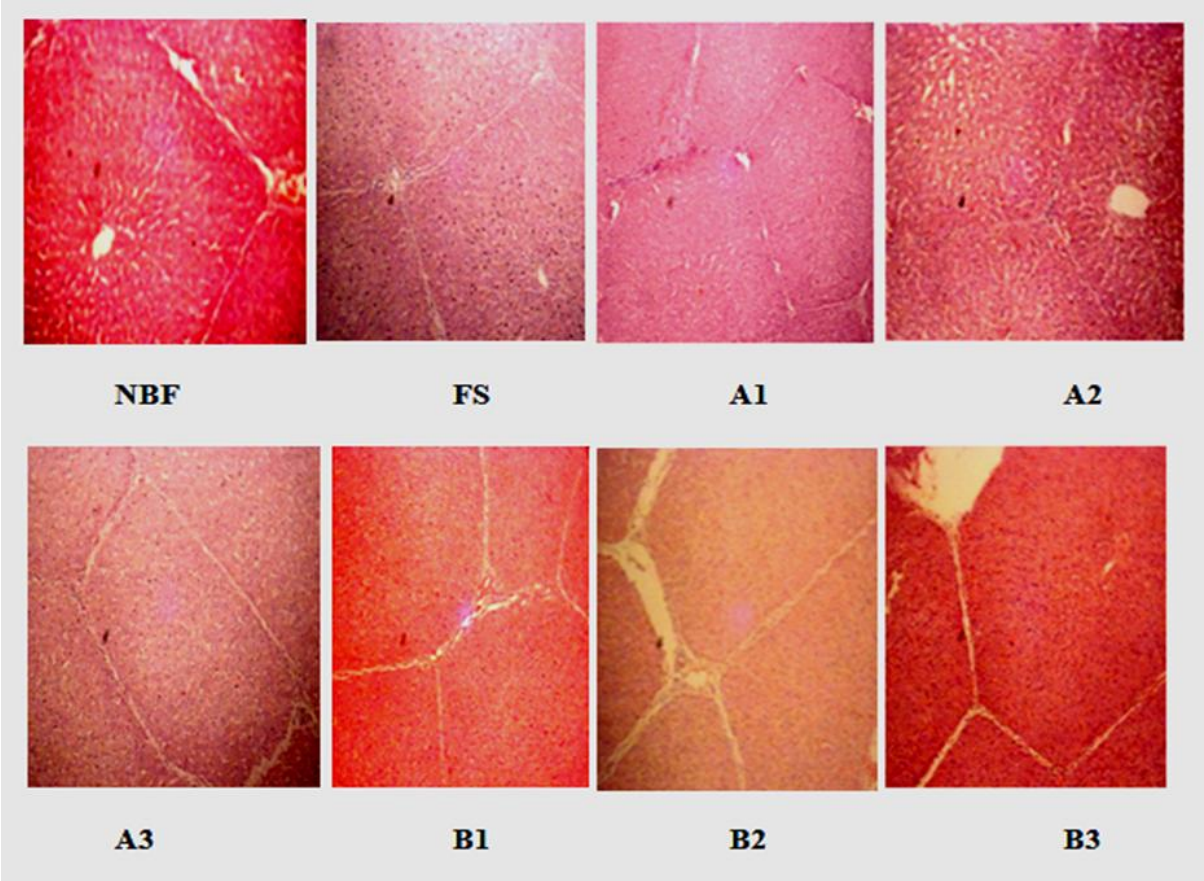


Plate 4.5: Photomicrographs of liver for all fixatives (H&E X 200)

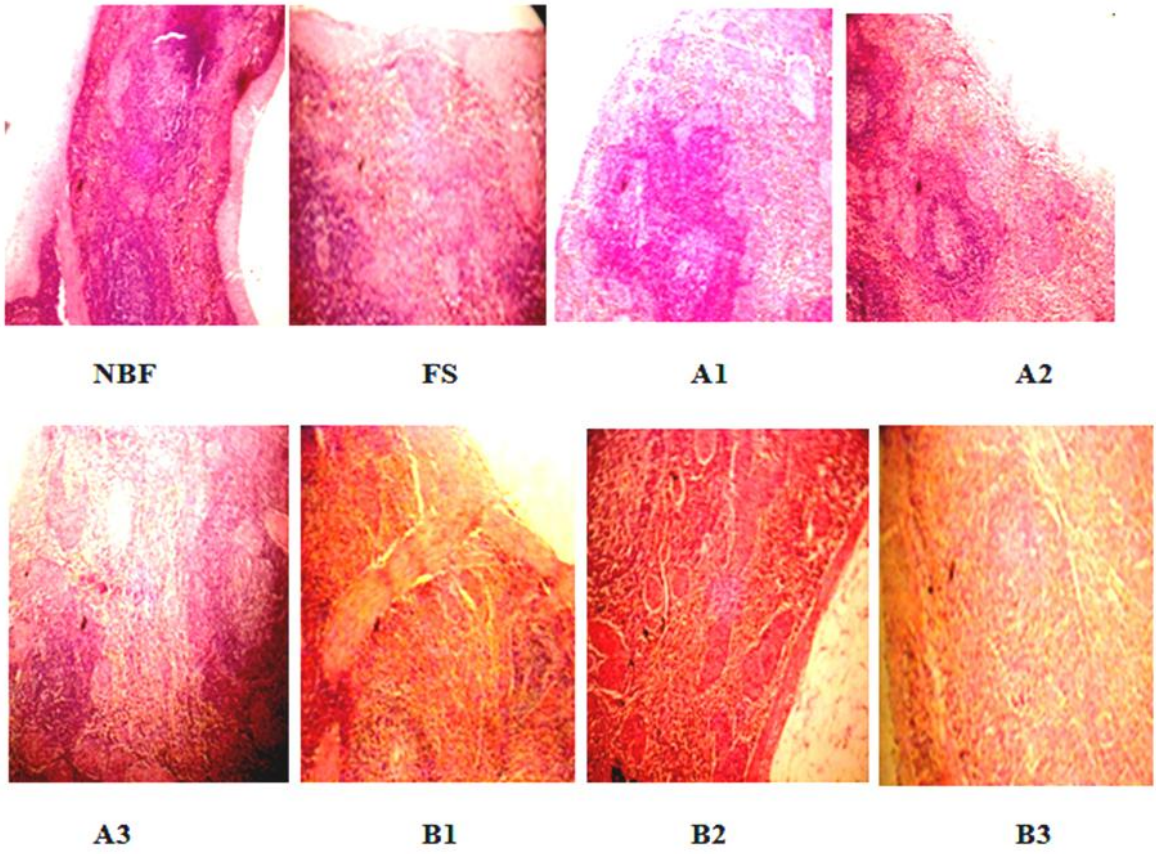


Plate 4.6: Photomicrographs of H&E on spleen for all fixatives (H&E X 200)

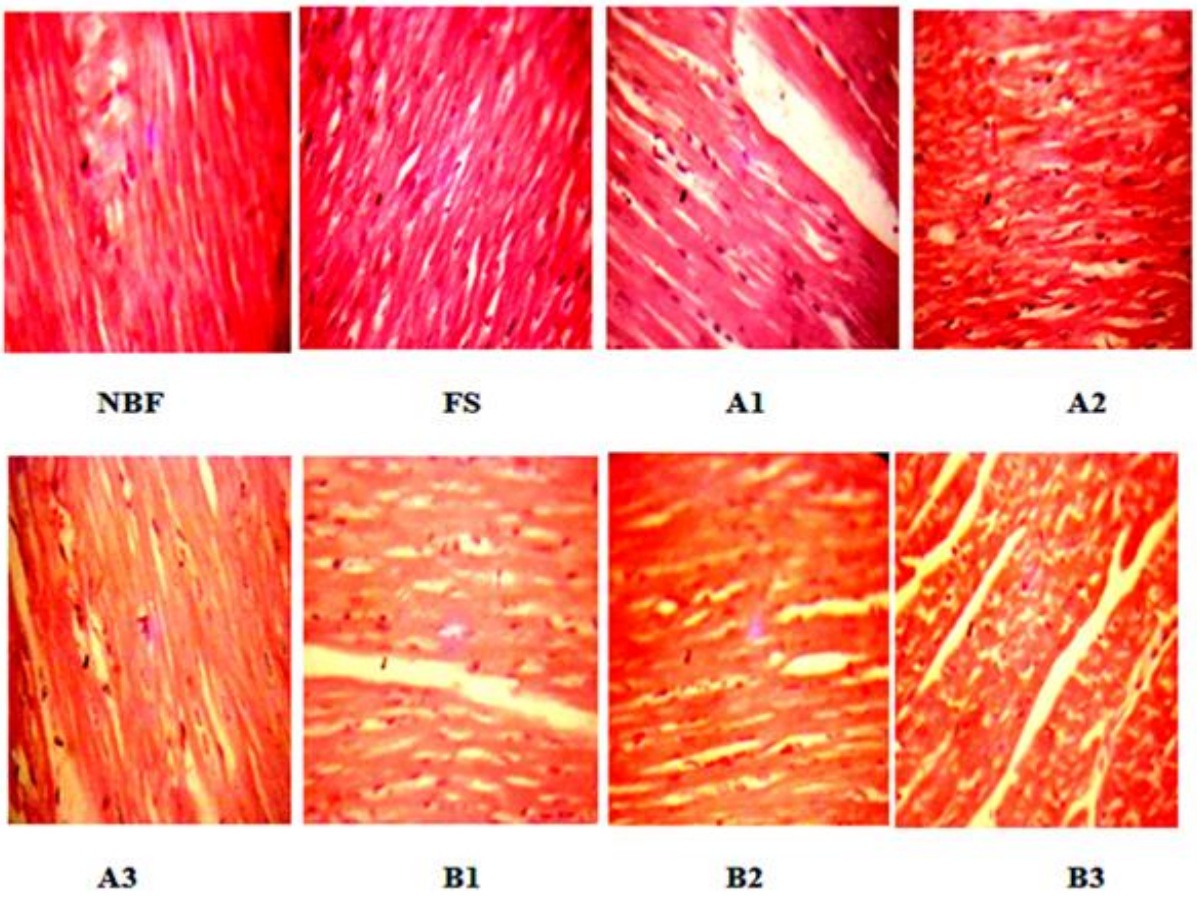


Plate 4.7: Photomicrographs of H&E on heart for all fixatives (H&E X 400)

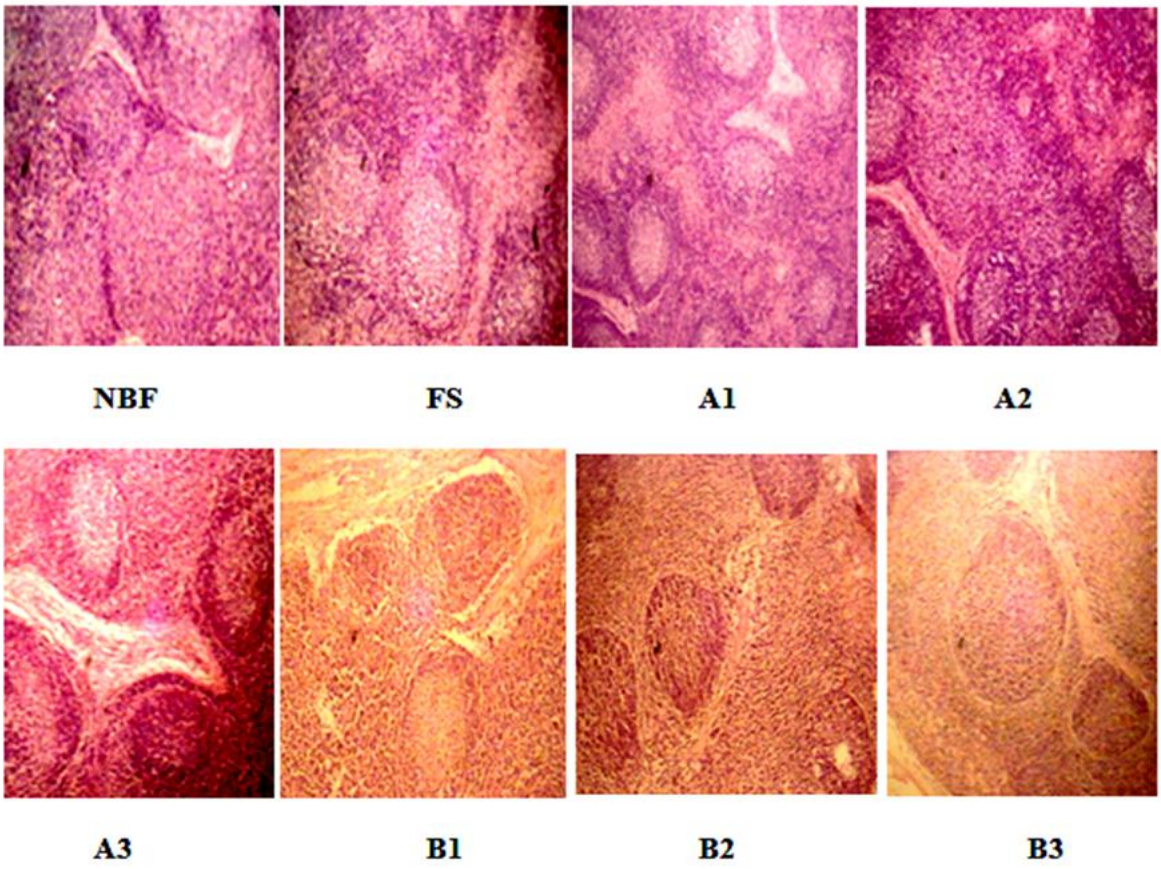
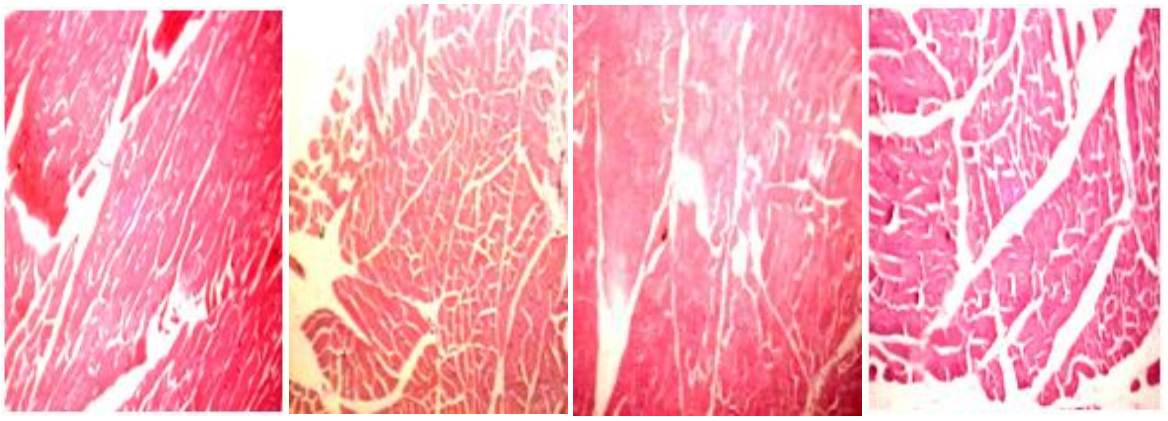
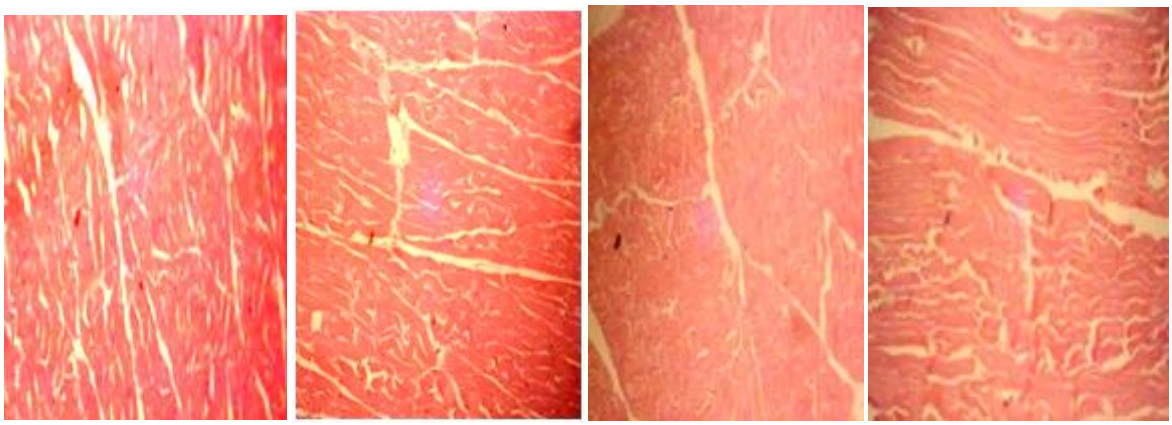


Plate 4.8: Photomicrographs of lymph nodes for all fixatives (H&E X 200)

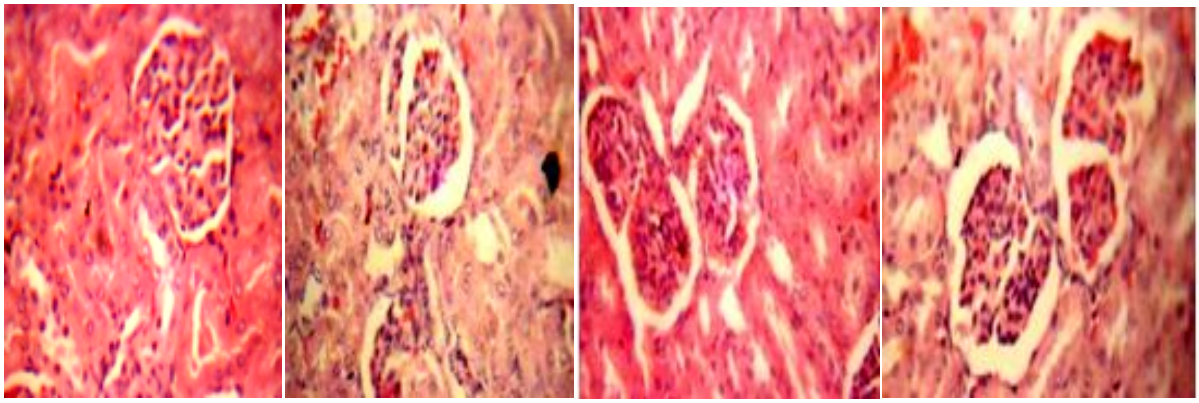


NBF **FS** **A1** **A2**

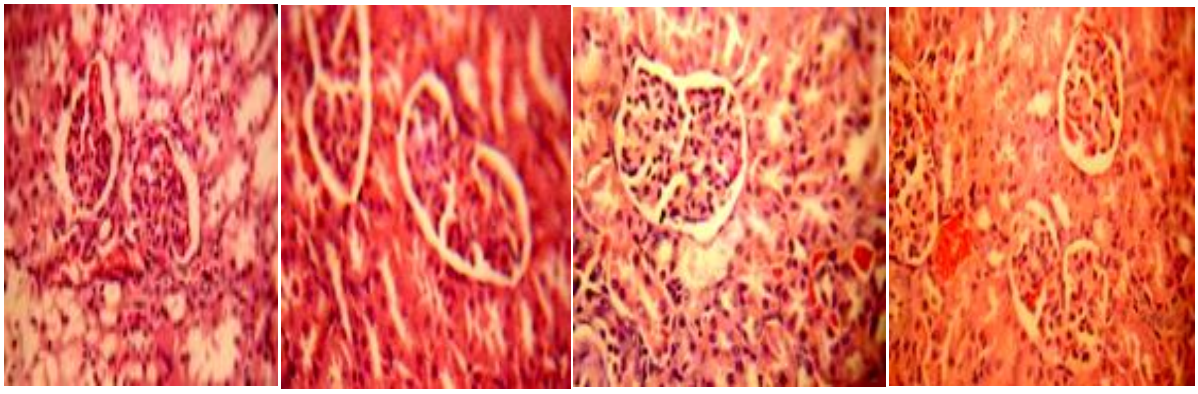


A3 **B1** **B2** **B3**

Plate 4.9: Photomicrographs of skeletal muscle for all fixatives (H&E X 200)



NBF **FS** **A1** **A2**



A3 **B1** **B2** **B3**

Plate 4.10: Photomicrographs of kidney for all fixatives (H&E X 400)

Table 4.3. Showing the overall average score, mean & median of averages, variance, standard deviation and p values of each fixative.

	NBF	FS	A1	A2	A3	B1	B2	B3
SKIN	**3.2**	**3.4**	**3.1**	**3.3**	**3.2**	**3.2**	**3.0**	**3.2**
BRAIN	**3.1**	**2.9**	**2.8**	**3.0**	**3.1**	**3.3**	**2.9**	**3.2**
LUNGS	**3.3**	**3.3**	**3.0**	**2.9**	**3.0**	**3.2**	**3.2**	**3.0**
HEART	**3.4**	**2.8**	**2.5**	**3.0**	**2.8**	**3.1**	**2.9**	**2.9**
LIVER	**3.3**	**2.9**	**2.8**	**2.8**	**2.8**	**3.1**	**2.8**	**3.1**
SPLEEN	**3.0**	**3.2**	**3.1**	**2.9**	**3.0**	**2.9**	**3.1**	**3.0**
KIDNEY	**3.3**	**3.0**	**3.1**	**3.3**	**3.3**	**2.8**	**3.5**	**3.1**
LYMPHNODES	**3.2**	**3.4**	**3.2**	**3.4**	**3.4**	**3.0**	**3.2**	**3.2**
SKELETAL MUSCLES	**3.4**	**3.1**	**3.2**	**2.7**	**3.2**	**3.0**	**3.0**	**2.9**
AVERAGE	**3.2**	**3.1**	**3.0**	**3.0**	**3.1**	**3.1**	**3.1**	**3.1**
VARIANCE	**0.02**	**0.05**	**0.05**	**0.06**	**0.04**	**0.03**	**0.04**	**0.02**
SD	**0.13**	**0.23**	**0.23**	**0.24**	**0.21**	**0.16**	**0.21**	**0.12**
P	**0.087**	**0.148**	**0.152**	**0.160**	**0.136**	**0.103**	**0.139**	**0.080**
MEAN OF AVERAGE	**3.1**							
MEDIAN OF AVERAGE	**3.1**							

Poor=1, Suboptimal=2, Good=3, Very Good=4

Table 4.4: showing mean, variance and p value of each fixative.

FIXATIVES	MEAN	VARIANCE	P
NBF	3.2	0.02	0.087
FS	3.1	0.05	0.148
A1	3.0	0.05	0.152
A2	3.0	0.06	0.160
A3	3.1	0.04	0.136
B1	3.1	0.03	0.103
B2	3.1	0.04	0.139
B3	3.1	0.02	0.080

Poor=1, Suboptimal=2, Good=3, Very Good=4

Table 4.5: Hypothesis testing using SPSS21 gave the following results: for individual fixatives;

NULL HYPOTHESIS	TEST	SIG.	DECISION
The categories of NBF occur with equal probabilities.	One-Sample Chi-Square Test	0.817	Retain the null hypothesis
The categories of FS occur with equal probabilities.	One-Sample Chi-Square Test	0.981	Retain the null hypothesis
The categories of A1 occur with equal probabilities.	One-Sample Chi-Square Test	0.817	Retain the null hypothesis
The categories of A2 occur with equal probabilities.	One-Sample Chi-Square Test	0.963	Retain the null hypothesis
The categories of A3 occur with equal probabilities.	One-Sample Chi-Square Test	0.963	Retain the null hypothesis
The categories of B1 occur with equal probabilities.	One-Sample Chi-Square Test	0.963	Retain the null hypothesis
The categories of B2 occur with equal probabilities.	One-Sample Chi-Square Test	0.963	Retain the null hypothesis
The categories of B3 occur with equal probabilities.	One-Sample Chi-Square Test	0.954	Retain the null hypothesis

Asymptotic significances are displayed. The significance level is 0.05

Table 4.6: For average scores of fixatives: Hypothesis Test Summary

NULL HYPOTHESIS	TEST	SIG.	DECISION
The categories of Fixatives occur with equal probabilities.	One-Sample Chi-Square Test	1.000	Retain the null hypothesis
The distribution of Scores is normal with mean 3.088 and standard deviation 0.06.	One-Sample Kolmogorov-Smirnov Test	0.358	Retain the null hypothesis

Asymptotic significances are displayed. The significance level is 0.05

Table 4.7: For total percentages of fixatives: Hypothesis Test Summary

NULL HYPOTHESIS	TEST	SIG.	DECISION
The categories of Fixatives occur with equal probabilities.	One-Sample Chi-Square Test	1.000	Retain the null hypothesis
The distribution of Scores is normal with mean 12.488 and standard deviation 0.32.	One-Sample Kolmogorov-Smirnov Test	0.752	Retain the null hypothesis

Asymptotic significances are displayed. The significance level is 0.05

Table 4.8: Two-Way ANOVA test for total percentages of tissue preservation qualities and qualities of staining: Hypothesis Test Summary

NULL HYPOTHESIS	TEST	SIG.	DECISION
The distributions of NBF, FS, A1, A2, A3, B1, B2 and B3 are the same.	Related Samples Friedman's Two-Way Analysis of Variance by Ranks	0.129	Retain the null hypothesis

Asymptotic significances are displayed. The significance level is 0.05

4.8. H&E RESULTS BASED ON TISSUE TYPE.

Table 4.9: Quality of tissue preservation grade of each fixative on skin.

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE (%)
I-Sk-15	0	0	12	4	16	12.40
L-Sk-15	0	0	6	12	18	13.95
A1-Sk-15	0	2	9	4	15	11.63
A2-Sk-15	0	0	9	8	17	13.18
A3-Sk-15	0	0	9	8	17	13.18
B1-Sk-15	0	0	15	0	15	11.63
B2-Sk-15	0	0	15	0	15	11.63
B3-Sk-15	0	0	12	4	16	12.40
TOTAL	**0**	**2**	**87**	**40**	**129**	**100.00**

Table 4.10: Quality of staining of skin (grading)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE (%)
I-Sk-15	0	0	12	4	16	12.60
L-Sk-15	0	0	12	4	16	12.60
A1-Sk-15	0	0	12	4	16	12.60
A2-Sk-15	0	0	12	4	16	12.60
A3-Sk-15	0	0	15	0	15	11.81
B1-Sk-15	0	0	9	8	17	13.39
B2-Sk-15	0	0	15	0	15	11.81
B3-Sk-15	0	0	12	4	16	12.60
TOTAL	0	0	99	28	127	100.00

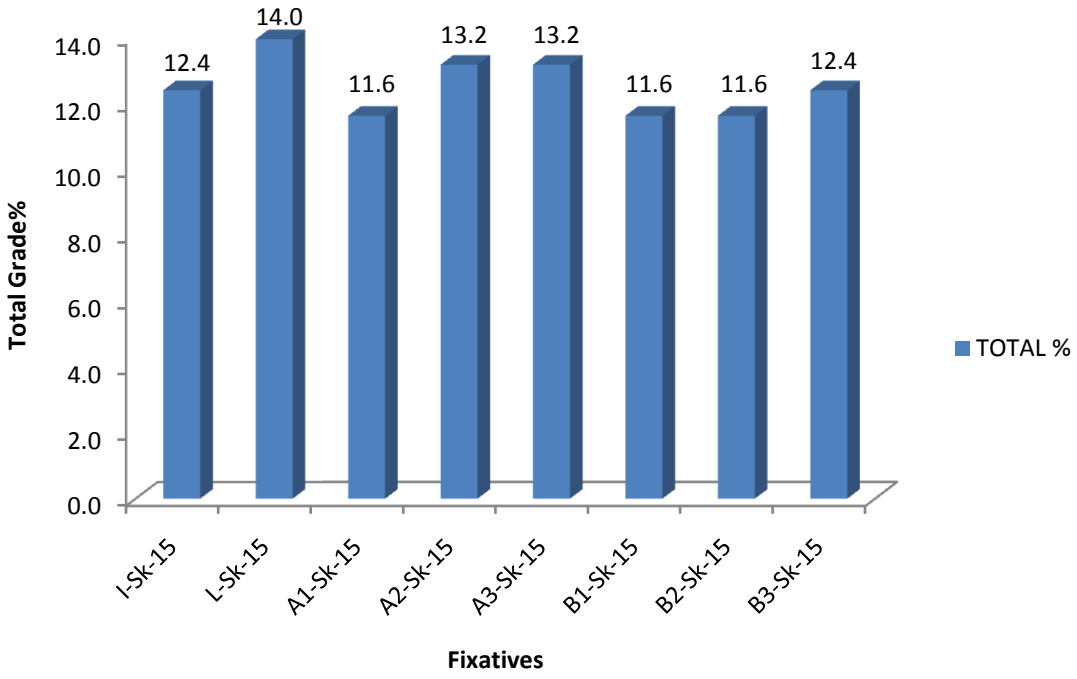


Fig.4.3: Bar chart showing quality of tissue preservation total grade% (skin)

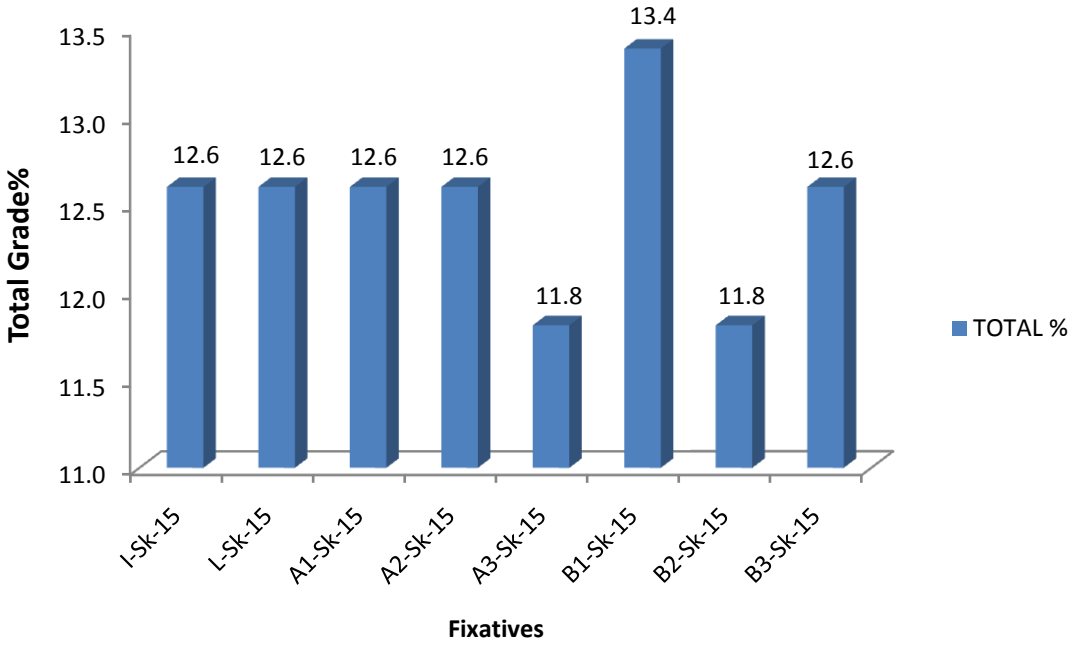


Fig.4.4: Bar chart showing quality of staining grading (skin)

Table 4.11: Quality of tissue preservation on the brain (grading)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE (%)
I-Br-15	0	0	12	4	16	12.90
L-Br-15	0	2	9	4	15	12.10
A1-Br-15	0	0	15	0	15	12.10
A2-Br-15	0	2	9	4	15	12.10
A3-Br-15	0	0	15	0	15	12.10
B1-Br-15	0	0	9	8	17	13.71
B2-Br-15	0	0	15	0	15	12.10
B3-Br-15	0	0	12	4	16	12.90
TOTAL	0	4	96	24	124	100.00

Table 4.12: Quality of staining of the brain (grading)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE (%)
I-Br-15	0	0	15	0	15	12.61
L-Br-15	0	2	12	0	14	11.76
A1-Br-15	0	4	9	0	13	10.92
A2-Br-15	0	0	15	0	15	12.61
A3-Br-15	0	0	12	4	16	13.45
B1-Br-15	0	0	12	4	16	13.45
B2-Br-15	0	2	12	0	14	11.76
B3-Br-15	0	0	12	4	16	13.45
TOTAL	0	8	99	12	119	100.00

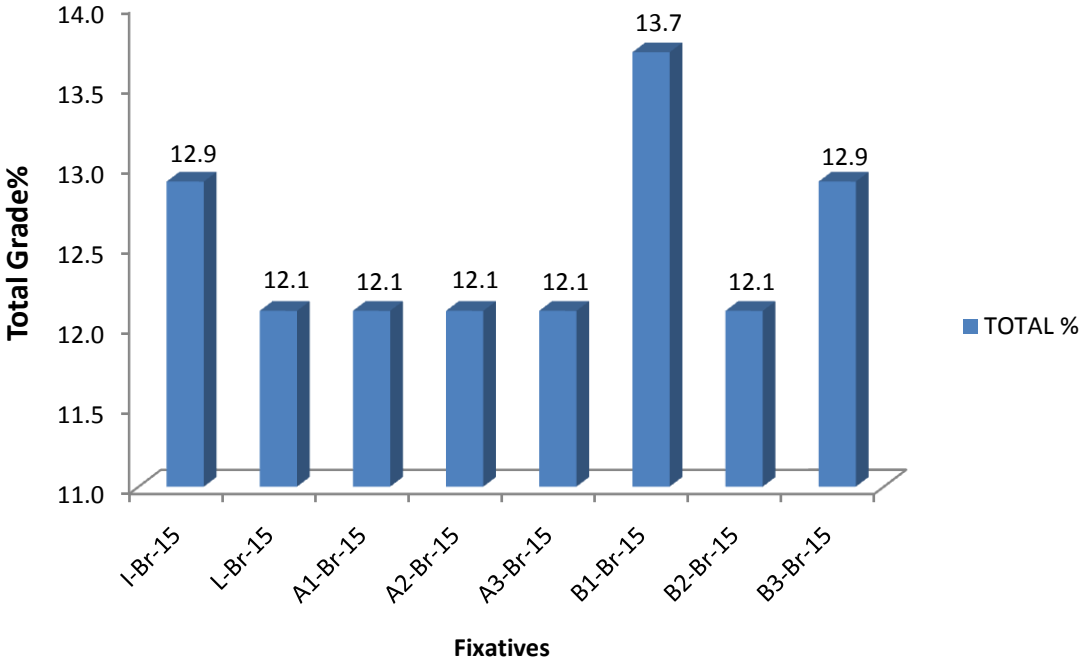


Fig. 4.5: Bar chart showing quality of tissue preservation grade% (brain)

The brain; for tissue preservation properties B1 was the best with a percentage grade of 13.7%, second best NBF and B3 12.9% each, third best a were others with 11.6% each.

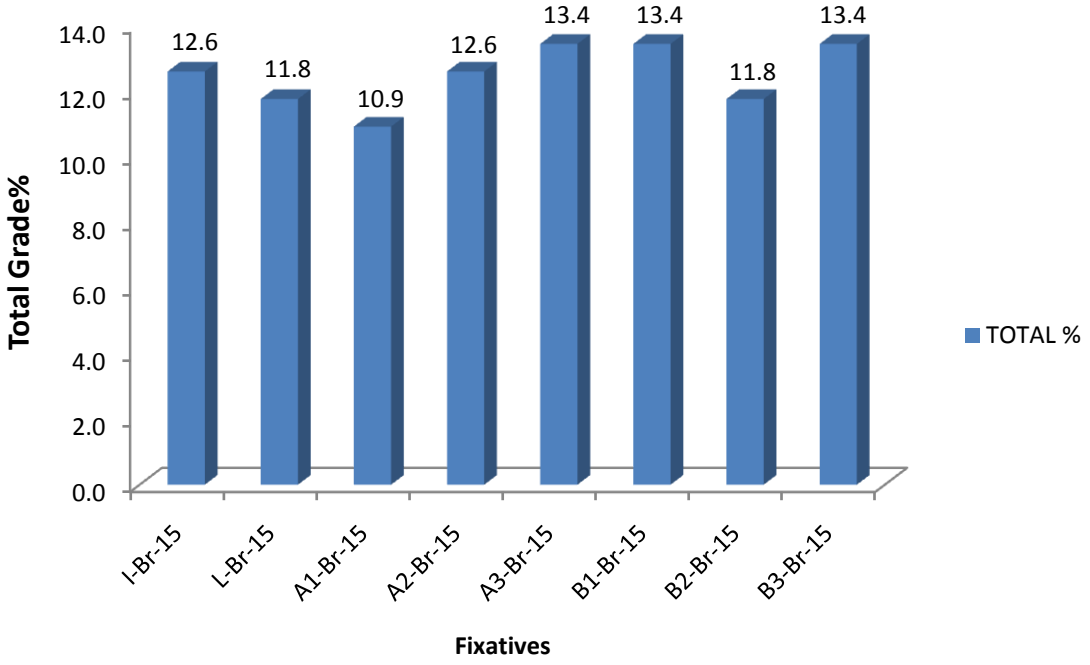


Fig. 4.6: Bar chart showing quality of staining grading (brain)

Table 4.13: Quality of tissue preservation of the lungs (grading)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE (%)
I-Lu-15	0	2	12	0	14	11.67
L-Lu-15	0	0	9	8	17	14.17
A1-Lu-15	0	2	9	4	15	12.50
A2-Lu-15	0	2	12	0	14	11.67
A3-Lu-15	0	0	15	0	15	12.50
B1-Lu-15	0	0	12	4	16	13.33
B2-Lu-15	0	0	15	0	15	12.50
B3-Lu-15	0	2	12	0	14	11.67
TOTAL	0	8	96	16	120	100.00

Table 4.14: Quality of staining of the lungs (grading)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE (%)
I-Lu-15	0	0	3	16	19	14.73
L-Lu-15	0	0	12	4	16	12.40
A1-Lu-15	0	0	15	0	15	11.63
A2-Lu-15	0	0	15	0	15	11.63
A3-Lu-15	0	0	15	0	15	11.63
B1-Lu-15	0	0	12	4	16	12.40
B2-Lu-15	0	0	9	8	17	13.18
B3-Lu-15	0	0	12	4	16	12.40
TOTAL	0	0	93	36	129	100.00

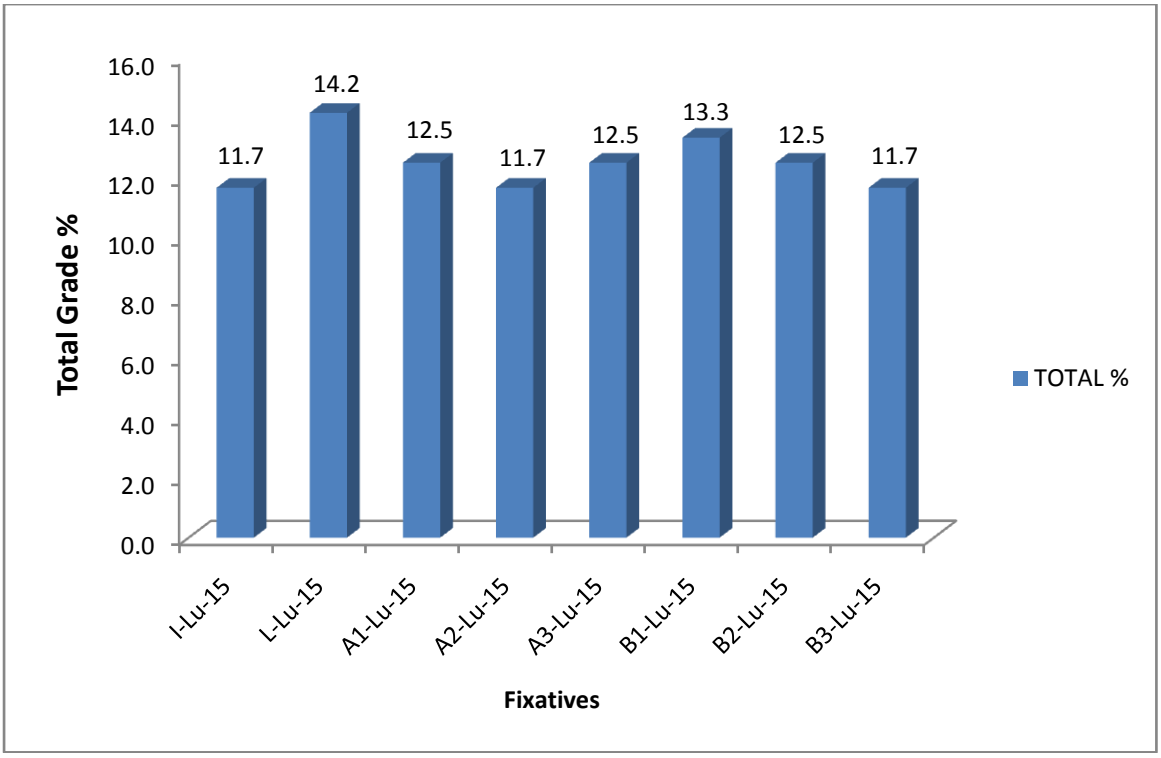


Fig. 4.7: Bar chart showing quality of tissue preservation grade% (lungs)

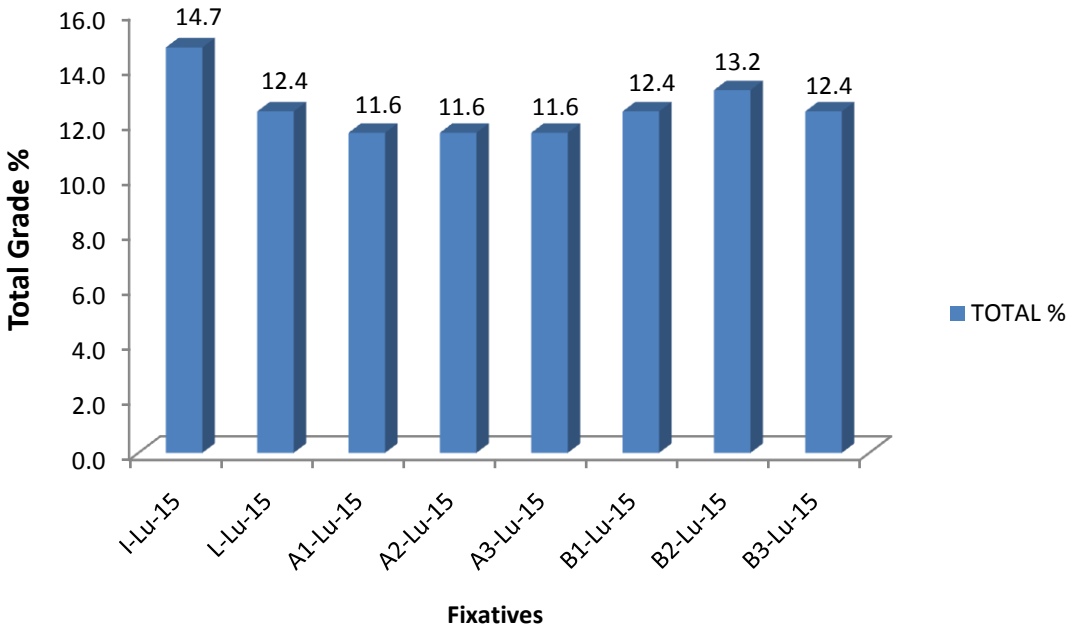


Fig. 4.8: Bar chart showing quality of staining grade% (lungs)

Table 4.15: Quality of tissue preservation of the liver (grading)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE (%)
I-Li-15	0	0	9	8	17	14.41
L-Li-15	0	2	12	0	14	11.86
A1-Li-15	0	0	15	0	15	12.71
A2-Li-15	0	2	12	0	14	11.86
A3-Li-15	1	0	12	0	13	11.02
B1-Li-15	0	0	15	0	15	12.71
B2-Li-15	0	2	12	0	14	11.86
B3-Li-15	0	0	12	4	16	13.56
TOTAL	1	6	99	12	118	100.00

Table 4.16: Quality of staining of the liver (grading)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE (%)
I-Li-15	0	0	12	4	16	13.56
L-Li-15	0	0	15	0	15	12.71
A1-Li-15	0	4	9	0	13	11.02
A2-Li-15	0	2	12	0	14	11.86
A3-Li-15	0	0	15	0	15	12.71
B1-Li-15	0	0	12	4	16	13.56
B2-Li-15	0	2	12	0	14	11.86
B3-Li-15	0	0	15	0	15	12.71
TOTAL	0	8	102	8	118	100.00

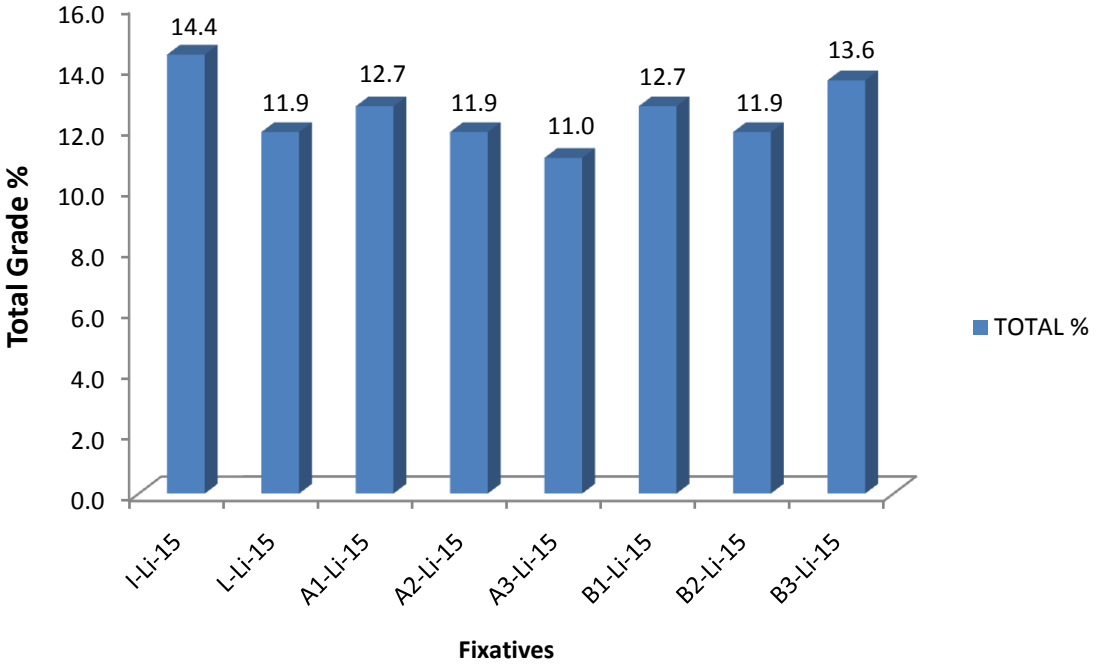


Fig. 4.9: Bar chart showing quality of tissue preservation grading (liver)

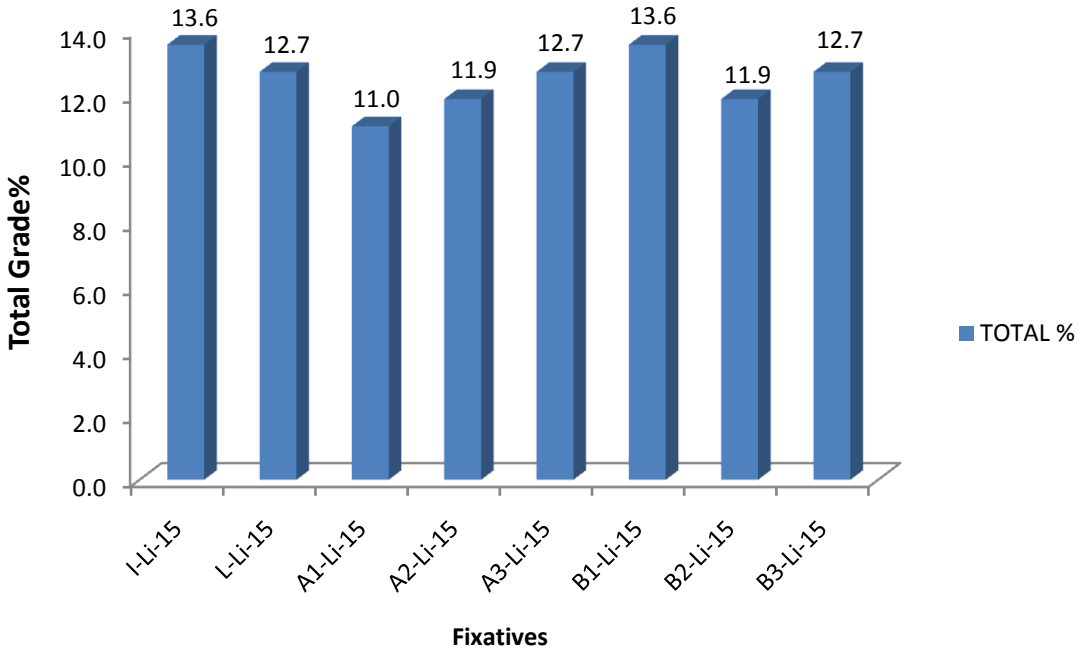


Fig. 4.10: Bar chart showing quality of staining grading (liver)

Table 4.17: Quality of tissue preservation of the spleen (grading)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE (%)
I-Sp-15	0	0	15	0	15	12.40
L-Sp-15	0	0	12	4	16	13.22
A1-Sp-15	0	0	12	4	16	13.22
A2-Sp-15	0	2	12	0	14	11.57
A3-Sp-15	0	0	15	0	15	12.40
B1-Sp-15	0	2	12	0	14	11.57
B2-Sp-15	0	0	12	4	16	13.22
B3-Sp-15	0	2	9	4	15	12.40
TOTAL	0	6	99	16	121	100.00

Table 4.18: Quality of staining of the spleen (grading)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE (%)
I-Sp-15	0	0	15	0	15	12.40
L-Sp-15	0	0	12	4	16	13.22
A1-Sp-15	0	0	15	0	15	12.40
A2-Sp-15	0	0	15	0	15	12.40
A3-Sp-15	0	0	15	0	15	12.40
B1-Sp-15	0	0	15	0	15	12.40
B2-Sp-15	0	0	15	0	15	12.40
B3-Sp-15	0	2	9	4	15	12.40
TOTAL	0	2	111	8	121	100.00

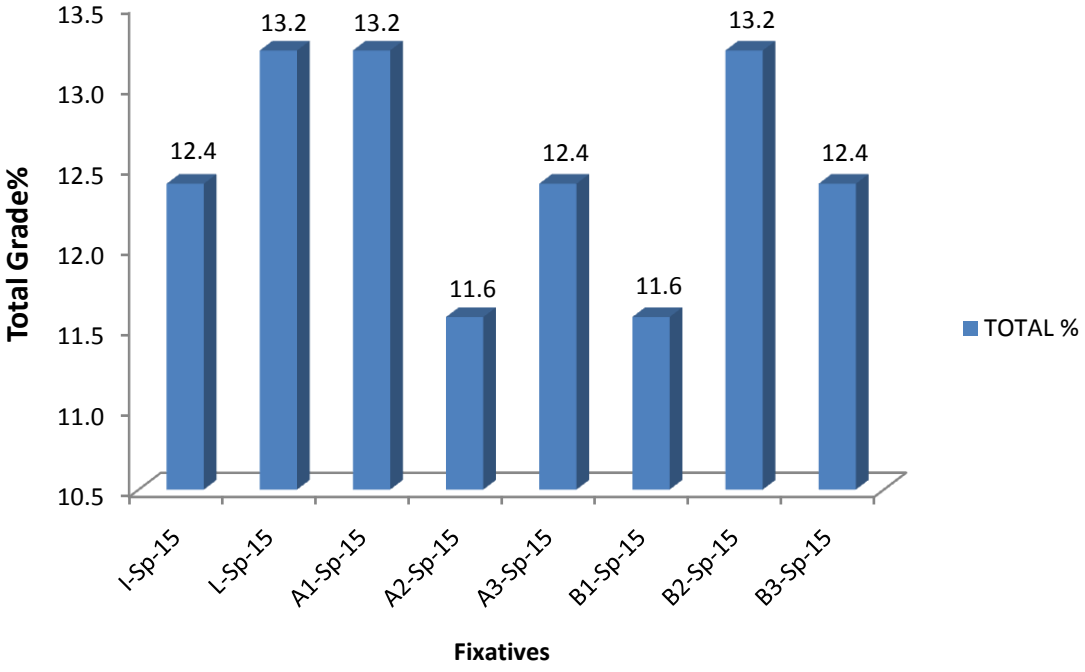


Fig. 4.11: Bar chart showing quality of tissue preservation grading (spleen)

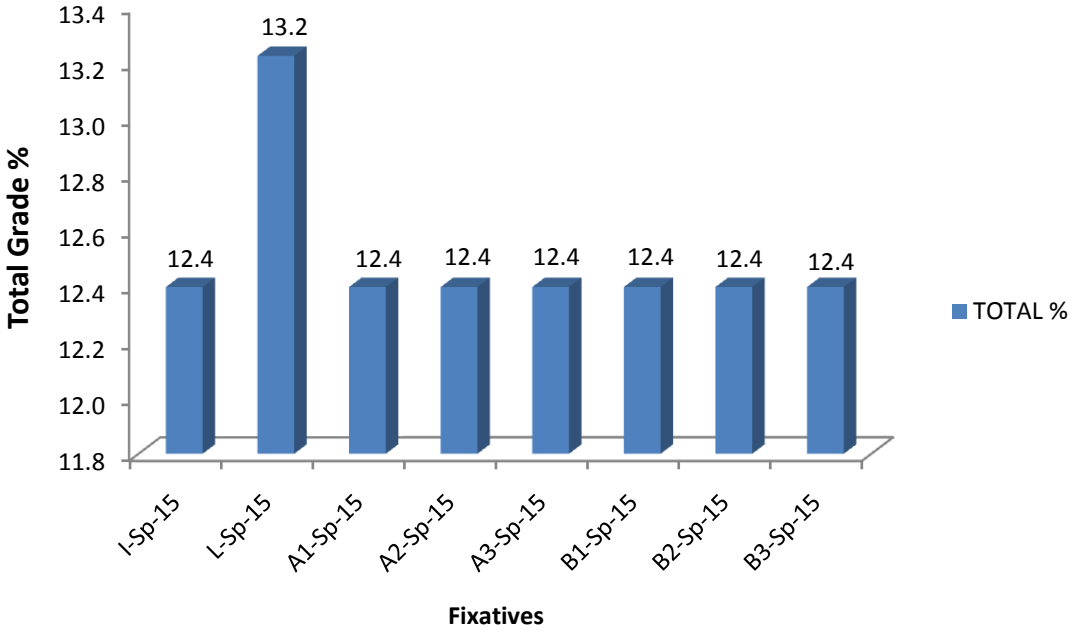


Fig. 4.12: Bar chart showing quality of staining grading (spleen)

Table 4.19: Quality of tissue preservation of the heart (grading)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE (%)
I-Ht-15	0	0	6	12	18	15.25
L-Ht-15	0	2	12	0	14	11.86
A1-Ht-15	1	2	9	0	12	10.17
A2-Ht-15	0	2	6	8	16	13.56
A3-Ht-15	0	2	12	0	14	11.86
B1-Ht-15	0	0	12	4	16	13.56
B2-Ht-15	0	2	12	0	14	11.86
B3-Ht-15	0	2	12	0	14	11.86
TOTAL	1	12	81	24	118	100.00

Table 4.20: Quality of staining of heart (grading)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE (%)
I-Ht-15	0	0	12	4	16	13.79
L-Ht-15	0	2	12	0	14	12.07
A1-Ht-15	0	4	9	0	13	11.21
A2-Ht-15	0	2	12	0	14	12.07
A3-Ht-15	0	2	12	0	14	12.07
B1-Ht-15	0	0	15	0	15	12.93
B2-Ht-15	0	0	15	0	15	12.93
B3-Ht-15	0	0	15	0	15	12.93
TOTAL	0	10	102	4	116	100.00

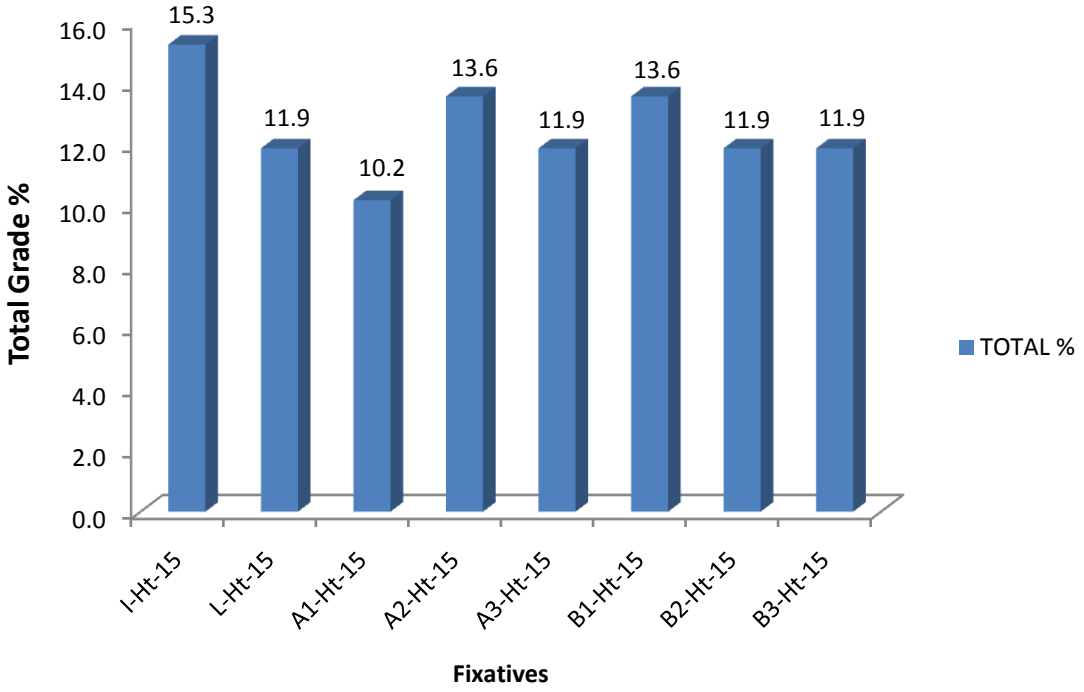


Fig. 4.13: Bar chart showing quality of tissue preservation grade% (heart)

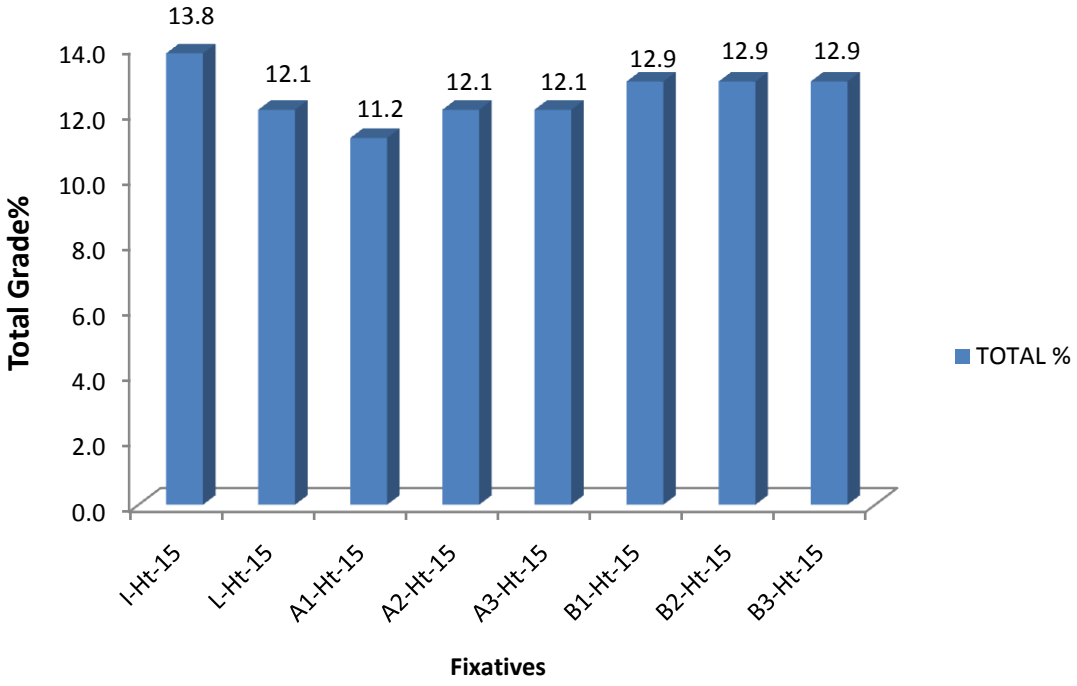


Fig. 4.14: Bar chart showing quality of staining grade % (heart)

Table 4.21: Quality of tissue preservation of the lymph node (grading)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE (%)
I-Ln-15	0	2	6	8	16	12.40
L-Ln-15	0	0	9	8	17	13.18
A1-Ln-15	0	0	12	4	16	12.40
A2-Ln-15	0	0	12	4	16	12.40
A3-Ln-15	0	0	9	8	17	13.18
B1-Ln-15	0	0	15	0	15	11.63
B2-Ln-15	0	0	12	4	16	12.40
B3-Ln-15	0	0	12	4	16	12.40
TOTAL	0	2	87	40	129	100.00

Table 4.22: Quality of staining of the lymph node (grading)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE (%)
I-Ln-15	0	0	12	4	16	12.21
L-Ln-15	0	0	9	8	17	12.98
A1-Ln-15	0	0	12	4	16	12.21
A2-Ln-15	0	0	6	12	18	13.74
A3-Ln-15	0	0	9	8	17	12.98
B1-Ln-15	0	0	15	0	15	11.45
B2-Ln-15	0	0	12	4	16	12.21
B3-Ln-15	0	0	12	4	16	12.21
TOTAL	0	0	87	44	131	100.00

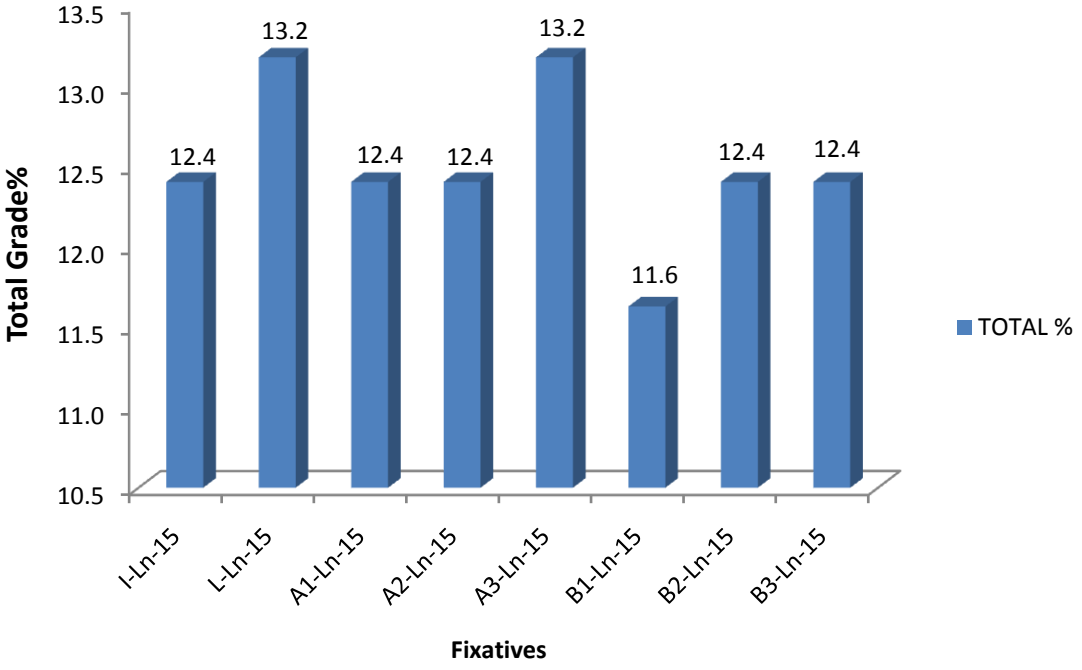


Fig. 4.15: Bar chart showing quality of tissue preservation grade% (lymph node)

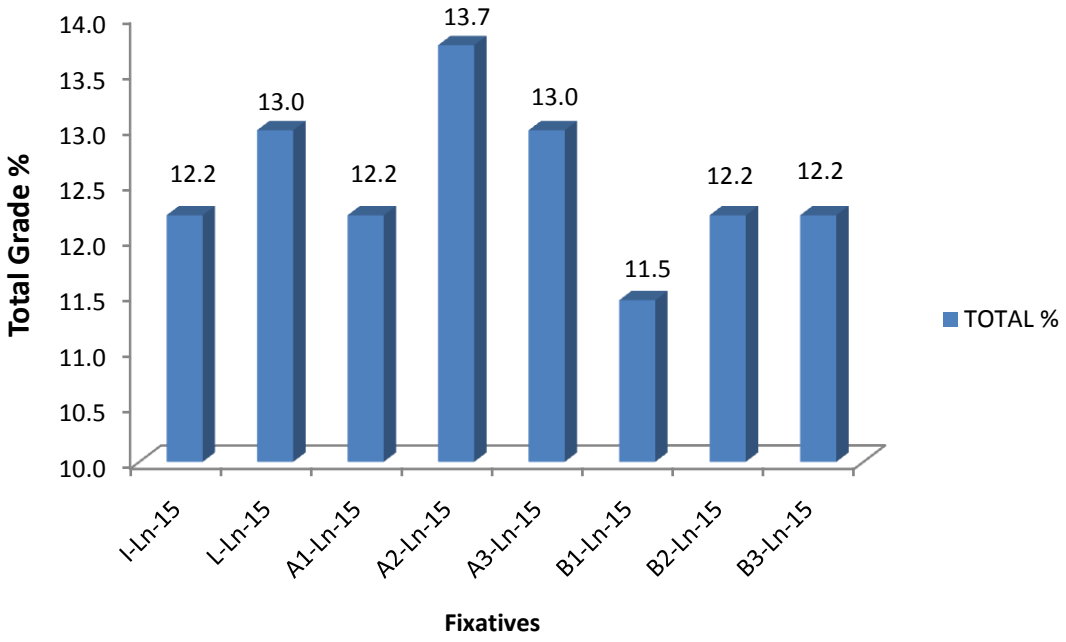


Fig. 4.16: Bar chart showing quality of staining grade % (lymph node)

Table 4.23: Quality of tissue preservation of the skeletal muscle (grading)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE (%)
I-Sm-15	0	0	6	12	18	14.75
L-Sm-15	0	2	9	4	15	12.30
A1-Sm-15	0	0	12	4	16	13.11
A2-Sm-15	0	2	12	0	14	11.48
A3-Sm-15	0	0	15	0	15	12.30
B1-Sm-15	0	0	15	0	15	12.30
B2-Sm-15	0	0	15	0	15	12.30
B3-Sm-15	0	2	12	0	14	11.48
TOTAL	0	6	96	20	122	100.00

Table 4.24: Quality of staining of the skeletal muscle (grading)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE (%)
I-Sm-15	0	0	12	4	16	13.01
L-Sm-15	0	0	12	4	16	13.01
A1-Sm-15	0	0	12	4	16	13.01
A2-Sm-15	1	0	12	0	13	10.57
A3-Sm-15	0	0	9	8	17	13.82
B1-Sm-15	0	0	15	0	15	12.20
B2-Sm-15	0	0	15	0	15	12.20
B3-Sm-15	0	2	9	4	15	12.20
TOTAL	1	2	96	24	123	100.00

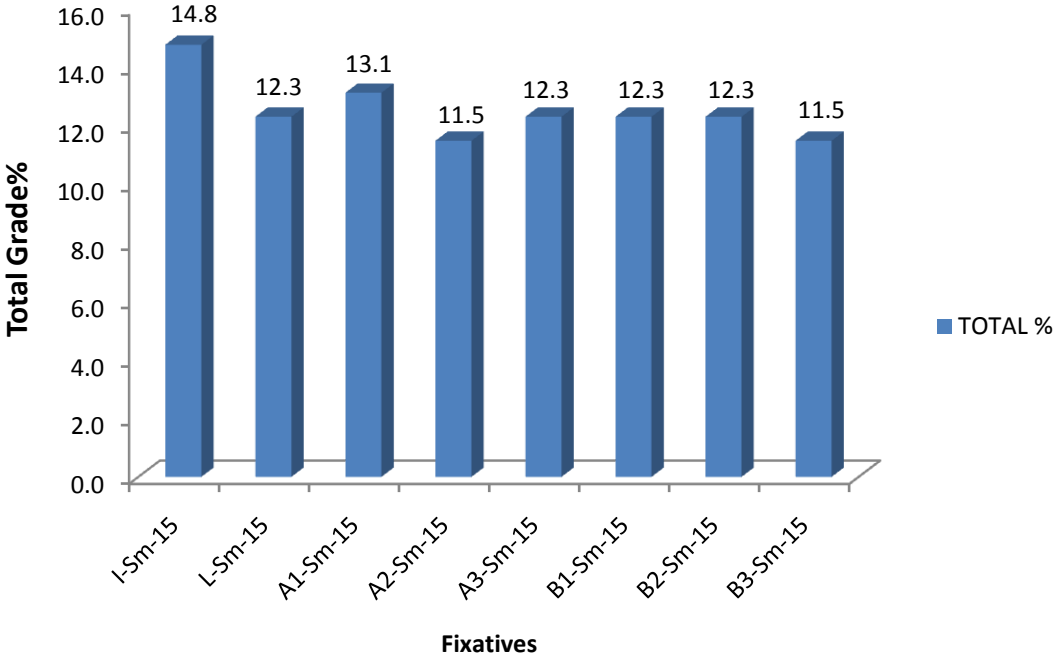


Fig. 4.17: Bar chart showing quality of tissue preservation grade% (skeletal muscle)

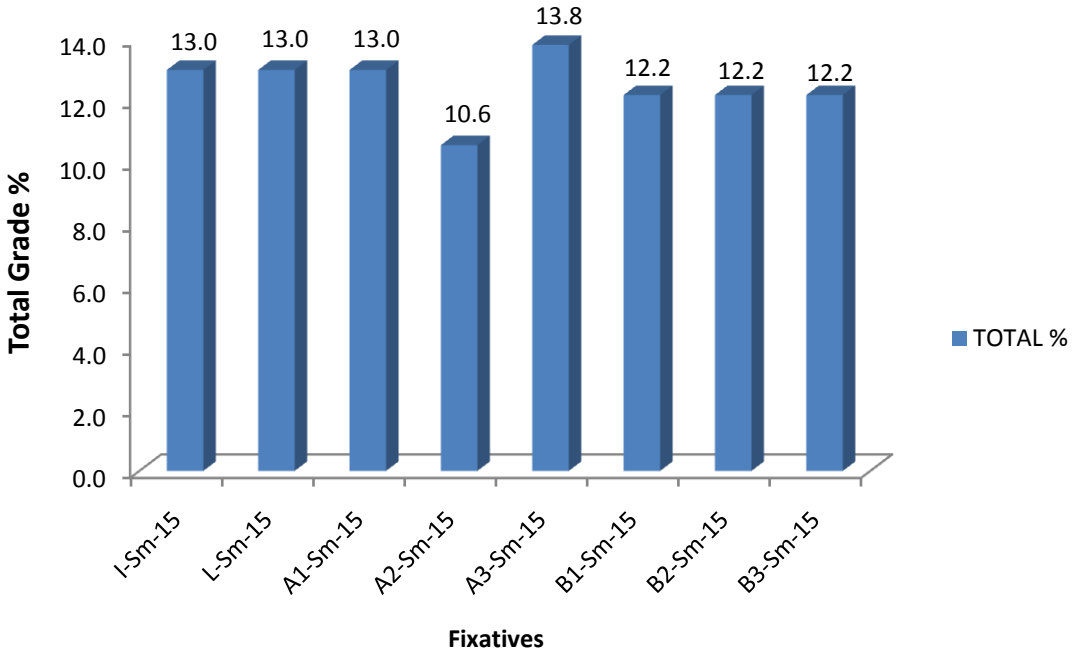


Fig. 4.18: Quality of staining grade % (skeletal muscle)

Table 4.25: Quality of tissue preservation of the kidney (grading)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE (%)
I-Kd-15	0	2	6	8	16	12.60
L-Kd-15	0	0	12	4	16	12.60
A1-Kd-15	0	2	9	4	15	11.81
A2-Kd-15	0	0	12	4	16	12.60
A3-Kd-15	0	0	12	4	16	12.60
B1-Kd-15	0	2	12	0	14	11.02
B2-Kd-15	0	0	6	12	18	14.17
B3-Kd-15	0	0	12	4	16	12.60
TOTAL	0	6	81	40	127	100.00

Table 4.26: Quality of staining grading of the kidney (grading)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE (%)
I-Kd-15	0	2	6	8	16	12.60
L-Kd-15	0	0	12	4	16	12.60
A1-Kd-15	0	2	9	4	15	11.81
A2-Kd-15	0	0	12	4	16	12.60
A3-Kd-15	0	0	12	4	16	12.60
B1-Kd-15	0	2	12	0	14	11.02
B2-Kd-15	0	0	6	12	18	14.17
B3-Kd-15	0	0	12	4	16	12.60
TOTAL	0	6	81	40	127	100.00

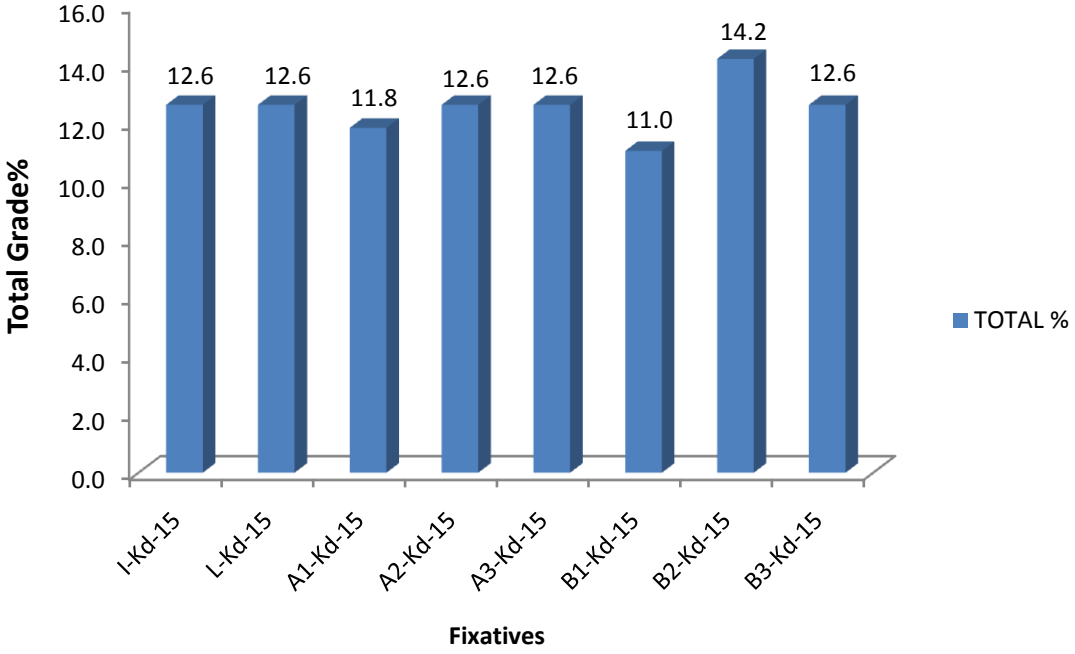


Fig. 4.19: Bar chart showing quality of tissue preservation grade% (kidney)

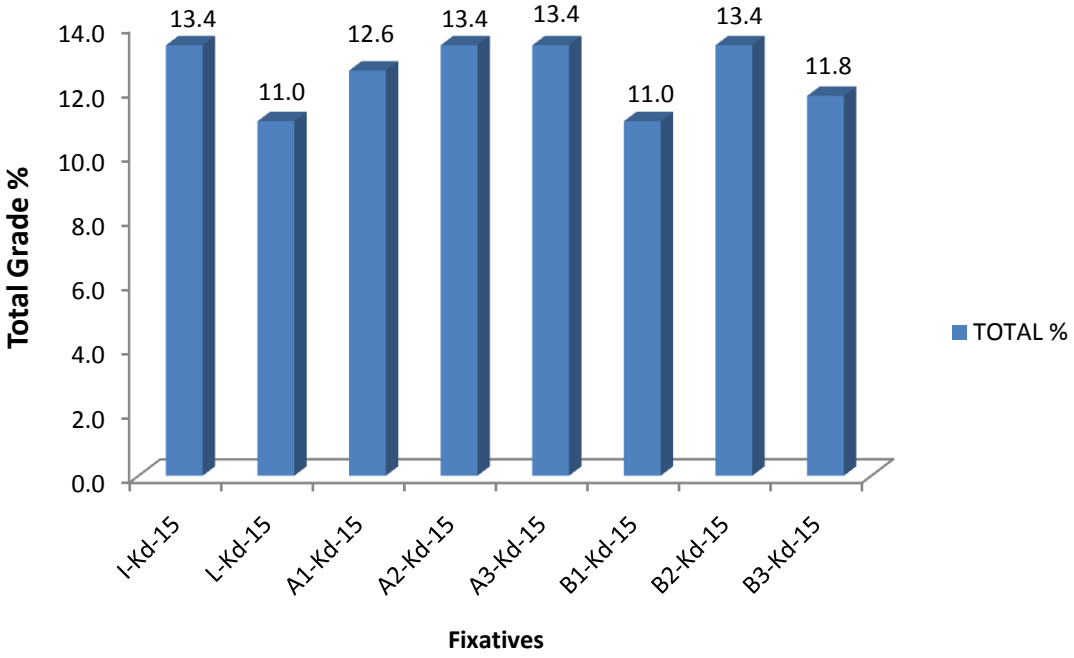


Fig. 4.20: Bar chart showing quality of staining grade % (kidney)

4.9. SPECIAL STAINS RESULTS (PAS & MASSONS FONTANA)

Table 4.27: Quality of tissue preservation grading on special stains

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE (%)
IP-Li-15	0	0	12	4	16	6.78
LP-Li-15	0	0	15	0	15	6.36
A1P-Li-15	0	0	15	0	15	6.36
A2P-Li-15	0	2	12	0	14	5.93
A3P-Li-15	0	2	9	4	15	6.36
B1P-Li-15	0	2	9	4	15	6.36
B2P-Li-15	0	2	9	4	15	6.36
B3P-Li-15	0	2	12	0	14	5.93
IM-Sm-15	0	0	12	4	16	6.78
LM-Sm-15	0	0	15	0	15	6.36
A1M-Sm-15	0	2	12	0	14	5.93
A2M-Sm-15	0	2	12	0	14	5.93
A3M-Sm-15	0	0	15	0	15	6.36
B1M-Sm-15	0	2	12	0	14	5.93
B2M-Sm-15	0	0	15	0	15	6.36
B3M-Sm-15	0	2	12	0	14	5.93
TOTAL	0	18	198	20	236	100

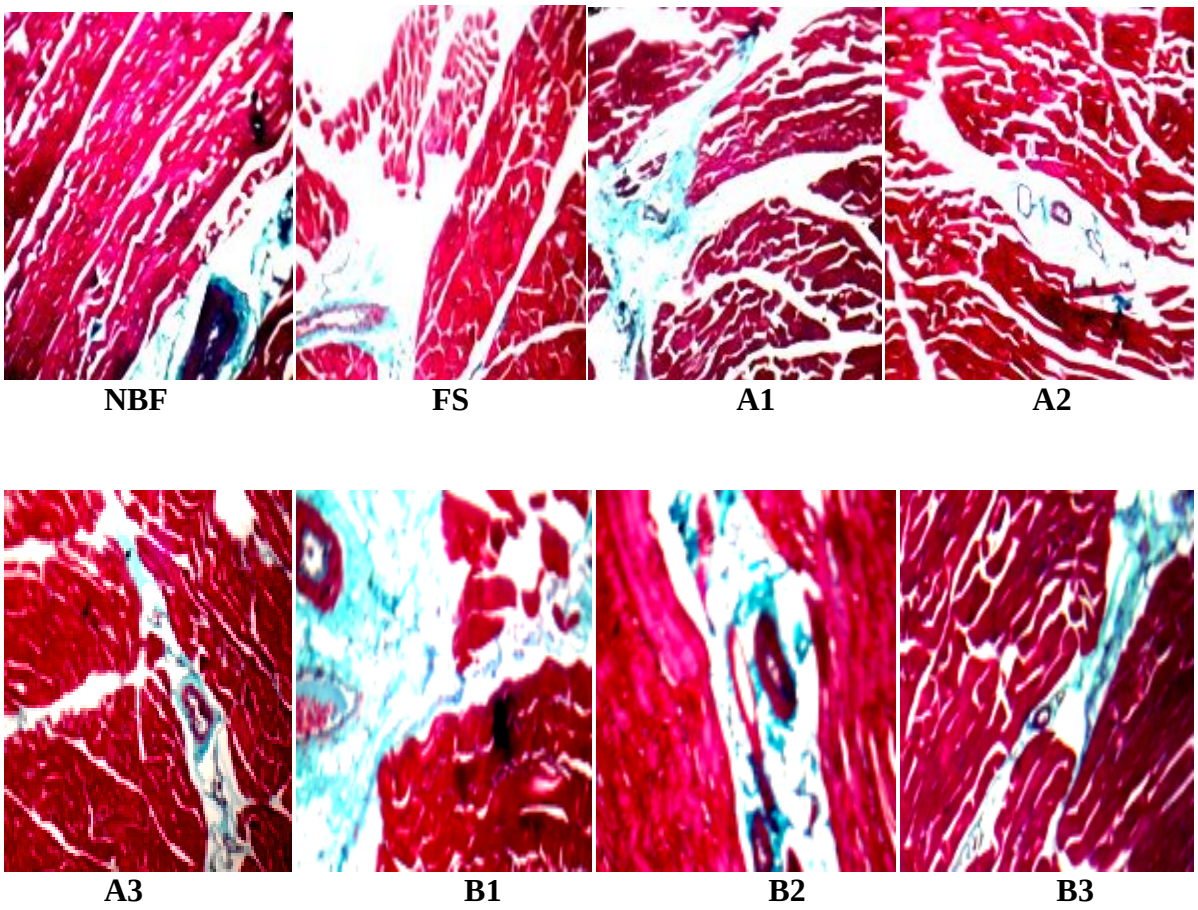


Plate 4.11: Photomicrographs of special stains (MT) on skeletal muscle for all the fixatives (x200)

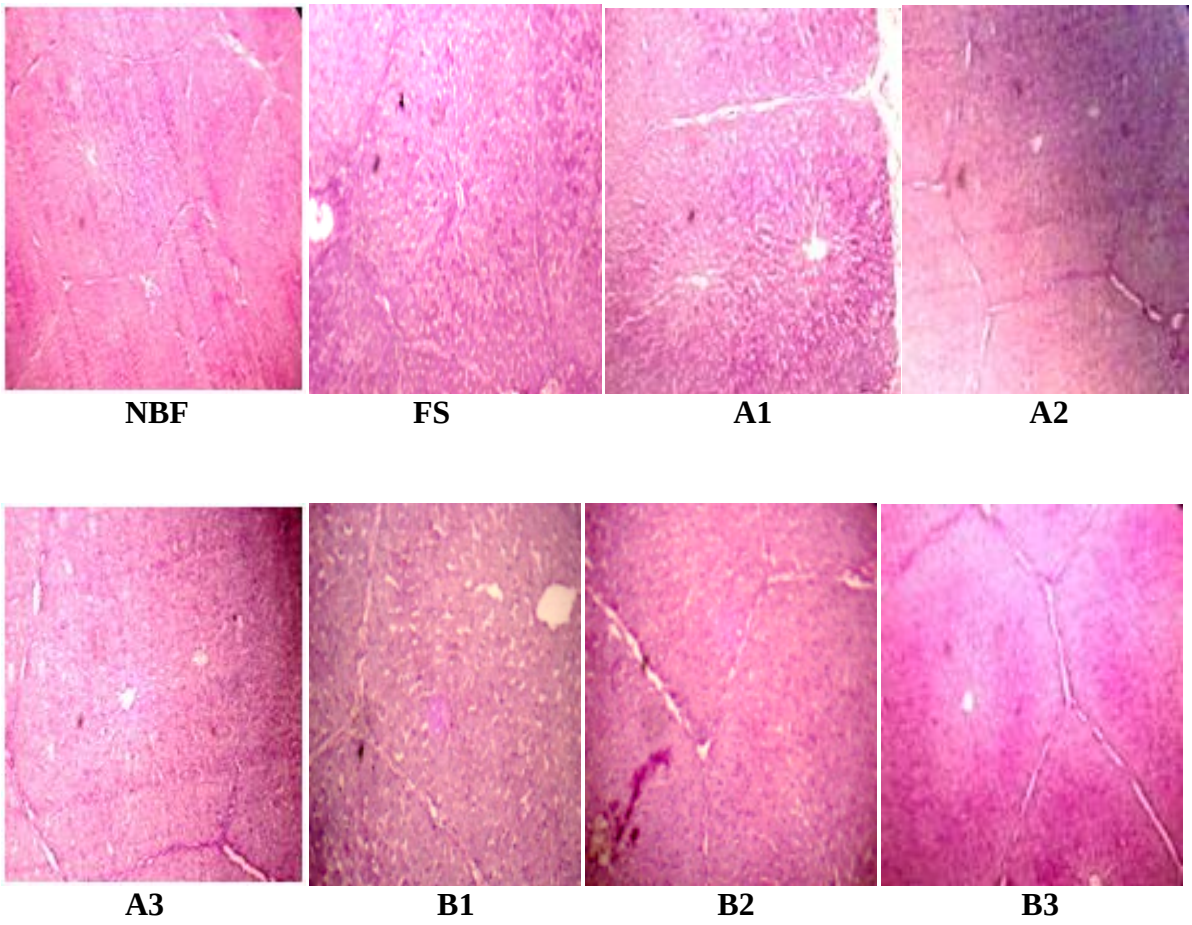


Plate 4.12: Photomicrographs of special stains (PAS) on liver for all the fixatives (x200)

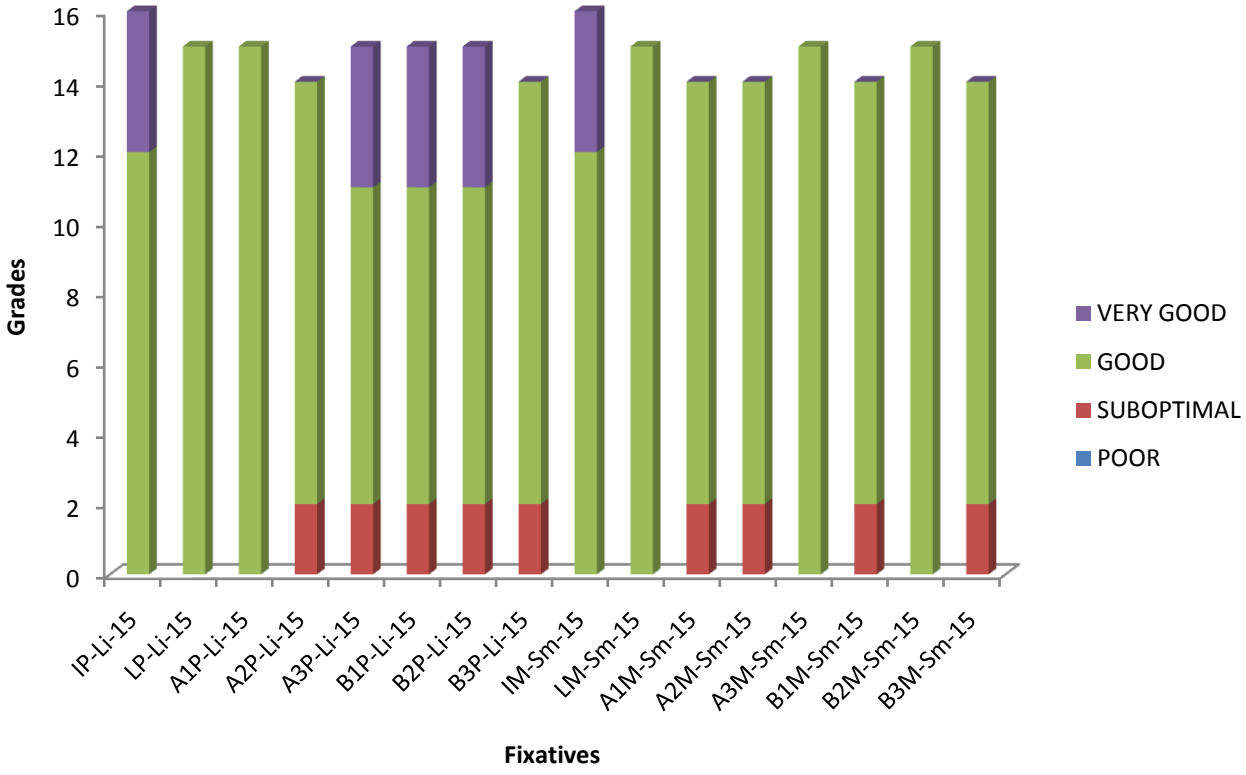


fig. 4.21: Bar chart showing quality of tissue preservation grading of each fixative on special stains.

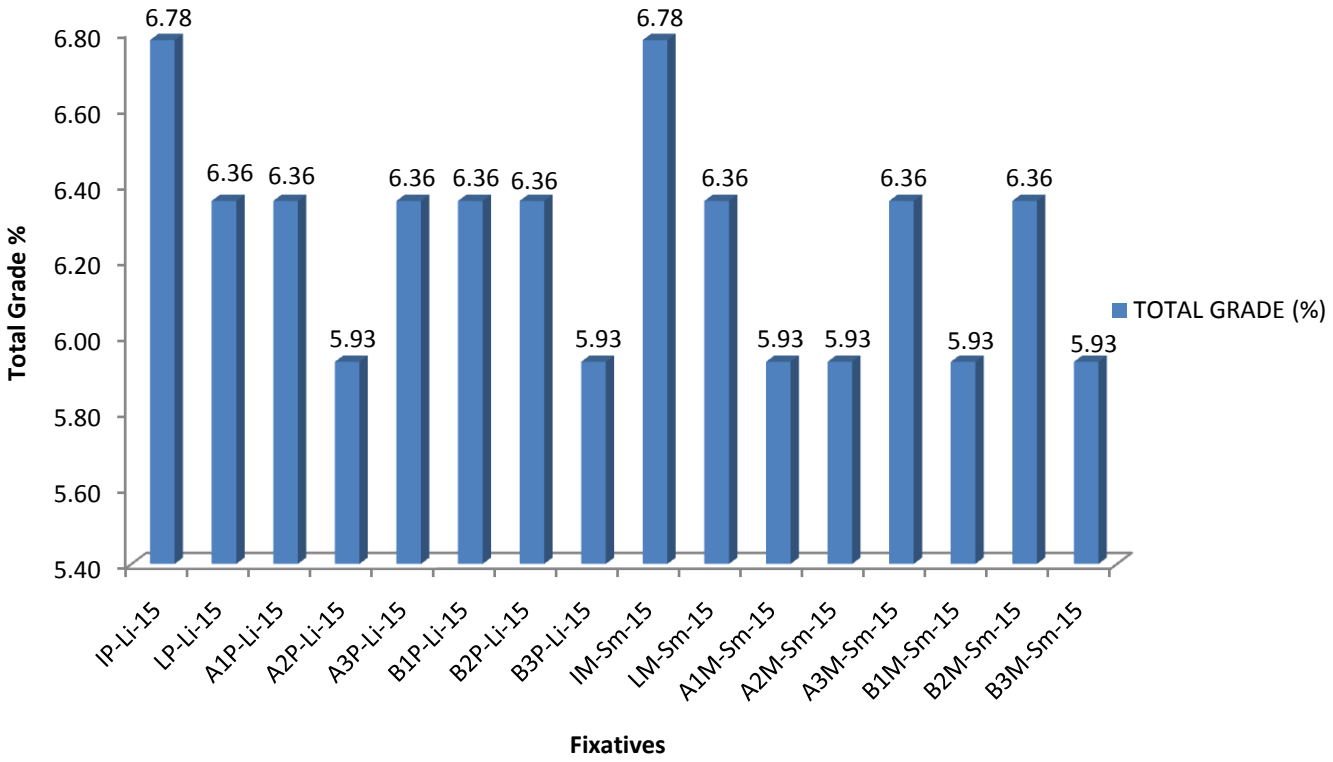


Fig. 4.22: Special stains quality of tissue preservation total grade (%)

Table 4.28: Quality of staining of PAS & MT (grading)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE(%)
IP-Li-15	0	0	15	0	15	5.93
LP-Li-15	0	0	12	4	16	6.32
A1P-Li-15	0	0	15	0	15	5.93
A2P-Li-15	0	0	15	0	15	5.93
A3P-Li-15	0	2	12	0	14	5.53
B1P-Li-15	0	2	9	4	15	5.93
B2P-Li-15	0	0	3	16	19	7.51
B3P-Li-15	0	2	12	0	14	5.53
IM-Sm-15	0	0	9	8	17	6.72
LM-Sm-15	0	0	6	12	18	7.11
A1M-Sm-15	0	0	9	8	17	6.72
A2M-Sm-15	0	0	12	4	16	6.32
A3M-Sm-15	0	0	12	4	16	6.32
B1M-Sm-15	0	0	9	8	17	6.72
B2M-Sm-15	0	2	12	0	14	5.53
B3M-Sm-15	0	0	15	0	15	5.93
TOTAL	0	8	177	68	253	100.00

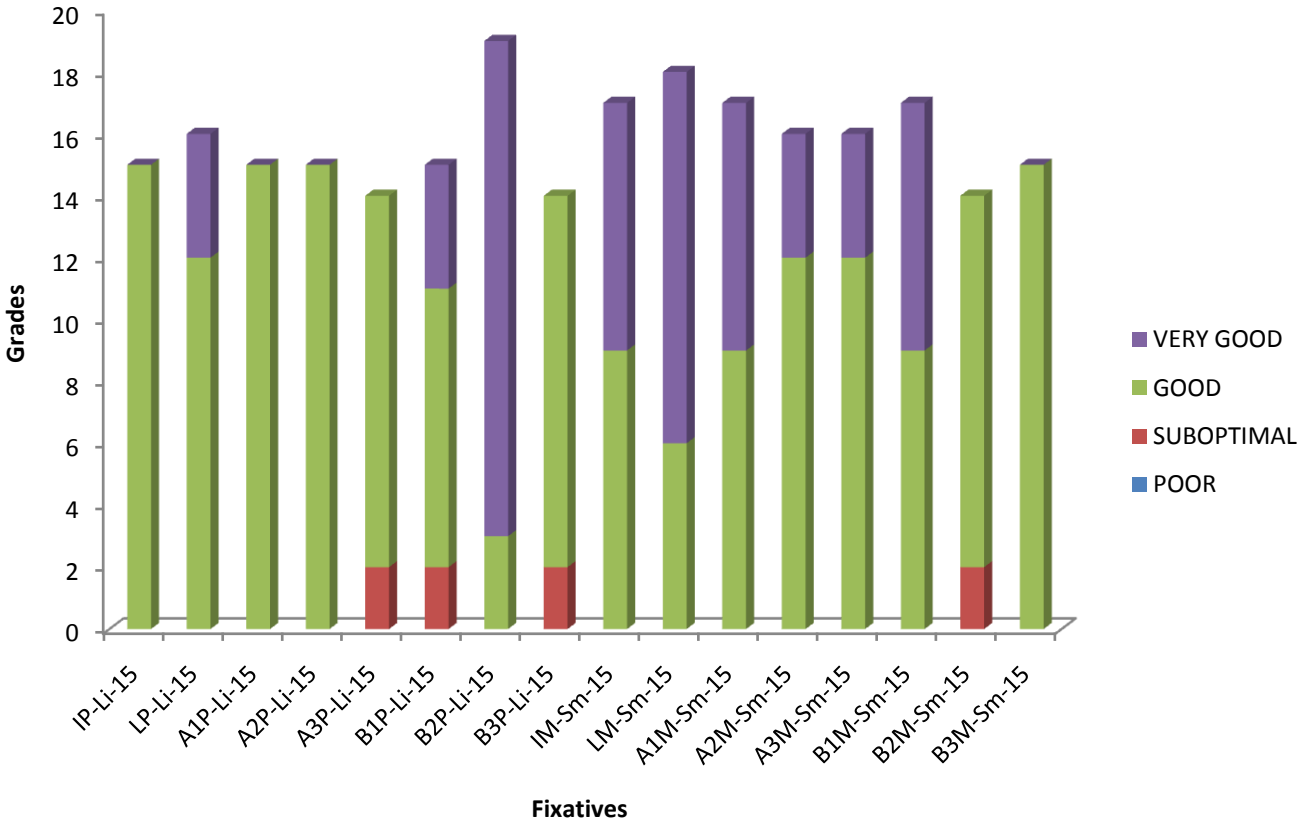


Fig. 4.23: Bar chart showing quality of staining grading (PAS & MT)

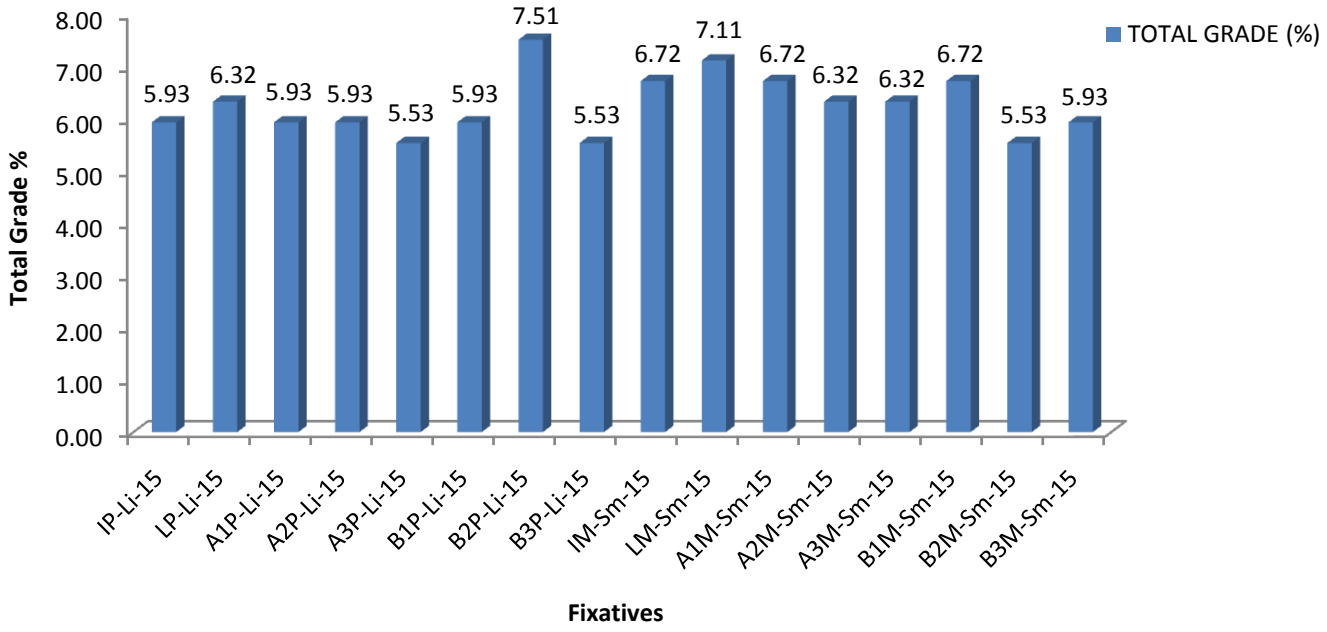


Fig. 4.24: Bar chart showing quality of staining grade% (PAS & MT)

4.10. IMMUNOHISTOCHEMISTRY (EMA & CD23) RESULTS

Table 4.29: Quality of tissue preservation grading (EMA & CD23)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE(%)
IE-Sk-15	0	0	12	4	16	6.18
LE-Sk-15	0	0	9	8	17	6.56
A1E-Sk-15	0	0	9	8	17	6.56
A2E-Sk-15	0	0	9	8	17	6.56
A3E-Sk-15	0	0	12	4	16	6.18
B1E-Sk-15	0	0	12	4	16	6.18
B2E-Sk-15	0	0	12	4	16	6.18
B3E-Sk-15	0	0	12	4	16	6.18
IC-Ln-15	0	0	12	4	16	6.18
LC-Ln-15	0	0	12	4	16	6.18
A1C-Ln-15	0	0	12	4	16	6.18
A2C-Ln-15	0	0	12	4	16	6.18
A3C-Ln-15	0	0	12	4	16	6.18
B1C-Ln-15	0	0	12	4	16	6.18
B2C-Ln-15	0	0	12	4	16	6.18
B3C-Ln-15	0	0	12	4	16	6.18
TOTAL	0	0	183	76	259	100.00

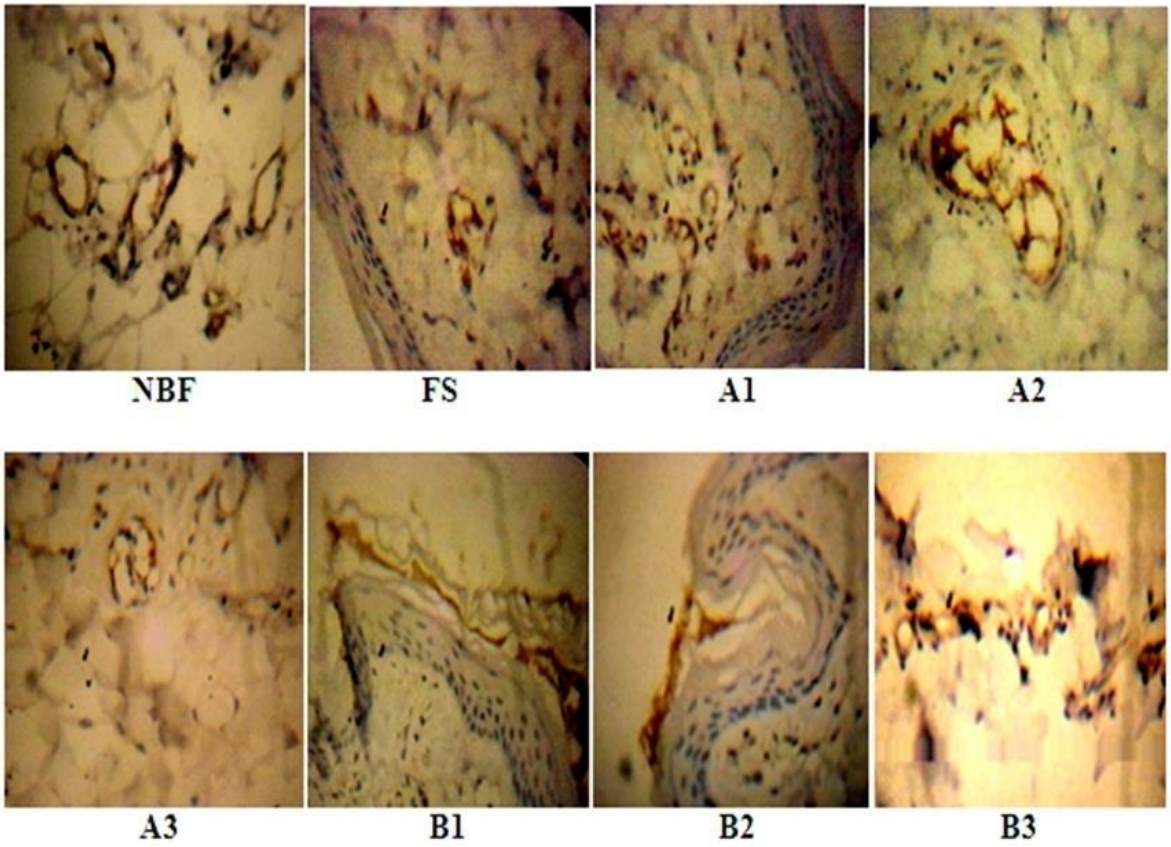


Plate 4.13: Photo micrographs of immunohistochemistry EMA on skin for all the fixatives (x200).

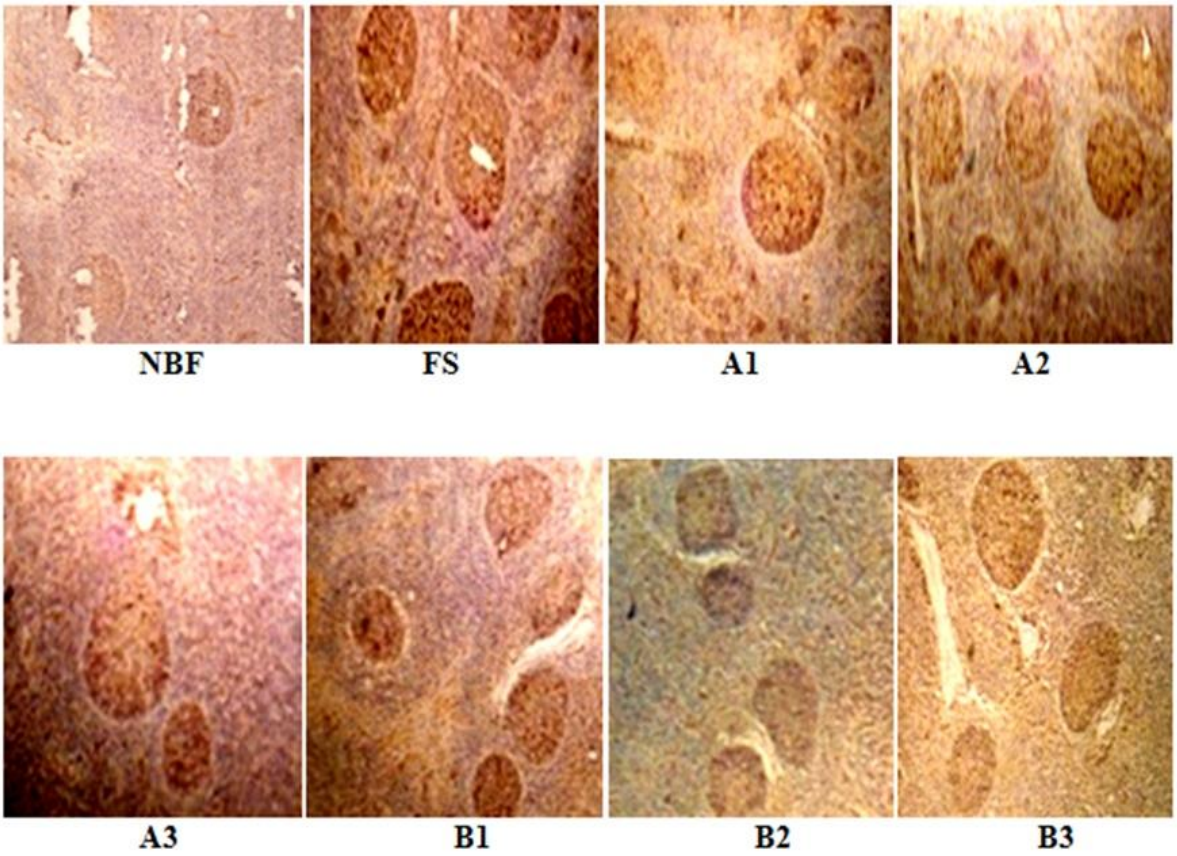


Plate 4.14: Photomicrographs of immunohistochemistry CD23 on mesenteric lymph nodes for all the fixatives (x200).

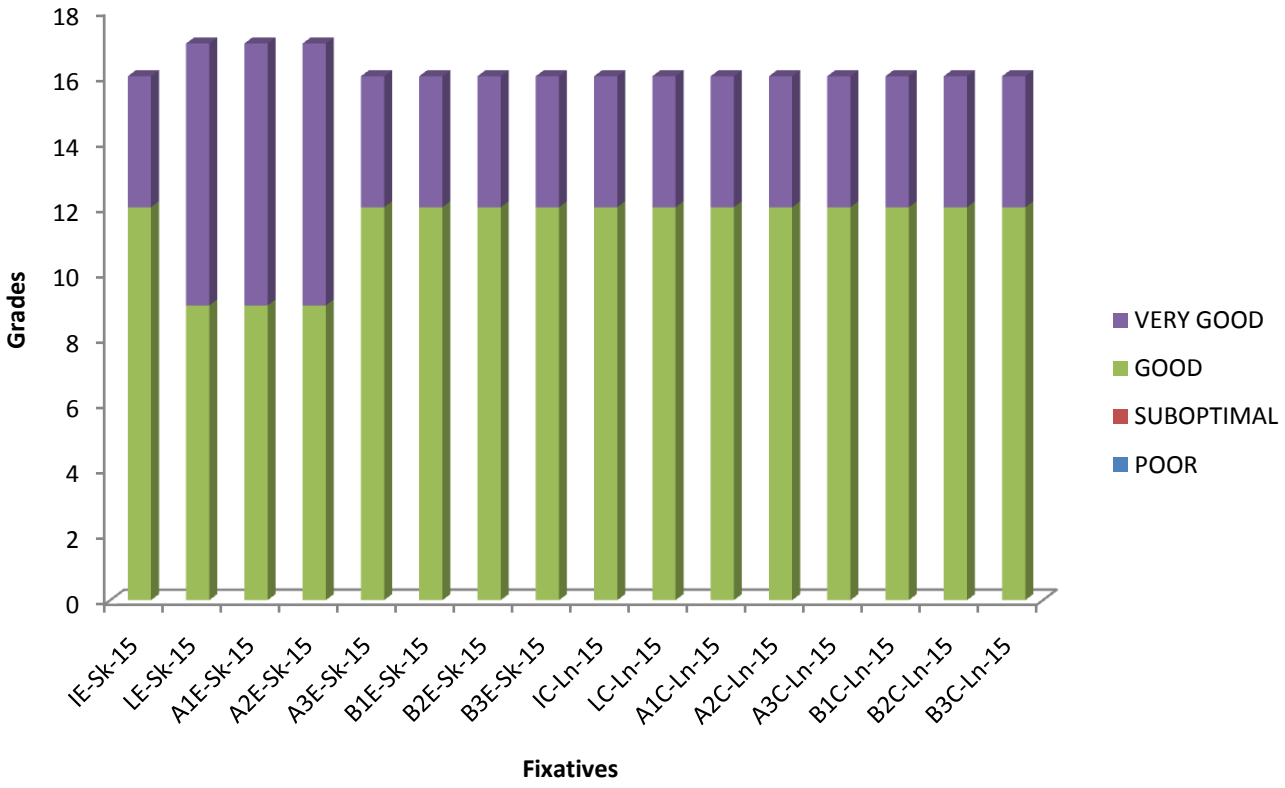


Fig. 4.25: Quality of tissue preservation grading (EMA & CD23)

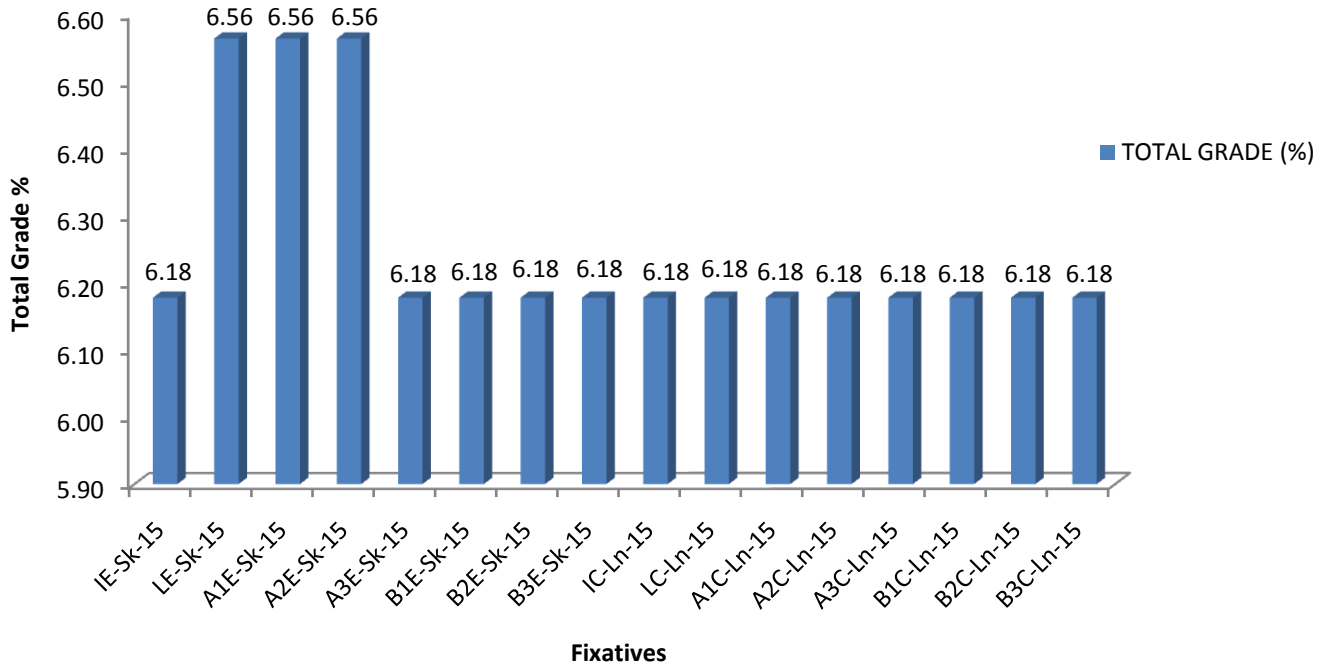


Fig. 4.26: IHC quality of tissue preservation total grade (%)

Table 4.30: Quality of staining of the EMA & CD23 (grading)

SLIDE NO	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE(%)
IE-Sk-15	0	0	15	0	15	6.02
LE-Sk-15	0	0	15	0	15	6.02
A1E-Sk-15	0	2	12	0	14	5.62
A2E-Sk-15	1	0	12	0	13	5.22
A3E-Sk-15	0	0	12	4	16	6.43
B1E-Sk-15	0	0	12	4	16	6.43
B2E-Sk-15	0	0	12	4	16	6.43
B3E-Sk-15	0	0	9	8	17	6.83
IC-Ln-15	0	0	12	4	16	6.43
LC-Ln-15	0	0	12	4	16	6.43
A1C-Ln-15	0	0	12	4	16	6.43
A2C-Ln-15	0	0	12	4	16	6.43
A3C-Ln-15	0	0	9	8	17	6.83
B1C-Ln-15	0	0	9	8	17	6.83
B2C-Ln-15	0	0	12	4	16	6.43
B3C-Ln-15	0	0	9	4	13	5.22
TOTAL	1	2	186	60	249	100.00

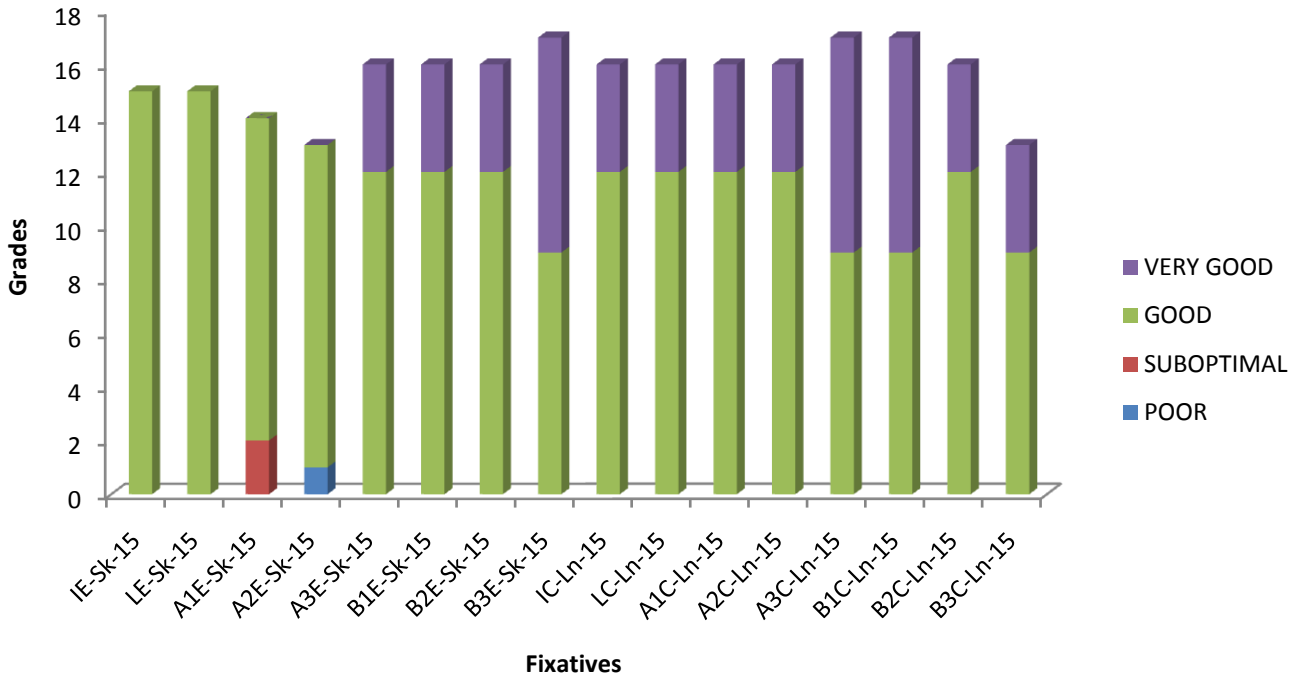


Fig. 4.27: Quality of staining grading (EMA & CD23)

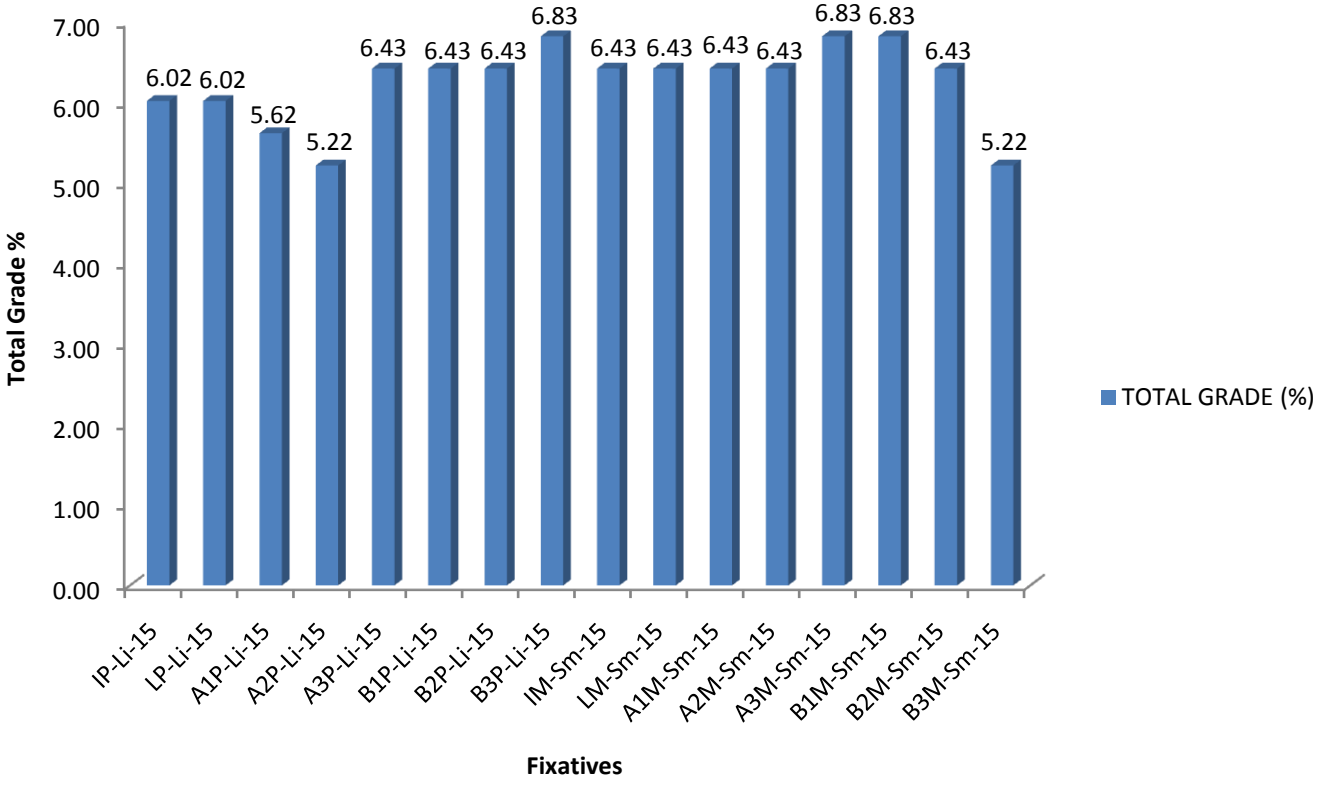


Fig. 4.28: IHC quality of staining total grade (%)

4.11. OVERALL H&E RESULTS

Table 4.31: H&E cumulative result on the quality of tissue preservation scores

	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL
NBF	0	3	28	14	45
FS	0	4	30	11	45
A1	1	4	34	6	45
A2	0	6	32	7	45
A3	1	1	38	5	45
B1	0	2	39	4	45
B2	0	2	38	5	45
B3	0	4	35	6	45
TOTAL	**2**	**26**	**274**	**58**	**360**

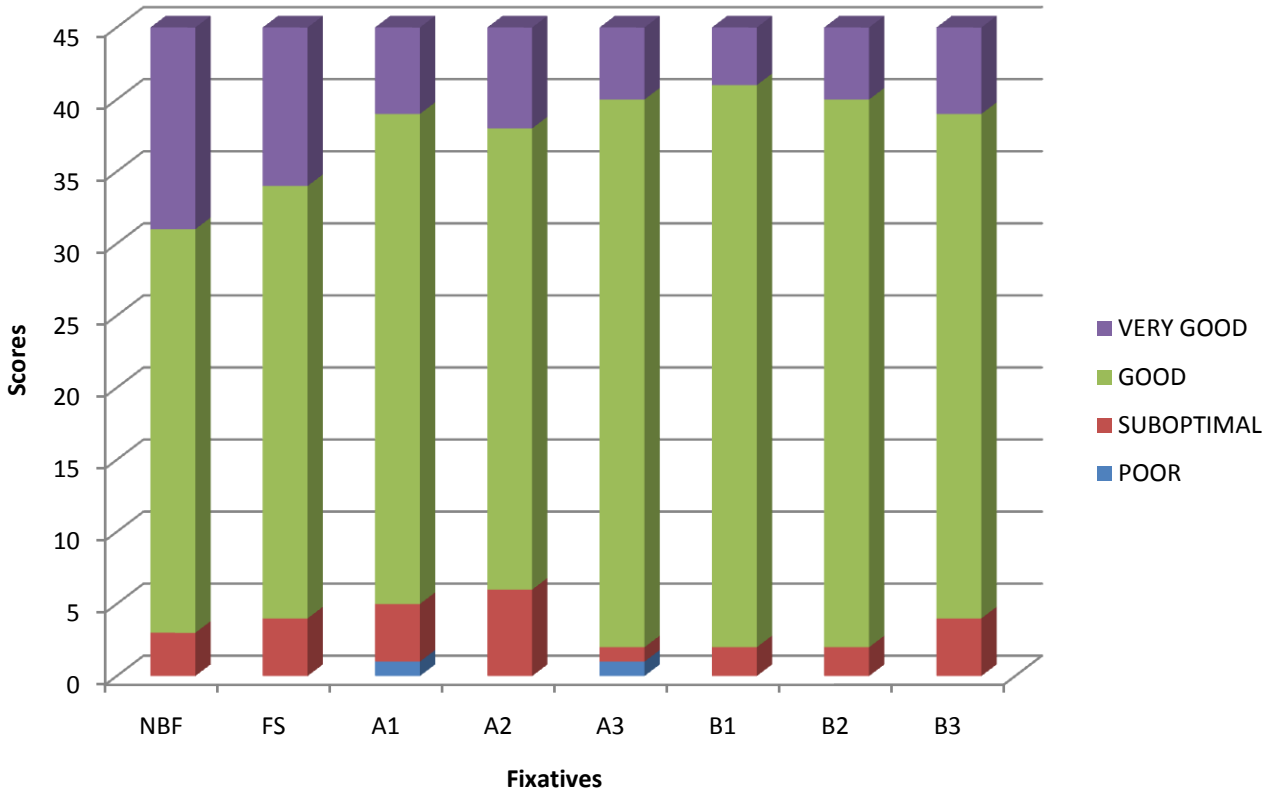


Fig. 4.29: H&E cumulative result on the quality of tissue preservation scores

Table 4.32: H&E cumulative result on the quality of tissue preservation grades

	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL %
NBF	0	6	84	56	146	13.2
FS	0	8	90	44	142	12.8
A1	1	8	102	24	135	12.2
A2	0	12	96	28	136	12.3
A3	1	2	114	20	137	12.4
B1	0	4	117	16	137	12.4
B2	0	4	114	20	138	12.5
B3	0	8	105	24	137	12.4
TOTAL	**2**	**52**	**822**	**232**	**1108**	**100.0**

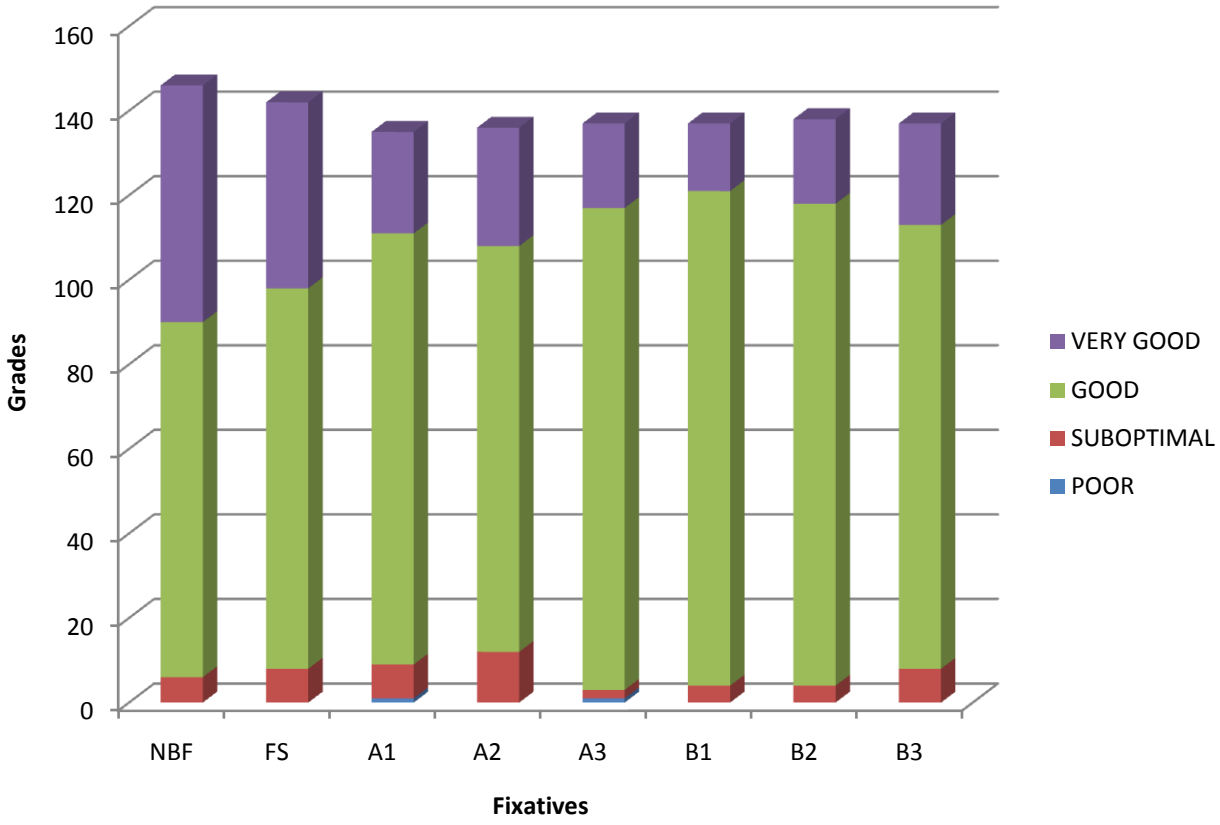


Fig. 4.30: H&E cumulative result on the quality of tissue preservation grades

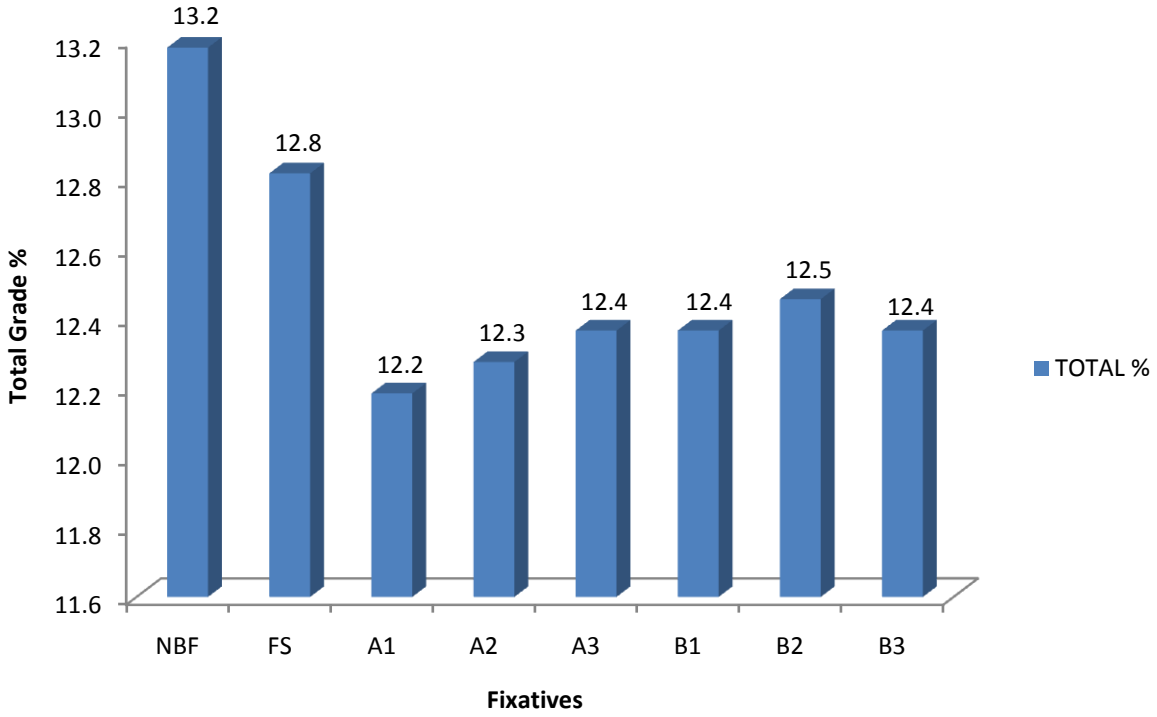


Fig. 4.31: Showing H&E cumulative result on the quality of tissue preservation total % grades

Table 4.33: H&E cumulative result on the quality of tissue staining scores

	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL
NBF	0	0	34	11	45
FS	0	3	36	6	45
A1	0	6	35	4	45
A2	1	2	36	6	45
A3	0	1	37	7	45
B1	0	1	39	5	45
B2	0	2	38	5	45
B3	0	2	37	6	45
TOTAL	**1**	**17**	**292**	**50**	**360**

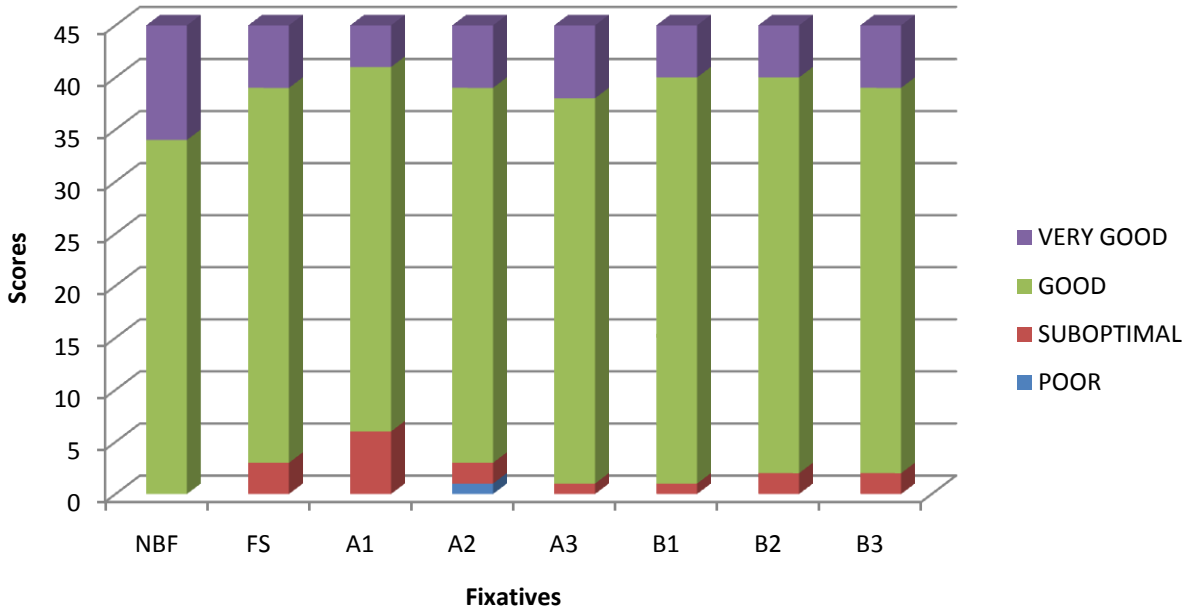


Fig. 4.32: H&E cumulative result on the quality of tissue staining

Table 4.34: H&E cumulative result on the quality of tissue staining

FIXATIVES	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL GRADE %
NBF	0	0	102	44	146	13.1
FS	0	6	108	24	138	12.4
A1	0	12	105	16	133	12.0
A2	1	4	108	24	137	12.3
A3	0	2	111	28	141	12.7
B1	0	2	117	20	139	12.5
B2	0	4	114	20	138	12.4
B3	0	4	111	24	139	12.5
TOTAL	**1**	**34**	**876**	**200**	**1111**	**100.0**

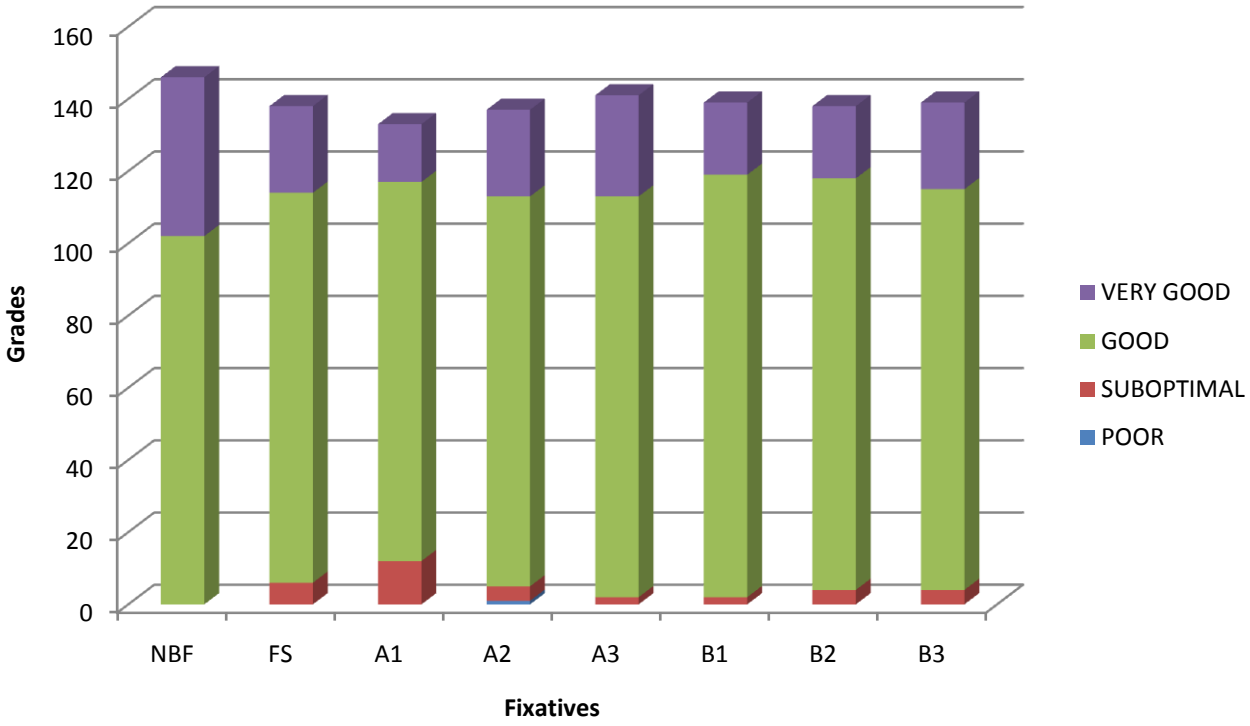


Fig.4.33: H&E cumulative result on the quality of tissue staining

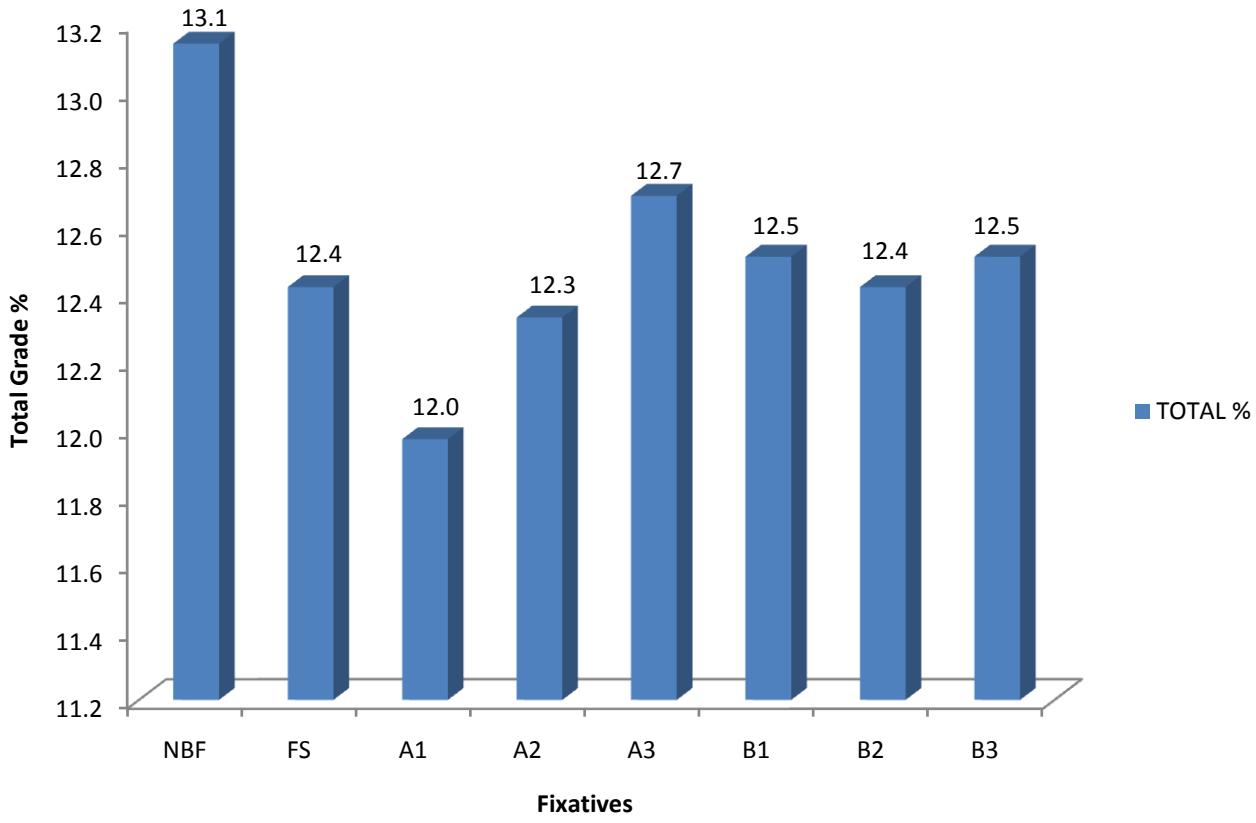


Fig. 4.34: H&E cumulative result on the quality of tissue staining total grade %

Table 4.35: overall H&E cumulative result score

	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL
NBF	0	3	62	25	90
FS	0	7	66	17	90
A1	1	10	69	10	90
A2	1	8	68	13	90
A3	1	2	75	12	90
B1	0	3	78	9	90
B2	0	4	76	10	90
B3	0	6	72	12	90
TOTAL	**3**	**43**	**566**	**108**	**720**

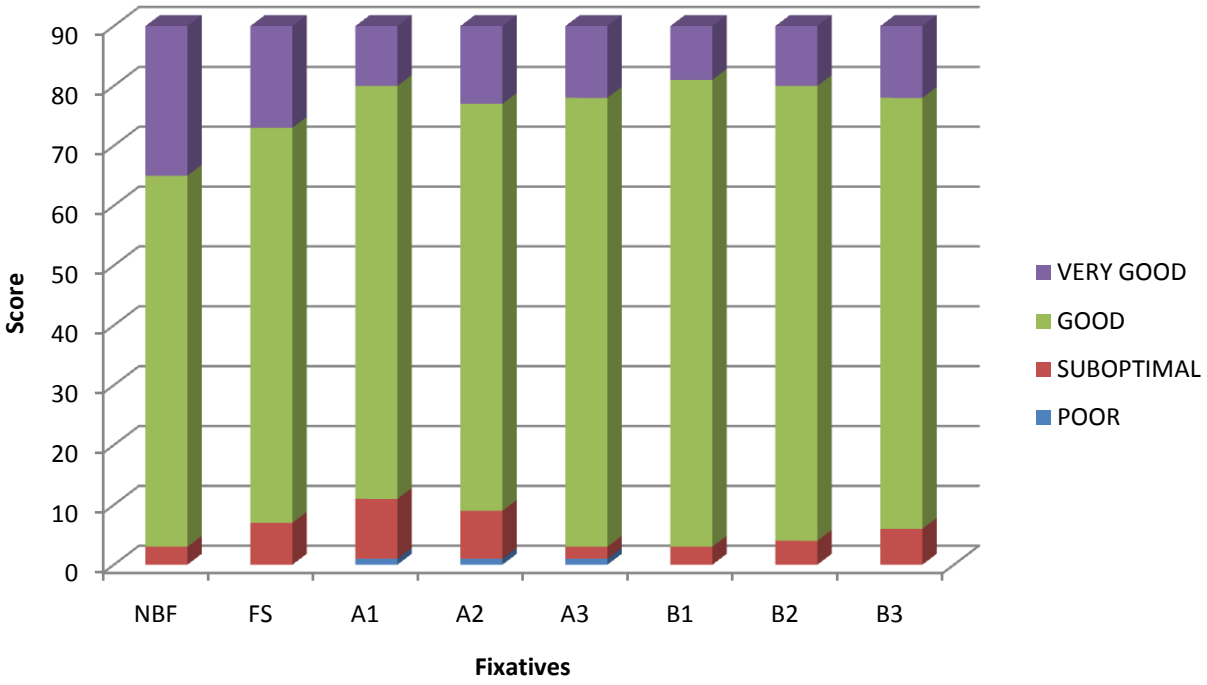


Fig. 4.35: Overall H&E cumulative result score

Table 4.36: Qverall H&E cumulative result grades

	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL	TOTAL %
NBF	0	6	186	100	292	13.2
FS	0	14	198	68	280	12.6
A1	1	20	207	40	268	12.1
A2	1	16	204	52	273	12.3
A3	1	4	225	48	278	12.5
B1	0	6	234	36	276	12.4
B2	0	8	228	40	276	12.4
B3	0	12	216	48	276	12.4
TOTAL	**3**	**86**	**1698**	**432**	**2219**	**100.0**

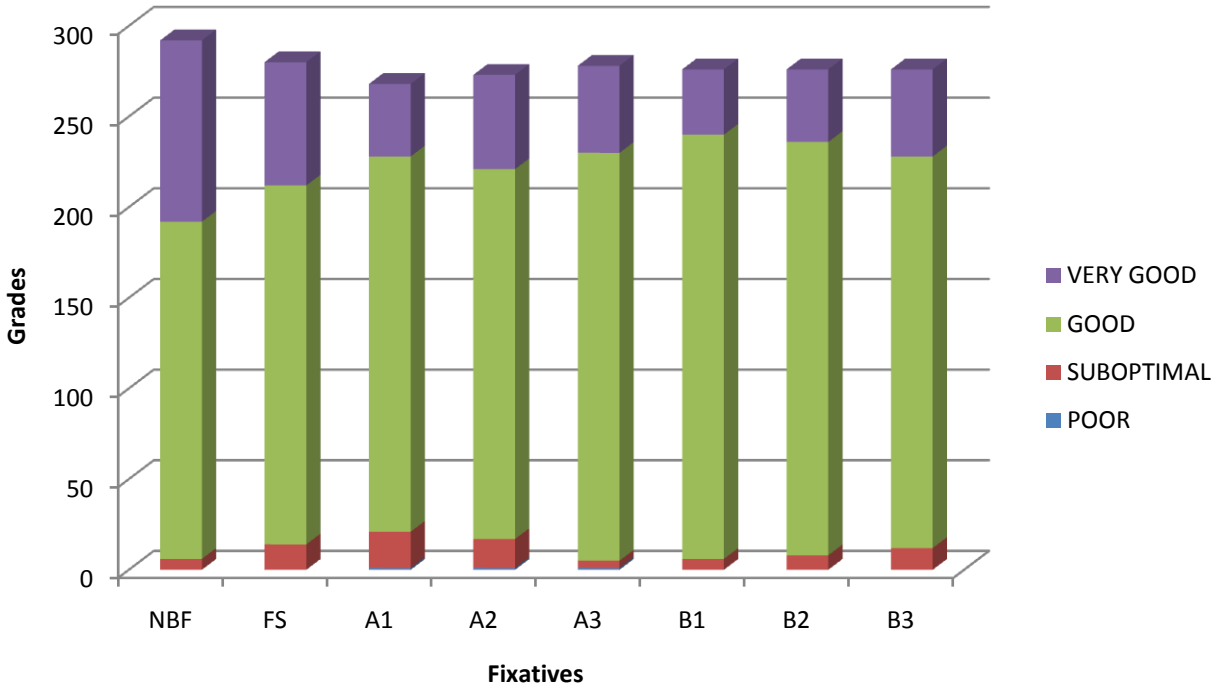


Fig. 4.36: Overall H&E cumulative result grades

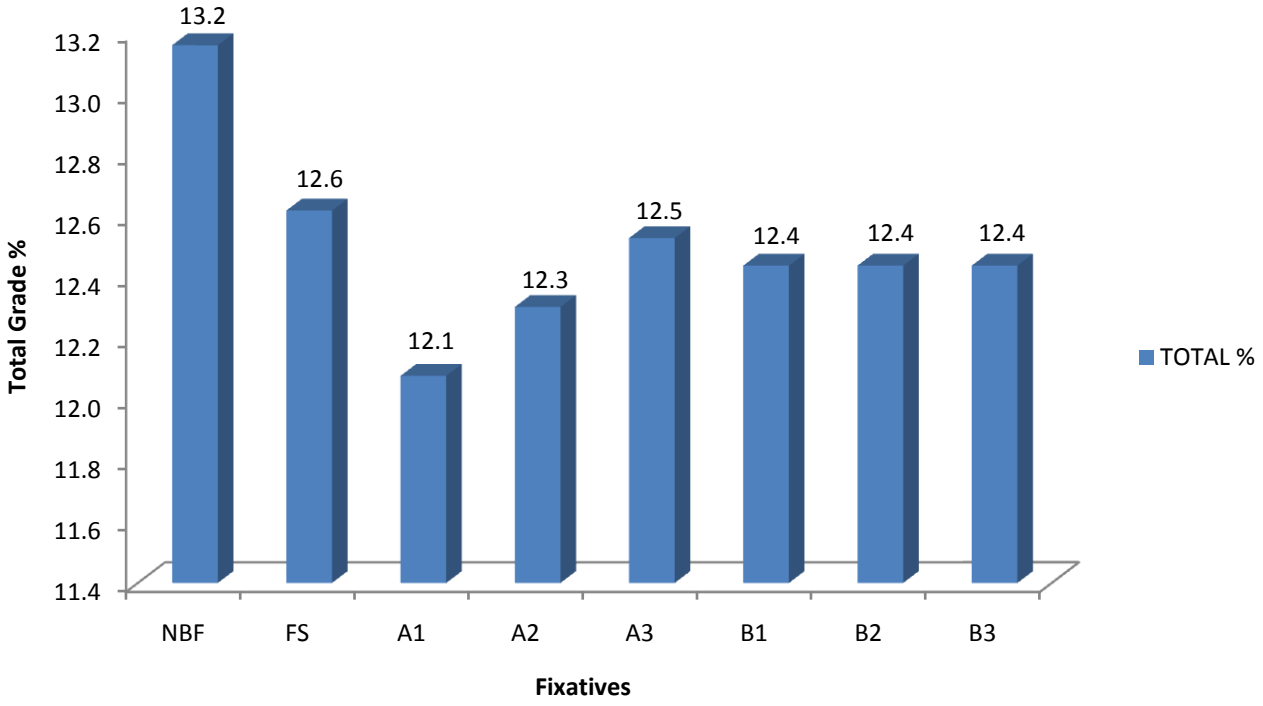


Fig. 4.37: Overall H&E cumulative result grade total %

Table 4.37: Overall percentage grade responses

	POOR	SUBOPTIMAL	GOOD	VERY GOOD	TOTAL
SPECIAL STAIN	0	8	177	68	**253**
IHC STAIN	1	2	186	64	**253**
H&E STAIN	1	34	876	200	**1111**
TISSUE QUALITY	2	52	822	232	**1108**
TOTAL	4	96	2061	564	**2725**
%	**0.15**	**3.52**	**75.63**	**20.70**	**100.00**

CHAPTER FIVE

5.0. DISCUSSION

This study was carried out to explore the functionally active range of concentration of formaldehyde (formalin) i.e. 2%-20% (Cecil et al, 1985) that would still give good histomorphologic outcome. A concentration of 4%, 6% and 8% as opposed to the 10% formaldehyde that is being used in the laboratories were explored, like a study done by Ferdinand Blum in 1985 using rat kidney fixed in 1, 2, 5, 10, 20, and 40% formaldehyde solutions from a commercial reagent for 24 hr and all these concentration gave desirable results except for 40% formaldehyde (Cecil et al, 1985).

Phenol increases the fixing abilities of formaldehyde (Anthony 2000), it is being used in products containing formaldehyde to reduce formaldehyde fumes in air i.e. prevent the release of formaldehyde fumes into the atmosphere hence making it more available in solution. When passing fresh tissues into the fixatives grossly the phenol containing fixatives show rapid fixation of tissues as oppose to the others as seen in plate 4.1. Other fixatives were looking bloody but the phenol containing solutions were clearer. Phenol also cause tissue softening, an effect that was noticed in this study while sectioning tissues fixed with phenol containing fixatives(B groups) hence more precaution was taken to section these tissues during microtomy.

The results from the microscopic assessment by the pathologists, show virtually an unremarkable difference in the performances of the combination formalin based fixatives as compared to the local and international standards as the overall difference between the best grade and the lowest grade percentage is 1.1% (Table 4.36, Fig 4.36 & Fig 4.37). No artifact attributed to fixation was noted for all the fixatives.

The fixing abilities of these fixatives varies for individual tissue types. The fixative with the best fixing abilties for the heart, liver and skeletal muscle was NBF, the skin, lungs and lymph nodes was formol saline, while for the brain was B1, for the spleen were A1, A2 and B2 and for the kidney was B2. The overall best performance for tissue fixing abilities was NBF. A similar outcome was seen in a study by Cathy et al, 2011, they used different fixatives other than formalin based on these range of tissue types; placenta, liver, brain, esophagus, stomach, duodenum, colon, omentum, lung, breast, adrenal gland, kidney, lymph node, thyroid, tonsil, spleen, and gallbladder for H&E staining, histochemical stains and immunohistochemical stains. The result also showed none was overall comparable to NBF with regard to macroscopy, morphologic evaluation, and immunohistochemical studies.

The most compatible with H&E stain was NBF but of the experimental fixatives it was A3, for the skin it was B1, for the brain they were A3, B1 and B3, for the lungs and heart it was NBF, for the liver were NBF and B1, for the spleen its formol saline, for the kidney were NBF, A2, A3 and B2, for the lymph nodes its A2 and for skeletal muscle was A3. In a blind study by Titford et al; (2012), formalin fixation provided the highest histomorphologic quality for tissue stained with H&E.

The most compatible with PAS stain of the experimental fixatives was B2, which did better than both standards but A1, A2 and B1 did as good as the international standard. While for MT, the most compatible with this stain was formol saline, of the experimental fixatives were A1 and B1, which did equivalent to the international standards. These agree with the reports of Melissa et al; (2005), Cathy et al; (2011) and Titford et al; (2012), who also noted that some fixatives not necessarily formalin based did better than NBF in stains.

The most compatible with EMA IHC stain of the experimental fixatives is B3, which did better than the standards likewise A3, B1 and B2 also did better than both standards. While

for CD23, the most compatible with this IHC stain were A3, B1 and B3, they did better than both standards while the others did equivalent to the standards. This outcome was similar to a study done by Adrian et al; (2000) where immunohistochemical staining was generally enhanced using F13 fixed tissue without the use of antigen retrieval as compared to formalin fixed tissue. In study like Nietner et al; (2012) reported that none of the alternative fixatives for IHC was comparable to formalin.

Interestingly one would expect a decreasing performance based on the concentration of formaldehyde and the effects of the additive as follows:

NBF, FS, B1, B2, B3, A1, A2, A3. But rather this was what was obtained:

NBF, FS, A3, B1, B2, B3, A2, A1.

Similar results were seen in a study done by Cathy et al; (2011) they used different types of fixatives mainly alcohol based and yet the NBF did better. In a blind study by Titford et al; (2012), formalin fixation provided the highest histomorphologic quality for tissue stained with H&E and examined for diagnostic surgical pathology. It was also noted that should the use of formalin be discontinued, pathologists will have to familiarize themselves with a different set of microscopic details associated with the replacement fixatives.

The average overall score and total percentage grades of each fixatives were tested for normalcy and both the mean and median were the same i.e. 3.1 indicating a normal distribution. Using alpha=0.05, confidence interval level=95, ANOVA and chi-square tests were performed and the results supports the acceptance of the null hypothesis. i.e. there is no significant difference between the experimental combination formalin fixatives to the formol saline use for routine surgical pathology procedures. Similar conclusions were drawn in studies by Anissian 2012 and Gatta 2012, where the alternative fixatives were found to have desired results comparable to formalin and was concluded that they could be used as suitable

alternative to formalin. More so in this study the overall result of responses for the fixatives in table 37 showed 96.33% of the pathologists' responses were graded good and very good.

CHAPTER SIX

6.0. CONCLUSION

i. All the formalin based combination fixatives used in this study provided good and desirable histomorphologic quality for the different stained tissues as compared to the standards, with an overall percentage total grades of 13.2%, 12.6%, 12.5%, 12.4%, 12.4%, 12.4%, 12.3% and 12.1% for neutral buffered formalin, formol saline, A3, B1, B2, B3, A2 and A1 respectively.

ii. Individually, some of the formalin based combination fixatives performed better than the international and local standards in some tissues for H&E, immunohistochemical and special stains inclusive.

iii. The result of the hypothesis tests (ANOVA and Chi-Square) supported maintenance of the null hypothesis and with p values greater than 0.05 implies any one of the experimental formalin based combination fixatives can be used as potentially less toxic suitable substitution to formol saline.

6.1. RECOMMENDATIONS

- From the outcome of the study, any of the experimental formalin based fixative can be used as an alternative for the 10% formol saline used for routine work in the laboratory.
- Further studies could be done exploring lesser percentage concentration of formaldehyde with or without additive and/or the long time effects of the various fixatives on tissue(s) during tissue preservation.

6.2. CONTRIBUTIONS TO KNOWLEDGE

- Formol saline of 4% (A3) concentration is significantly ($p = 0.21$) comparable to be the best alternative to 10% NBF and 10% formol saline as a tissue fixative.
- Formol saline of 8% concentration with (B1) or without phenol l (A1) was most compatible with MT but that was not replicable with PAS.
- Formol saline of 4% concentration with phenol (B3) was most compatible with EMA; Formol saline of 8% concentration with (B3) or without phenol (A1) and Formol saline of 6% concentration with phenol (B2) were most compatible with CD23.

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APPENDICES

APPENDIX 1: CLASSIFICATION OF CARCINOGENS BY VARIOUS BODIES

INTERNATIONAL AGENCY FOR RESEARCH ON CANCER (IARC) (fixation, 2015)

Since 1971 it has published a series of *Monographs on the Evaluation of Carcinogenic Risks to Humans* that have been highly influential in the classification of possible carcinogens.

Group 1: the agent (mixture) is definitely carcinogenic to humans. The exposure circumstance entails exposures that are carcinogenic to humans.
Group 2A: the agent (mixture) is probably carcinogenic to humans. The exposure circumstance entails exposures that are probably carcinogenic to humans.
Group 2B: the agent (mixture) is possibly carcinogenic to humans. The exposure circumstance entails exposures that are possibly carcinogenic to humans.
Group 3: the agent (mixture or exposure circumstance) is not classifiable as to its carcinogenicity to humans.
Group 4: the agent (mixture) is probably not carcinogenic to humans.

GLOBALLY HARMONIZED SYSTEM

The Globally Harmonized System of Classification and Labelling of Chemicals (GHS) 2009 around the world. It classifies carcinogens into two categories, of which the first may be divided again into subcategories if so desired by the competent regulatory authority:

Category 1: known or presumed to have carcinogenic potential for humans
Category 1A: the assessment is based primarily on human evidence
Category 1B: the assessment is based primarily on animal evidence
Category 2: suspected human carcinogens

U.S. NATIONAL TOXICOLOGY PROGRAM

The National Toxicology Program of the U.S. Department of Health and Human Services is mandated to produce a biennial *Report on Carcinogens*. As of June 2011, the latest edition was the 12th report (2011). It classifies carcinogens into two groups:

Known to be a human carcinogen
Reasonably anticipated to be a human carcinogen

AMERICAN CONFERENCE OF GOVERNMENTAL INDUSTRIAL HYGIENISTS

The American Conference of Governmental Industrial Hygienists (ACGIH) is a private organization best known for its publication of threshold limit values (TLVs) for occupational exposure and monographs on workplace chemical hazards. It assesses carcinogenicity as part of wider assessment of the occupational hazards of chemicals.

Group A1: Confirmed human carcinogen
Group A2: Suspected human carcinogen

Group A3: Confirmed animal carcinogen with unknown relevance to humans
Group A4: Not classifiable as a human carcinogen
Group A5: Not suspected as a human carcinogen

EUROPEAN UNION

The European Union classification of carcinogens is contained in the Dangerous Substances Directive and the Dangerous Preparations Directive. It consists of three categories:

Category 1: Substances known to be carcinogenic to humans.
Category 2: Substances which should be regarded as if they are carcinogenic to humans.
Category 3: Substances which cause concern for humans, owing to possible carcinogenic effects but in respect of which the available information is not adequate for making a satisfactory assessment.
This assessment scheme is being phased out in favor of the GHS scheme (see above), to which it is very close in category definitions.

SAFE WORK AUSTRALIA

Under a previous name, the NOHSC, in 1999 Safe Work Australia published the Approved Criteria for Classifying Hazardous Substances [NOHSC:1008(1999)]. This classification consists of three categories:

Category 1: Substances known to be carcinogenic to humans.
Category 2: Substances that should be regarded as if they were carcinogenic to humans.
Category 3: Substances that have possible carcinogenic effects in humans but about which there is insufficient information to make an assessment

APPENDIX 2: PHYSICAL PROPERTIES OF FORMALDEHYDE

PHYSICAL PROPERTIES OF FORMALDEHYDE (HSDB, 1994; CRC, 1994)
Description Colorless gas
Molecular formula CH2O
Molecular weight 30.03 g/mol
Density 0.815 g/L @ -20°C
Boiling point -19.1°C
Melting point -92°C
Vapor pressure 220 kPa @ 0°C
Solubility Soluble in water, ethanol, ether, acetone
Conversion factor 1 ppm = 1.23-1.25 mg/m^3 @ 25°C

Physicochemical properties of formaldehyde

Name	Formaldehyde
International Union for Pure and Applied Chemistry name	Formaldehyde
Synonyms	Formic aldehyde Methanal Methyl aldehyde Methylene oxide Oxomethane Oxymethylene
Chemical Abstracts Service Index name	Formaldehyde
CASRN	50-00-0
Formula	HCHO
Molecular weight	30.03
Density	Gas: 1.067 (air = 1) Liquid: 0.815 g/mL at –20°C
Vapor pressure	3,883 mm Hg at 25°C
Log K_{ow}	–0.75 to 0.35
Henry's law constant	3.4×10^{-7} atm-m^3/mol at 25°C 2.2×10^{-2} Pa-m^3/mol at 25°C
Conversion factors (25°C, 760 mm Hg)	1 ppm = 1.23 mg/m^3 (v/v) 1 mg/m^3 = 0.81 ppm (v/v)
Boiling point	–19.5°C at 760 mm Hg
Melting point	–92°C
Flash point	60°C; 83°C, closed cup for 37 %, methanol-free aqueous solution; 50°C closed cup for 37% aqueous solution with 15% methanol
Explosive limits	73% upper; 7% lower by volume in air
Autoignition temperature	300°C
Solubility	Very soluble in water; soluble in alcohols, ether, acetone, benzene
Reactivity	Reacts with alkalis, acids and oxidizers

APPENDIX 3: COMPOSITIONS OF FIXATIVES USED IN THIS STUDY

Concentrated (Conc.) formalin = 40% formaldehyde in solution (ready-to-use)
Saline solution = 9g NaCl in1000 ml tap water

10% BUFFERED NEUTRAL FORMALIN

Conc. Formalin	100 ml
Distilled water	900 ml
Sodium dihydrogen orthophosphate	4 g
Disodium hydrogen orthophosphate (anhydrous)	6.5 g

Fixation time: 24-72 hours.

FORMOL SALINE (10%)

Conc. Formalin	100 ml
Saline solution	900 ml

SOLUTION A

A1 (8%)		**A2 (6%)**		**A3 (4%)**	
Conc. Formalin	80 ml	Conc. Formalin	60 ml	Conc. Formalin	40 ml
Saline solution	920 ml	Saline solution	940 ml	Saline solution	960 ml

SOLUTION B

B1 (8%)		**B2 (6%)**		**B3 (4%)**	
Conc. Formalin	80 ml	Conc. Formalin	60 ml	Conc. Formalin	40 ml
Saline solution	920 ml	Saline solution	940 ml	Saline solution	960 ml
Phenol	20 g	Phenol	20 g	Phenol	20 g

APPENDIX 4: TWA FOR CHEMICAL USED IN THE LABORATORY

Eight hours of TWA for some chemicals frequently used in the histology laboratory

Toxic level at	Chemical substance
0.01 ppb	Silver nitrate (silver metal dust/fumes)
0.02 ppb	Osmium tetroxide
0.05 ppb	Potassium dichromate; uranyl nitrate[a]
0.1 ppb	Iodine; picric acid (explosive)
0.2 ppb	Potassium permanganate
0.5 ppb	Chromium trioxide (chromic acid)
1 ppb	Ferric chloride; oxalic, phosphotungstic, and sulfuric acids
2 ppb	Hydroquinone; paraffin wax fumes; sodium hydroxide
10 ppb	Aluminum hydroxide; glycerin mist
0.1 ppm	Potassium iodide; sodium barbital
0.2 ppm	Glutaraldehyde (mutagenic agent)
0.5 ppm	Chlorine
0.75 ppm	Formalin; paraformaldehyde (both carcinogens)
1 ppm	Hydrogen peroxide
2 ppm	Nitric acid; sodium hydroxide
5 ppm	Formic and hydrochloric acids; phenol
10 ppm	Acetic acid
25 ppm	Ammonium hydroxide
100 ppm	Xylene

ppb = parts per billion (equivalent to mg/m3); ppm = parts per million (equivalent to g/m3, 1 ppm = 1000 ppb).

a One hundred milliliter of 1% aqueous solution of uranyl nitrate undergoes about 12000 disintegrations/s (a specific activity of 123 Bq/mL) equivalent to 0.26 μg of radium

APPENDIX 5 : MODIFIED ALLRED SCORING

MODIFIED ALLRED SCORING

Proportion score
0—No cells are stained.
1—≤ 1% of cells are stained.
2—1–10% of cells are stained.
3—11–33% of cells are stained.
4—34–66% of cells are stained.
5—67–100% of cells are stained.

Intensity score
0—Negative.
1—Weak.
2—Intermediate.
3—Strong.

Score
0–1—Poor.
2–3—Suboptimal.
4–6—Good.
7–8—Very good.

Grade
1—Poor.
2—Suboptimal.
3—Good.
4—Very good.

Segen's Medical Dictionary. 2012 Farlex, Inc.

APPENDIX 6 : ADDENDUM TO THE 12TH REPORT ON CARCINOGENS

Addendum to the 12th Report on Carcinogens

Published by the U.S. Department of Health and Human Services, National Toxicology Program

The twelfth edition of the National Toxicology Program (NTP) Report on Carcinogens (RoC) contains a change in the listing status of formaldehyde. Prior editions of the RoC had listed formaldehyde as *reasonably anticipated to be a human carcinogen*, and following a rigorous scientific review, formaldehyde is now reassigned to the category *known to be a human carcinogen*. Concurrently, the Environmental Protection Agency (EPA) prepared and released a draft Integrated Risk Information System (IRIS) assessment of formaldehyde. At the request of EPA Administrator Jackson, a National Academy of Sciences (NAS) committee was convened to conduct an independent review of the draft EPA IRIS assessment of formaldehyde. The draft EPA IRIS assessment is a comprehensive health effects assessment and provides quantitative estimates of human risks of adverse human health outcomes from specific levels of exposure to formaldehyde.

The NAS committee was not charged with performing its own health effects assessment, conducting its own literature searches, or formulating its own conclusions regarding cancer causality for formaldehyde. The charge to the NAS consisted of specific questions, one of which (Is the weight-of-evidence *narrative* in the EPA IRIS assessment scientifically supported?) was related to the hazard identification component of the IRIS document and was relevant to the IRIS formaldehyde cancer assessment; the other charge questions were specific for the risk assessment component of the IRIS document.

The NAS produced a peer-review report of the EPA IRIS assessment's justifications for its conclusions for health effects, including cancer (NRC 2011). Because the NAS document is not an independent hazard assessment, it has limited applicability to the NTP's RoC evaluation of formaldehyde. The RoC evaluation involved a multistep comprehensive assessment of the literature, and resulted in a narrative justification for the NTP's conclusions that was developed independently from the EPA IRIS assessment. Neither the NTP listing process nor the justification for the listing of formaldehyde in the RoC was reviewed by the NAS. Nonetheless, the NTP has carefully reviewed the key scientific issues raised in the NAS peer-review report for potential relevance to the NTP's conclusions on the carcinogenicity of formaldehyde.

Listing for formaldehyde in the 12th RoC

The NTP's decision to list formaldehyde in the 12th RoC as *known to be a human carcinogen* was the result of a rigorous scientific review process that included many opportunities for public involvement and comment. The NTP solicited advice from three independent review groups: a non-government expert scientific panel, a government interagency scientific panel, and a National Institute of Environmental Health Sciences (NIEHS)–NTP scientific panel. The criterion for listing as *known to be a human carcinogen* in the RoC is "sufficient evidence of carcinogenicity from studies in humans, which indicates a causal relationship between exposure to the agent, substance, or mixture, and human cancer" (see page 4 of the 12th RoC). The NTP's decision to list formaldehyde as *known to be a human carcinogen* is

based on the points below and is consistent with the recommendations from each of the three independent review groups.1 Point A alone satisfies the RoC criteria to list a substance as *known to be a human carcinogen*.

1The expert panel's recommendation on listing status is available at http://ntp.niehs.nih.gov/ntp/roc/twelfth/2009/November/FA_PartB.pdf.

A. Sufficient evidence of cancer from studies in humans: nasopharyngeal cancer, sinonasal cancer, and lymphohematopoietic cancer, specifically myeloid leukemia.

B. Supporting evidence from (a) cancer studies in experimental animals (primarily tumors of the nasal cavity, but also tumors at other sites) and (b) mechanistic data, which are data that determine how a (typically) adverse health outcome occurs.

The information that the NTP used to reach a decision on the listing status for formaldehyde is presented in a publicly available Background Document, which was peer reviewed by the non-government expert panel and was discussed in detail by each of the three review groups charged with making a listing recommendation. For the purpose of listing a substance as *known to be a human carcinogen* in the RoC, the literature must provide sufficient information to establish that significant exposure to the substance occurs or has occurred in the United States, and to allow a determination of cancer causation from scientific evidence from studies in humans. Appreciation of "mode of action," or an understanding of how exposure to a given substance might lead to cancer, is an important piece of supporting evidence, but is not a requirement for listing in the RoC. Empirical evidence of cancer causation in humans is sufficient to satisfy the listing criterion.

Causality from studies in humans is defined by the RoC listing criteria as a credible association that cannot be explained by chance, bias, or confounding. Several of the guidelines proposed by Bradford-Hill — strength of the association (e.g., magnitude 3 of the risk estimate, consistency across findings, exposure-response relationships, and temporality) — are used to evaluate whether an association is credible and whether chance, bias, and confounding can be ruled out (Hill 1965). Emphasis is placed on "informative studies," which are studies of high quality with a design and methodologies to detect an effect and to rule out potential confounding from exposure to other carcinogens. Characteristics of these studies are sufficient statistical power, robust exposure assessments, evaluation of exposure-response relationships, and adequate reporting to allow a full consideration of methodological limitations.

The scientific decision to list a substance in the RoC is based on the entire body of literature, including both positive and negative studies. The evidence that supports the listing status is captured in the substance profile and includes (1) the identification of informative studies and (2) the critical findings from those studies that allow one to rule out chance, bias, and confounding, such as consistency across studies in tumor sites of interest, consistency in increased risk in different populations, statistical significance, and positive exposure-response relationships.

Conclusions of the EPA IRIS assessment regarding formaldehyde as a human carcinogen

The conclusions for formaldehyde reached by the EPA IRIS evaluation are generally consistent with those of the NTP RoC evaluation. The EPA IRIS and the NTP RoC concluded that human epidemiologic studies demonstrate that formaldehyde exposure causes nasopharyngeal cancer, sinonasal (nasal and paranasal) cancer, and myeloid leukemia.

The EPA IRIS also stated there was a causal association with all leukemias and lymphohematopoietic cancers as a group, and there was strong, but not sufficient, evidence

for a causal association for other upper-respiratory tract cancers, Hodgkin's lymphoma, or multiple myeloma. The NTP recognizes that there is scientific disagreement over whether it is appropriate to group lymphohematopoietic cancers and, therefore, evaluated data on both grouped and individual subtypes of leukemia. With regard to these cancers, the RoC's listing of formaldehyde as a known human leukemogen is based on formaldehyde's causal association with myeloid leukemia only, not lymphohematopoietic cancers as a group.

NAS conclusions from the committee's review of the EPA IRIS assessment of formaldehyde

The NAS report includes the following conclusions:

A. The NAS committee stated that the EPA narrative justifies EPA's conclusion that formaldehyde causes cancers of the nose, nasal cavity, and nasopharynx in humans, which is consistent with the NTP's listing of formaldehyde as *known to be a human carcinogen* in the 12th€ RoC.

4B. The NAS committee stated that the EPA narrative does not justify the IRIS assessment's conclusion that formaldehyde causes specific subtypes of lymphohematopoietic cancers, such as myeloid leukemia. Because the NAS committee did not conduct its own independent assessment, it did not offer an opinion on whether the evidence from studies in humans supports a causal relationship. The NTP has concluded that the scientific evidence from studies in humans is sufficient to conclude that exposure to formaldehyde causes myeloid leukemia.

C. The NAS committee stated that the EPA narrative does justify the IRIS assessment's conclusion that formaldehyde causes genetic damage (damage to DNA and chromosomes) in exposed humans, which is a presumed mechanism by which formaldehyde causes cancer. The NTP evaluation for the 12th RoC concurs.

The NAS and RoC conclusions specific for lymphohematopoietic cancers

As mentioned above, the NAS did not specifically state an opinion on the strength of the epidemiologic evidence for an association of formaldehyde and myeloid leukemia in exposed humans. They noted that while a well-established mode of action is not required to make causal inference, they did not believe it was plausible, based on the demonstrated rapid reactivity of formaldehyde with blood components, that inhaled formaldehyde could gain direct access to the bone marrow. The NAS also disagreed with the grouping of all lymphohematopoietic cancers in the EPA IRIS assessment because they believed that they are different diseases. They commented that the narrative provided in the draft IRIS assessment did not support the EPA's determination that formaldehyde causes lymphohematopoietic cancers and recommended that the EPA clarify its arguments that support determinations of causality, including describing the criteria that were used to weigh evidence and assess causality.

The NTP used the approach described above in its hazard evaluation for myeloid leukemia for the RoC. A limitation in the body of literature of human studies on formaldehyde and lymphohematopoietic cancers is that only a subset reported risk estimates specific for myeloid leukemia. The scientific information supporting the NTP's conclusions is summarized in the RoC substance profile. The substance profile identifies the high-quality, informative studies on formaldehyde that allow one to rule out chance, bias, and confounding; describes the study populations and findings; and discusses why they were considered to be the most useful studies. The evidence supporting causality in human studies is (1) consistent findings of increased risk among the highest exposed workers across studies,

(2) positive exposure-response relationships, and (3) adequacy to rule out confounding. A recent meta-analysis finding a positive association among workers with the highest exposure (RR = 2.47, 95% CI = 1.31 to 2.67) confirmed the consistency of the findings across studies (Schwilk *et al*. 2010).

The NAS committee, the EPA IRIS document, and the 12th RoC formaldehyde substance profile are consistent on the point that the mechanism(s) by which formaldehyde causes leukemia are not known, but agree that formaldehyde causes genetic damage in exposed humans, which is a key event in cancer formation. Most substances that cause leukemia are thought to do so by directly damaging stem cells (cells that give rise to blood and lymphoid cells) in the bone marrow. Formaldehyde is highly reactive; thus, some scientists have questioned whether it is plausible for formaldehyde inhaled through the nasal cavity to cause adverse health effects in tissues, such as bone marrow, that are far from the site of entry. The 12th RoC substance profile acknowledges the current lack of understanding of the biological mechanism(s) by which formaldehyde causes cancer at distal sites, including the evidence in laboratory animals failing to demonstrate systemic delivery. In addition, the substance profile cites direct evidence of genetic damage in circulating lymphocytes (white blood cells) and evidence of suppression of blood-forming elements in workers exposed to formaldehyde as support for the plausibility that formaldehyde causes myeloid leukemia. At the current time, it is not known how formaldehyde causes genetic damage to the stem cells, leading to hematological changes, and cancer; however, plausible hypotheses have been advanced on ways that formaldehyde might damage stem cells in the nose or blood without directly damaging the bone marrow and they are discussed in the substance profile. The mechanism(s) by which a substance listed in the RoC causes cancer are typically not known and, as noted above, mode of action is not a requirement for listing. Empirical evidence of cancer causation in humans satisfies the criterion for listing as *known to be a human carcinogen*.

Conclusion

Studies in humans have shown that formaldehyde causes nasopharyngeal cancer, sinonasal cancer, and myeloid leukemia. The NTP's recommendation for listing formaldehyde in the 12th RoC is the result of a rigorous scientific review process that provided many opportunities for public and scientific input, including an independent assessment of the literature by external experts and peer review of the science supporting the NTP's listing decision.

Although the NAS report is an important document, it has limited applicability to the NTP review of formaldehyde, because it is a peer-review report of the EPA IRIS assessment's justifications for its conclusions on carcinogenicity and other health effects and not an independent hazard assessment of the scientific literature on formaldehyde. Nevertheless, the NAS report concurs with the EPA's conclusions that formaldehyde causes cancer of the nose, nasal cavity, and nasopharynx and genetic damage (a general mechanism by which substances are thought to cause cancer), which is supportive of the NTP's listing for formaldehyde as *known to be a human* 6

carcinogen. With respect to myeloid leukemia, the NAS report questioned the plausibility that formaldehyde could cause this specific type of cancer by currently known mechanism(s), but was silent on whether the epidemiologic data from cancer studies in humans show a causal relationship. The NTP acknowledges uncertainty regarding the mechanism by which formaldehyde causes myeloid leukemia. The NTP's conclusion that formaldehyde causes myeloid leukemia is based on the human epidemiologic data and is consistent with the RoC listing criterion.

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Schwilk E, Zhang L, Smith MT, Smith AH, Steinmaus C. 2010. Formaldehyde and leukemia: an updated meta-analysis and evaluation of bias. *J Occup Environ Med* 52(9): 878-886.

APPENDIX 7: TISSUE SELECTION BASED ON ORGAN SYSTEM

SYSTEMS	**TISSUES**
NERVOUS SYSTEM	BRAIN
CARDIOVASCULAR SYSTEM	HEART
SKIN	SKIN
LYMPHATICS	LYMPH NODE/SPLEEN
GENITOURINARY SYSTEM	KIDNEY
RESPIRATORY SYSTEM	LUNGS
GASTROINTESTINAL SYETEM	LIVER
MUSCULOSKELETAL SYSTEM	SKELETAL MUSCLE

APPENDIX 8: PRO-FORMA FOR RESPONSES FROM THE PATHOLOGISTS

SLIDE NO	**Physical Quality**	**QUALITY OF TISSUE PRESERVATION SCORE**				**QUALITY OF STAINING SCORE**				
	Artifacts Associated with fixation (mention)	**POOR**	**SUB OPTI MAL**	**GOOD**	**VERY GOOD**	Artifacts Associated with fixation (mention)	**POOR**	**SUB OPTI MAL**	**GOOD**	**VERY GOOD**
I-Sk-15										
I-Br-15										
I-Lu-15										
I-Li-15										
I-Sp-15										
I-Ht-15										
I-Ln-15										
I-Sm-15										
I-Kd-15										
L-Sk-15										
L-Br-15										
L-Lu-15										
L-Li-15										
L-Sp-15										
L-Ht-15										
L-Ln-15										
L-Sm-15										
L-Kd-15										
A1-Sk-15										
A1-Br-15										
A1-Lu-15										
A1-Li-15										
A1-Sp-15										
A1-Ht-15										
A1-Ln-15										
A1-Sm-15										
A1-Kd-15										
A2-Sk-15										
A2-Br-15										
A2-Lu-15										
A2-Li-15										
A2-Sp-15										
A2-Ht-15										
A2-Ln-15										
A2-Sm-15										
A2-Kd-15										
A3-Sk-15										

A3-Br-15										
A3-Lu-15										
A3-Li-15										
A3-Sp-15										
A3-Ht-15										
A3-Ln-15										
A3-Sm-15										
A3-Kd-15										
B1-Sk-15										
B1-Br-15										
B1-Lu-15										
B1-Li-15										
B1-Sp-15										
B1-Ht-15										
B1-Ln-15										
B1-Sm-15										
B1-Kd-15										
B2-Sk-15										
B2-Br-15										
B2-Lu-15										
B2-Li-15										
B2-Sp-15										
B2-Ht-15										
B2-Ln-15										
B2-Sm-15										
B2-Kd-15										
B3-Sk-15										
B3-Br-15										
B3-Lu-15										
B3-Li-15										
B3-Sp-15										
B3-Ht-15										
B3-Ln-15										
B3-Sm-15										
B3-Kd-15										

SLIDE NO	**Physical Quality**	**QUALITY OF TISSUE PRESERVATION SCORE**				**QUALITY OF STAINING SCORE**				
	Artifacts Associated with fixation (mention)	**POOR**	**SUB OPTI MAL**	**GOOD**	**VERY GOOD**	Artifacts Associated with fixation (mention)	**POOR**	**SUB OPTI MAL**	**GOOD**	**VERY GOOD**
IP-Li-15										
LP-Li-15										
A1P-Li-15										
A2P-Li-15										
A3P-Li-15										
B1P-Li-15										
B2P-Li-15										
B3P-Li-15										
IM-Sm-15										
LM-Sm-15										
A1M-Sm-15										
A2M-Sm-15										
A3M-Sm-15										
B1M-Sm-15										
B2M-Sm-15										
B3M-Sm-15										

SLIDE NO	**Physical Quality**	**QUALITY OF TISSUE PRESERVATION SCORE**				**QUALITY OF STAINING SCORE**				
	Artifacts Associated with fixation (mention)	**POOR**	**SUB OPTI MAL**	**GOOD**	**VERY GOOD**	Artifacts Associated with fixation (mention)	**POOR**	**SUB OPTI MAL**	**GOOD**	**VERY GOOD**
IE-Sk-15										
LE-Sk-15										
A1E-Sk-15										
A2E-Sk-15										
A3E-Sk-15										
B1E-Sk-15										
B2E-Sk-15										
B3E-Sk-15										
IC-Ln-15										
LC-Ln-15										
A1C-Ln-15										
A2C-Ln-15										
A3C-Ln-15										
B1C-Ln-15										
B2C-Ln-15										
B3C-Ln-15										

APPENDIX 9: KEY TO SLIDES' LEBELLING

SLIDES LABELLING: KEY

I	International standard i.e. Neutral Buffered Formalin,
Sk	Skin,
15	The year 2015
Br	Brain
Lu	Lung
Li	Liver
Sp	Spleen
Ht	Heart
Ln	Lymph node
Sm	Skeletal muscle
Kd	Kidney
L	Local Standard i.e. 10% Formol Saline
A1	Fixative A1
A2	Fixative A2
A3	Fixative A3
B1	Fixative B1
B2	Fixative B2
B3	Fixative B3
P	PAS
M	Massons Trichrome
E	EMA
C	CD23

APPENDIX 10: TARGET AND CHEMICAL FIXATIVE DO'S AND DON'TS

(Fixation, 2014)

TARGET	FIXATIVE OF CHOICE	FIXATIVE TO AVOID
Proteins	Neutral Buffered Formalin, Paraformaldehyde	OsmiumTetroxide
Enzymes	Frozen sections	Chemical Fixatives
Lipids	Frozen Sections*, Glutaraldehyde/Osmium Tetroxide	Alcoholic fixatives, Neutral Buffered Formalin
Nucleic Acids	Alcohol fixatives	Aldehyde fixatives
Mucopolysaccharides	Frozen Sections	Chemical Fixatives
Biogenic Amines	Bouin Solution~, Neutral Buffered Formalin	
Glycogen	Alcoholic based fixatives	OsmiumTetroxide

- Frozen Sections preserve RNA and Lipids despite poor morphology. Compare to Paraffin sections, synonymous to Chemical Fixatives in the table, which destroy RNA and affect some antigens BUT give good morphology.

~ A picrate.

APPENDIX 11: ETHICAL CLEARANCE FOR THE STUDY