COMPARATIVE STUDY OF THE EFFECT OF TABLE SALT IN 10% FORMAL SALINE AND SATURATED SODIUM CHLORIDE SOLUTION AS TISSUE FIXATIVES

BY

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DEPARTMENT OF HUMAN ANATOMY

FACULTY OF MEDICINE

AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA

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DEPARTMENT OF HUMAN ANATOMY

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NIGERIA

MARCH, 2014

DECLARATION

I GBADAMOSI Monsurat Titi hereby declare that this Thesis titled "comparative study of the effect of table salt in 10% formal saline and saturated sodium chloride solution as tissue fixatives" was performed by me in the Department of Human Anatomy, under the supervision of Professor K V Shola and Dr W O Hamman. The Information derived from the literature has been duly acknowledged in the text and a list of the references is provided. No part of this research has been presented for another degree or diploma at any other Institution.

---------- ----------

Signature **Date**

CERTIFICATION

This project Report titled: "Comparative study of the effect of Table salt in 10% Formal Saline and Saturated Sodium Chloride Solution as tissue Fixatives" meets the regulation governing the award of the degree of Masters of Science of Ahmadu Bello University Zaria and is approved for it contribution to scientific knowledge and literary presentation.

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DEDICATION

This work is dedicated

To

GOD Almighty WHO is “the beginning and the end”

And

To the Best Siblings in the World, My Brothers!

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All thanks and Praises belong to Almighty Allah who has made it possible for me to complete my study successfully. My profound gratitude goes to my Supervisors, Dr K V Olorunshola and Dr W O Hamman for their Patience, understanding and tireless efforts in making this work a success.

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ABSTRACT

Formal saline and saturated sodium chloride solutions were evaluated and compared as tissue fixatives in a quest of finding a safer and cheaper alternative to formaldehyde. Livers and testes dissected from 20 male rats allotted to 4 groups of 5 animals each were fixed for 12hrs, 24hrs, 48hrs and 7days in either standard formal saline (control) or formal saline prepared with table salt or saturated sodium chloride solution prepared with laboratory salt or saturated sodium chloride solution prepared with table salt and labeled as groups A, B, C and D respectively. The livers and testes were processed routinely and stained with H&E.

The histological features (such as good staining, morphological appearance of the nucleus and cytoplasm etc) observed in the liver sections were not significantly different across the fixative groups at all fixative periods while those observed in the testes sections fixed in formal saline (both the standard and the one prepared with table salt) were significantly higher than those fixed in saturated sodium chloride solutions (both from laboratory and table salt) $P<0.001$. Histomorphometric evaluations of the Area and Diameter of seminiferous tubules were significantly higher in testes fixed in formal saline (prepared with table salt) and those fixed in saturated sodium chloride solutions (both from laboratory and table salt) than the control group of standard formal saline $P<0.05$. The height of germinal epithelium was significantly lower in testes fixed in formal saline with table salt and those in saturated sodium chloride solutions (both from laboratory and table salt) than the control group of standard formal saline $P<0.05$.

So we conclude that the saline in formal saline could be replaced with table salt to enhance its performance on the fixation of liver and testis tissues of adult male wistar rats, saturated sodium chloride solution (both from laboratory and table salt) could be

used to fix liver tissues for routine H&E demonstration for up to 48hours but not for testes of adult male wistar rats.

CHAPTER ONE

1.0 INTRODUCTION

Fixation is the single most influential factor in the long sequence of steps between procurement of the specimen and coverslipping the stained slide (Carson and Christa, 2009). Nearly any other step can be reversed to ameliorate a problem. Tissues can be reverse-processed and then reprocessed if a mistake or breakdown occurs in tissue processing. Most stains can be removed and reapplied to correct problem with intensity or specificity. Bubbles under the coverslip can be removed simply by removing and resetting the coverslip. In sharp contrast to these examples, errors in fixation are permanent. On the positive side, properly fixed tissue is nearly impervious to abuse during tissue processing and slide preparation. Understanding the role and mechanism of fixation is crucial to producing quality slides and interpreting artifacts (Carson and Christa, 2009). Once tissues are removed from the body, they 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 proteins and eventual liquefaction of the cell. Autolysis is more severe in tissues which are rich in enzymes, such as the liver, brain and kidney, and is less rapid in tissues such as elastic fibre and collagen.

Fixation is the preservation of tissues and cellular components in life state as close as possible to arrest autolysis which is self-degradation of tissues that commences immediately after death, prevent putrefaction which is bacterial degradation of tissue and to prepare the tissues for subsequent stages of processing without significant change. The objective of fixation is to preserve cells and tissue constituents in as close a life-like state as possible and to allow them to undergo further preparative procedures

without significant change. Fixation arrests autolysis and bacterial decomposition and stabilizes the cellular and tissue constituents so that they withstand the subsequent stages of tissue processing. 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 (Olszewski *et al*., 2004). The technique of using fixatives in the preparation of cytologic, histologic, or pathologic specimens for the purpose of maintaining the existing form and structure of all the constituent elements is termed TISSUE FIXATION while FIXATIVES are agents employed in the preparation of histologic or pathologic specimens for the purpose of maintaining the existing form and structure of all the constituent elements (Olszewski *et al.*, 2007).

1.1 BACKGROUND OF THE STUDY

A large variety of fixatives are now available but no single substance or known combination of substances has the ability to preserve and allow the demonstration of every tissue component. It is for this reason that some fixatives have only special and limited applications, and in other instances, a mixture of two or more reagents is necessary to employ the special properties of each. The selection of an appropriate fixative is based on considerations such as the structures and entities to be demonstrated and the effects of short-term and long-term storage (Puchtler *et al*., 1985). Each fixative has advantages and disadvantages; some are restrictive while others are multipurpose. Ferdinand Blum has been credited as the first person to use formaldehyde as a tissue fixative. Formaldehyde, as 4% buffered formaldehyde (10% buffered formalin), is the most widely employed universal fixative particularly for routine paraffin embedded sections.

There is a general consensus that formalin is the best fixative and there is therefore no need for improvement for any reason, generating what has been defined by some authors as " the formalin dogma" (Tbakhi *et al*., 1998), a somewhat fatalistic and additive approach that has severely hampered the search for alternatives to formalin in fixation procedures. The toxicity of formaldehyde is emerging as the main issue for its abolition as a general fixative used in large quantities in pathology and research laboratories (a medium- large structure may annually use more than 3500 litres of ready to use formalin) (Cristina *et al*., 2012).

The IARC report linked formaldehyde exposure to nasopharyngeal carcinoma (Cogliano *et al*., 2005), a successful link between formaldehyde and leukemia was put forward in 2009 (Baan *et al*., 2009) these statements were reinforced in the 2012 report (IARC, 2012) by the same agency. These data of the carcinogenicity of formaldehyde for humans should act as a potent stimulus to reconsider the "formalin dogma" and to evaluate with an open mind the use of alternative fixatives. Moreover, the formation of DNA-Protein cross-links possibly represents a permanent "signature of exposition to formaldehyde (Shaham *et al*., 2003). This information was largely ignored by the pathologists and researchers communities who assumed that the use of formaldehyde as a fixative is mandatory and thus completely safe when used with adequate procedures (Buesa, 2008). More recently chromosomal alterations have been detected in health workers and linked to formalin use in pathology wards (Santovito *et al*., 2011).

Commercially-available alternatives to formalin are fairly numerous and in different supply formats either as ready for use or in concentrated forms. While the ready to use formulas are very convenient, concentrated formulas may allow some degree of

manipulation and custom tailored modifications (Cristina *et al*., 2012). Less numerous are the non-proprietary formulas proposed in recent scientific journals and histochemistry textbooks may be worth revisiting as they give useful clues for new improvements. The availability of commercial alternatives to formalin is mainly restricted to western countries being widely spread in North America and to a lesser degree in Europe leaving out Africa and Asia. The possibility of preparing effective substitutes in the laboratory may be of particular interest considering the high cost of an effective and durable aspiration chain of formalin vapors (from aspiration hoods, aspirated cabinets and final proper disposal) and the limited economical resources of developing countries that frequently lack proper servicing instruments (Marianni-Constantini, 2011).

Indirect costs of formalin fixation (such as adequate disposal of used fixative and fixed specimens, potential toxicity of products from incineration of residual specimens and the imperative necessity of adequate health surveillance of workers exposed to a human carcinogen) should also be considered as an additional and non-trivial economic cost (Cristina *et al*., 2012). Another point to be considered is the use of formaldehyde in developing countries in which aspiration devices are rarely used and the disposal of toxic waste may also be inexistent or problematic (Lykidis *et al*., 2007). Thus, the use of alimentary grade compounds for fixation (as is the case of some "homemade" substitutes) may be of benefit in their laboratories and be economically sustainable even in low income environment.

1.2 AIM AND OBJECTIVES OF THE RESEARCH

The aim of the research is to comparatively evaluate the effect of table salt in 10% formal saline and saturated sodium chloride solution as tissue fixatives with the ultimate goal of finding a safer, cheaper and readily available alternative to formaldehyde. The objectives of the study include:

- To compare the effects of 10% formal saline and saturated sodium chloride solution on the fixation of liver and testes of adult male Wistar Rats.
- To compare the effects of table salt in 10%formal saline and saturated sodium chloride solution on the preservation of liver and testes of adult male Wistar Rats.
- To compare the effects of standard 10% formal saline and table salt in 10% formal saline on the preservation of liver and testes of adult male Wistar Rats.
- To compare the effects of Laboratory salt and table salt on the preservation of liver and testes of adult male Wistar Rats.

1.3 STATEMENT OF THE RESEARCH PROBLEM

The most widely used fixative in histology laboratory is 10% neutral buffered formalin and formal saline (Carson and Christa, 2009). The sodium chloride supplied to laboratory is expensive, so the cheaper edible table salt is sought as a replacement. Also, formaldehyde (more expensive than sodium chloride) has been implicated as a carcinogenic material, its total replacement by the cheaper, safer and readily available saturated solution of sodium chloride is thought to be worthy of investigation.

1.4 JUSTIFICATION OF THE STUDY

Household use of Sodium Chloride as a preservative has been recognized and documented since ancient times. However, its use as a fixative in the laboratory has not been well explored considering sparse literature available on the subject. Also with the numerous problems associated with the use of formaldehyde especially in our environment ranging from its toxicity, to carcinogenicity, availability and costs implications (direct and indirect). The need for an alternative to formaldehyde is apparent and this study will contribute to this all important quest.

1.5 SIGNIFICANCE OF THE STUDY

Findings from this study may lead to the development of cheaper, safer and readily available fixative for research, histological and pathological purposes.

1.6 RESEARCH HYPOTHESES

- The use of laboratory and table salt in the preparation of formal saline has similar effects on the fixation of Liver and Testes of Adult male Wistar Rats.
- Saturated sodium chloride solution prepared from Laboratory and Table Salt have similar effects on the Liver and Testes of Adult male Wistar Rats.
- Saturated sodium chloride solutions prepared from Laboratory and Table Salt have the ability to fix Liver and Testes of Adult male Wistar Rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 FIXATIVES

The concept of fixation of biological tissues in order to understand biological function and structure has led to the development of many types of fixatives over the last century. The mechanisms and principles by which specific fixatives act to harden and preserve tissues and to prevent the loss of specific molecules fall into broad categories. These include the covalent addition of reactive groups and of cross-links, dehydration, and the effects of acids, salt formation, and heat, along with combinations of these actions. Compound fixatives may function via several of these mechanisms. Each fixative has advantages and disadvantages. These include molecular loss from 'fixed' tissues, swelling or shrinkage of tissues during the process, variations in the quality of histochemical and immunohistochemical staining, the ability to perform biochemical analysis accurately, and varying capabilities to maintain the structures of cellular organelles (Eitoum *et al*., 2001a, 2001b). To date a universal or ideal fixative has not been identified. Fixatives are therefore selected based on their ability to produce a final product needed to demonstrate a specific feature of a specific tissue (Dapson, 1993). Several chemicals or combinations of chemicals can act as good fixatives and accomplish many of the stated goals of fixation. Some fixatives add covalent reactive groups which may induce cross-links between proteins, individual protein moieties, within nucleic acids, and between nucleic acids and proteins (Eitoum *et al*., 2001a, 2001b).The best examples of such 'cross-linking fixatives' are formaldehyde and Glutaraldehyde.

2.2 FORMALDEHYDE

The use of formalin as histological fixative dates back to the time when the synthesis of formaldehyde from methanol was successfully achieved by AW Hofmann in 1868. Since then, properties and potential use of this substance were intensively studied and formalin became attractive as an alternative reagent to the old established histological fixatives such as ethanol. Apart from tissue fixation, formaldehyde has become an important product for many applications, and its production reached large scales in most countries in Europe, America and Asia. Formaldehyde is sold as a saturated aqueous solution under the name *formalin* or *formaldehyde solution (37-40%)*. Alternatively, formaldehyde can be obtained as a white crystalline product, i.e. *paraformaldehyde* which is polymerized formaldehyde. The formalin in laboratories is usually used as 10% formal saline i.e. Formalin 100ml, Distilled water 900ml, and Sodium chloride 9g. Depending on the intended use, cells or tissues of different origin may need different fixation schedules. Fixation solutions for the study of fine structural details usually need more careful preparation than those for routine histopathological purpose.

Physical and chemical properties of formaldehyde include a molecular weight of 30.0[a]. Specific gravity of 0.815 at –20°C/4°C[b]. Melting point of –92°C[a]. Boiling point of –19.5°Ca and its solubility in water is 400 g/L at 25°Ca. it has a Log *K*ow of 0.35a, a vapor pressure of 3,890 mm Hg at 25°Ca, its vapor density relative to air is 1.067a and it has a Dissociation constant (p K_a) of 13.27 at 25°Ca (HSDB, 2009). Formaldehyde is a gas (formula HCHO). It dissolves readily into water which is called *formalin*. Formaldehyde dissolves into water to 37-40% (w/v), and this solution represents *100% formalin*. When diluting such a formalin solution, the final concentration is expressed

based on the 100% formalin reference: 10% formalin is a 1:10 dilution of 100% formalin. Four per cent formaldehyde or 10% buffered formalin is commonly prepared by adding 100 ml of 40% formaldehyde to 900 ml distilled water with 4 g sodium phosphate, monobasic and 6.5 g sodium phosphate, dibasic (anhydrous). To be effective, the specimen is completely submerged in five to twenty times its volume of fixative. 10% formal saline is prepared by adding 100ml of formalin to 900ml distilled water with 8.5g or 9g sodium chloride.

Formalin progressively hardens tissue, but is a mild coagulant of proteins; in fact it is so weak that it might be considered a non-coagulant. It neutralizes basic groups and increases the acidity of proteins. In consequence, formalin-fixed proteins will stain well in basic dyes, but less well in acidic dyes. Formalin has a moderate speed of penetration but its action is slow and somewhat incomplete unless tissues are left in it for some time. It reacts most efficiently at pH 7.5-8. Although formalin preserves the cells adequately it may not protect them completely, because it requires a long time to harden the tissue. Shrinkage, therefore, can take place if dehydration, clearing and infiltration are initiated before the hardening action is complete. Since these processes are not employed in the freezing technique, formalin is a satisfactory fixative for this method (Leong *et al.*, 1996). 10% buffered formalin and 10% formal saline are probably the simplest fixatives and have been in common use for many years, which should be thought of as a testament to their effectiveness (Eitoum *et al.*, 2001b). 10% formalin is not isotonic and there is a possibility that erythrocytes may be damaged by lysis. Formal saline is a little more effective than 10% formalin as it is isotonic and there is less likelihood that erythrocytes will be damaged. Both solutions are prone to the formation of formalin pigment, especially in bloody tissues, sometimes quite

rapidly. This is due to formic acid being produced from the formaldehyde causing the pH of the solution to be lowered which could be neutralized by storing the fixatives over a layer of marble chips or crushed oyster shells. Both are useful as fixatives for museum and photography specimens as they permit restoration of natural colour to the specimen (Khulmann, 2009).

2.2.1 SOURCES AND ROUTES OF EXPOSURE TO FORMALDEHYDE

The major source of exposure to exogenous formaldehyde is from occupational exposure, since it is produced and used in large quantities industrially. Formaldehyde is used in the manufacture of many permanent adhesives such as those used to produce plywood, fibreboard, particle board and carpet adhesives. Small amounts of formaldehyde occur naturally in the environment and may also be present in the emissions from the combustion of organic materials such as wood and tobacco smoke, and automobile emissions (Golden, 2005). However, the amounts of formaldehyde present from such sources are likely to be smaller than may be found in an occupational setting. Formaldehyde in solutions of approximately 5% in water is also used as a disinfectant and fumigant in hospitals. Its buffered 10% solution is used as a fixative in various tissue processing laboratories (Stayner *et al*., 1994). The major routes of occupational exposure to formaldehyde are by inhalation of vapour or by dermal contact with splashes of formaldehyde solutions (IPCS, 1989, ATSDR, 1999, and CCOHS, 2004).

2.2.2 METABOLISM OF FORMALDEHYDE

Formaldehyde is metabolized at the initial site of contact into formate, by formaldehyde dehydrogenase. Formaldehyde is naturally a metabolic intermediate produced in all

cells during the metabolism of serine, glycine, methionine and choline (Heck *et al*., 1982) also in the oxidative demethylation of xenobiotics. Neither formaldehyde nor its metabolites accumulate in any of the body tissues to any appreciable extent. However, the elimination of formate is slower than its formation from formaldehyde. Formaldehyde is eliminated mainly by urinary excretion as formic acid or exhaled as carbon dioxide (Grammer, 1990).

Inhaled formaldehyde reacts rapidly at the site of contact and is efficiently absorbed in the respiratory tract. A portion of the formaldehyde entering the fluid layer covering the respiratory epithelium, the respiratory tract lining fluid (RTLF), is reversibly hydrated to methylene glycol. Among other components, the RTLF is rich in antioxidants including glutathione (Cross *et al*., 1994) with which formaldehyde may reversibly react to form *S*-hydroxymethylglutathione. Both the hydrated and unreacted formaldehyde may be absorbed into the epithelial layer where there is further opportunity for formaldehyde to bind to glutathione. This glutathione conjugate in turn is oxidized to *S*-formylglutathione by formaldehyde dehydrogenase. Hydrolysis of *S*-formylglutathione yields formate and glutathione. Formic acid may be eliminated in urine and feces, or dehydrogenated to CO_2 and exhaled. The presence of glutathione and formaldehyde dehydrogenase in epithelial cells of the respiratory tract varies with location and influences the amount of formaldehyde reaching the blood. While glutathione-bound formaldehyde is rapidly metabolized, free formaldehyde in cells can form DNA-protein cross-links (Franks, 2005). Formaldehyde is rapidly metabolized by glutathione-dependent formaldehyde dehydrogenase (also known as alcohol dehydrogenase 5, ADH5) and *S*-formyl-glutathione hydrolase to formic acid, which enters the one-carbon pool and can be either excreted in the urine or oxidized to carbon

dioxide and exhaled. ADH5 has been detected in all human tissues at all stages of development, from embryo through adult (Thompson *et al.* 2009). Although formaldehyde is rapidly metabolized, it is an electrophile that reacts with a variety of endogenous molecules, including glutathione, proteins, nucleic acids, and folic acid (NTP 2010).

2.2.3 HEALTH EFFECTS OF FORMALDEHYDE EXPOSURE

The predominant effects following an acute inhalation exposure to formaldehyde are irritation and burning of the mucous membranes of the nose, mouth and upper respiratory tract (Shara *et al*., 1992). Some adverse effects following acute exposure to large amounts of formaldehyde may include weakness, headache, nausea, vomiting, pneumonia, dyspnoea, wheezing, coughing, laryngeal and pulmonary oedema, bronchospasm, laryngeal spasm, respiratory depression, obstructive tracheo-bronchitis, central nervous system depression, convulsions and coma (Nordmann *et al*., 1985). Acute ingestion of formaldehyde will lead to irritation and burns of the mouth and throat and burns and ulceration of the gastrointestinal tract, chest or abdominal pain, nausea, vomiting, diarrhoea, gastrointestinal haemorrhage and renal failure (Thrasher *et al*., 1990). Repeated or prolonged dermal exposure to splashes of solutions containing formaldehyde can lead to skin irritation and may also cause skin sensitization (allergic contact dermatitis) (Maibach, 1983).

No convincing evidence of genotoxic effects were detected in tissues other than those of the portal of entry. Formaldehyde formed DNA-Protein Crosslinks at the sites of first contact. DNA-Protein Crosslinks were found in the nasal mucosa of rats (Casanova *et al.*, 1984a), but there was no indication of an accumulation of DNA-

Protein Crosslinks in high-tumour sites of the noses. DNA-Protein Crosslinks were similar after acute and subchronic exposures, suggesting that rat nasal DNA-Protein Crosslinks are rapidly removed (Casanova *et al.*, 1984b). *In vitro*, formaldehyde is able to induce gene mutations and chromosomal aberrations in mammalian cells without (and also in presence of) external metabolic activation. DNA-protein crosslinks are a sensitive measure of DNA interaction by formaldehyde. *In vivo*, the overall evidence of available studies supports the conclusion that the genotoxic effects after exposure via relevant routes are limited to those cells which are in direct contact with formaldehyde and no effects are observed in distant-site tissues. This is consistent with formaldehyde's high reactivity with many cellular nucleophiles and its rapid metabolic degradation. Formaldehyde is not expected to cause reproductive or developmental toxicity at exposures below maternally toxic doses (IARC, 2006).

Formaldehyde was tested for carcinogenicity by inhalation in mice, rats and hamsters, by oral administration in drinking-water in rats, by skin application in mice, and by subcutaneous injection in rats. In additional studies in mice, rats and hamsters, modification of the carcinogenicity of known carcinogens was tested by administration of formaldehyde in drinking-water, by application to the skin or by inhalation and evidence of the carcinogenicity was observed in several of these studies particularly by the induction of squamous cell carcinomas in the nasal cavities (Shaham *et al*., 1996). A study in male rats demonstrated an increase in fore stomach papillomas. A further study in both male and female rats showed an increase in gastrointestinal leiomyosarcomas, particularly in the females, whilst another study identified an increased incidence in the male rats of malignant tumours, lymphomas, leukemias and testicular interstitial-cell adenomas (Mclaughlin, 1994).

Occupational exposure of workers to formaldehyde has been associated with a significant increase in mortality due to nasopharyngeal cancers compared with the US national population (IARC, 2006). The results from the largest and most informative cohort of industrial workers in the USA, supported by largely positive findings from other studies, provide sufficient evidence that formaldehyde causes nasopharyngeal cancer in humans. There was only limited epidemiological evidence that formaldehyde causes sinonasal cancer in humans (IARC, 2006). A successful link between formaldehyde exposure and leukemia was put forward in 2009 (Bann *et al*, 2009) and were reinforced in 2012 report (IARC, 2012) by the same agency. Overall, The International Agency for Research on Cancer (IARC) has evaluated that there is sufficient evidence for the carcinogenicity of formaldehyde in humans and experimental animals, and has therefore concluded that formaldehyde is carcinogenic to humans.

Formaldehyde is known to be a human carcinogen based on sufficient evidence of carcinogenicity from studies in humans and supporting data on mechanisms of carcinogenesis. Formaldehyde was first listed in the *Second Annual Report on Carcinogens* in 1981 as *reasonably anticipated to be a human carcinogen* based on sufficient evidence from studies in experimental animals. Since that time, additional cancer studies in humans have been published, and the listing status has been changed to *known to be a human carcinogen* in the *Twelfth Report on Carcinogens* (2011)**.**

2.3 SODIUM CHLORIDE

Sodium chloride, also known as common salt, table salt, or halite, is a chemical compound with the formula NaCl. Sodium chloride is the salt most responsible for the

salinity of the ocean and of the extracellular fluid of many multicellular organisms. As the major ingredient in edible salt, it is commonly used as a condiment and food preservative. The physical and chemical properties of sodium chloride include a Molecular formula Nacl, Molar Mass of 58.4277g/mol, Density of 2.16g/cm, solid, it appears as white or colorless crystals of powder and it is odourless. Its melting and boiling points are 801A C and 1738K respectively and it has a solubility of 35.9g/mol in water.

Salt is currently mass-produced by evaporation of sea water or brine from other sources, such as brine wells and salt lakes, and by mining rock salt, called halite. In 2002, world production was estimated at 210 million metric tonnes, the top five producers being the United States (40.3 million tonnes), China (32.9), Germany (17.7), India (14.5), and Canada (12.3) (Feldman, 2005). As well as the familiar uses of salt in cooking, salt is used in many applications, from manufacturing pulp and paper, to setting dyes in textiles and fabric, to producing soaps, detergents, and other bath products. It is the major source of industrial chlorine and sodium hydroxide, and used in almost every industry (Feldman, 2005).

In cold countries, large quantities of rock salt are used to help clear highways of ice during winter, although "Road Salt" loses its melting ability at temperatures below -15Â°C to -20Â°C (5Â°F to -4Â°F). Sodium chloride is sometimes used as a cheap and safe desiccant due to its hygroscopic properties, making salting an effective method of food preservation historically. Even though more effective desiccants are available, few are safe for humans to ingest (Finnish environmental institute, 2007).

In humans, a high-salt intake has long been known to generally raise blood pressure, especially in certain individuals. It was demonstrated to attenuate nitric oxide

production. Nitric oxide (NO) contributes to vessel homeostasis by inhibiting vascular smooth muscle contraction and growth, platelet aggregation, and leukocyte adhesion to the endothelium (Markel, 1987).

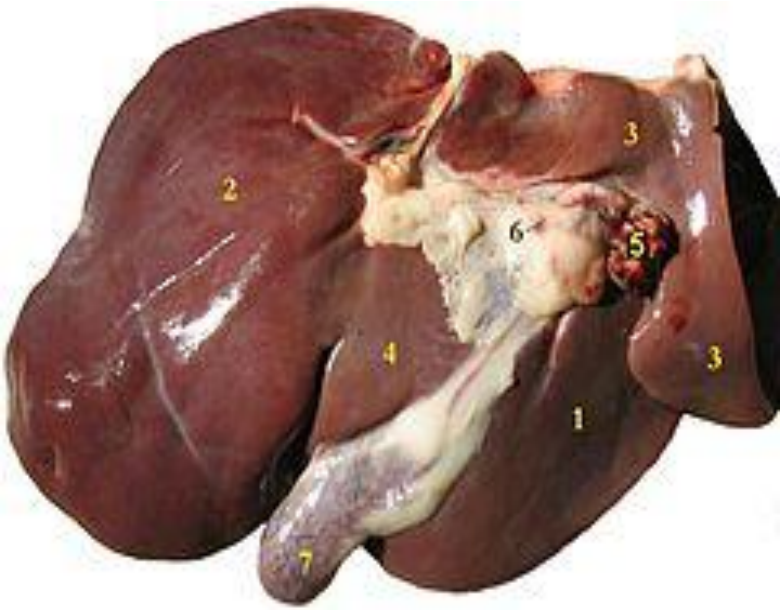
A method to overcome the problems of formaldehyde is to use an alternative fixative that is comparable to formaldehyde in action or better suited for the preservation of tissues which this study is hoped to contribute in solving. Researchers have tried various other agents from commercial formalin substitutes (Vladimir *et al*., 2003; Yak-Nam *et al*., 2011) to homemade fixatives (Zanini *et al*., 2012) and Honey (Ozkan *et al*., 2012) all in an effort to discover a safer fixative to replace formaldehyde, all presenting with good and encouraging results. A study concluded that the cheap saturated table salt solution can be used as an alternative to the formaldehyde in preserving the bodies of animals (Al Hubaity *et al.*, 2003). Fixation of skin and lymph node fragments in anhydric sodium chloride at room temperature for periods of weeks or months was found to preserve morphological structures and immunoreactivity (Olszewski *et al.*, 2004). Segments of rat's aorta were harvested, immersed in pulverized dehydrated sodium chloride, and stored for 1 week to 3 months. Thereafter, they were desalinated and transplanted. The tensile strength and maximum intraluminal pressures did not significantly differ from freshly harvested, transplanted aortic segments (Gewartowska *et al.*, 2007).

Al Saraj (2010), concluded his study by suggesting the use of saturated sodium chloride solution as an alternative fixative procedure which can be used in emergency cases when no other fixative is available (for pathologist, veterinarian, biologist…etc) in such cases for tissue preservation. Human skin fragments can be preserved in anhydric

sodium chloride at room temperature for periods of weeks or months and successfully transplanted, retaining normal morphological structure (Olszewski *et al.*, 2004).

2.4 THE LIVER

The liver is a reddish brown organ with four lobes of unequal size and shape. A human liver normally weighs 1.44–1.66 kg (3.2–3.7 lb), (Cotran *et al.*, 2005) and is a soft, pinkish-brown, triangular organ. It is both the largest internal organ (the skin being the largest organ overall) and the largest gland in the human body. It is located in the right upper quadrant of the abdominal cavity, resting just below the diaphragm. The liver lies to the right of the stomach and overlies the gall bladder. It is connected to two large blood vessels, the hepatic artery and the portal vein. The hepatic artery carries blood from the aorta while the portal vein carries blood containing digested nutrients from the entire gastrointestinal tract except the anal canal and also from the spleen and pancreas. These blood vessels subdivide into capillaries, which then lead to a lobule. Each lobule is made up of millions of hepatic cells which are the basic metabolic cells. Lobules are the functional units of the liver.



LIVER: (1) right lobe, (2) left lobe, (3) caudate lobe, (4) quadrate lobe, (5) hepatic artery and portal vein, (6) hepatic lymph nodes, (7) gall bladder.

2.4.1 FUNCTIONAL PARTS OF THE LIVER

Traditional gross anatomy divided the liver into four lobes based on surface features. The falciform ligament is visible on the front (anterior side) of the liver. This divides the liver into a left anatomical lobe, and a right anatomical lobe. If the liver is flipped over, to look at it from behind (the visceral surface), there are two additional lobes between the right and left. These are the caudate lobe (the more superior) and the quadrate lobe (the more inferior). At the visceral surface, the lobes are divided up by the ligamentum venosum and ligamentum teres (anything left of these is the left lobe), the transverse fissure (or porta_hepatis) divides the caudate from the quadrate lobe, and the right sagittal_fossa, which the inferior vena cava runs over, separates these two lobes from the right lobe. Each of the lobes is made up of lobules; a vein goes from the centre, which then joins to the hepatic vein to carry blood out from the liver. On the surface of the lobules, there are ducts, veins and arteries that carry fluids to and from

them. The central area where the common bile duct, hepatic portal vein, and hepatic artery proper enter is the hilum or "porta_hepatis". The duct, vein, and artery divide into left and right branches, and the portions of the liver supplied by these branches constitute the functional left and right lobes (Kmeic 2001).

The functional lobes are separated by an imaginary plane joining the gall bladder fossa to the inferior vena cava. The plane separates the liver into the true right and left lobes. The middle hepatic vein also demarcates the true right and left lobes. The right lobe is further divided into an anterior and posterior segment by the right hepatic vein. The left lobe is divided into the medial and lateral segments by the left hepatic vein. The fissure for the ligamentum_teres also separates the medial and lateral segments. The medial segment is also called the quadrate lobe. In the widely used Couinaud (or "French") system, the functional lobes are further divided into a total of eight subsegments based on a transverse plane through the bifurcation of the main portal vein (Kmeic, 2001).

2.4.2 VESSELS AND NERVES OF THE LIVER

The Liver receives blood from two sources i.e the portal vein (70%) and the hepatic artery (30%) (Dalley and Moore, 1999). The portal vein is a short wide vein formed by the superior mesenteric and splenic veins posterior to the neck of the pancreas and ascends superior to the inferior vena cava and divides at the end of the porta hepatis into right and left branches that ramify within the liver (Dalley and Moore, 1999). The hepatic artery is a branch of the celiac trunk and may be divided into the common hepatic and the hepatic artery proper (Dalley and Moore, 1999).

The hepatic artery carries well oxygenated blood from the aorta and the portal vein carries poorly oxygenated but nutrient-filled blood from the gastrointestinal tract

(except the inferior part of the anal canal) to the sinusoids of the liver (Dalley and Moore, 1999). At or close to the porta hepatis, the hepatic artery and portal vein terminates by dividing into right and left branches which supply the right and left parts of the liver respectively (Dalley and Moore, 1999).

The nerves of the liver are derived from the hepatic nerve plexus which is the largest derivative of the celiac plexus. The hepatic plexus accompanies the branches of the hepatic artery and portal vein to the liver (Dalley and Moore, 1999). It consists of fibers from the anterior and posterior vagal trunks. Nerve vessels accompany the vessels and bile ducts of the portal triad within the liver (Dalley and Moore, 1999).

2.4.3 LYMPHATIC DRAINAGE OF THE LIVER

The liver is a major lymph-producing organ during intra uterine life. It produces between one-quarter and one-half of the lymph received by the thoracic duct. The lymphatic vessels of the liver occur as superficial lymphatics in the subperitoneal fibrous capsule of the liver and as deep lymphatics in the connective tissue of that accompany the ramification of the portal triad and hepatic veins (Dalley and Moore, 1999). The lymphatics on the anterior aspects of the diaphragm and viscera surfaces of the liver and those accompanying the portal triad drain into the hepatic lymph nodes. Efferent nodes drain into celiac lymph nodes and the chyle cistern (Dalley and Moore, 1999). The lymphatics on the posterior aspects of the diaphragmatic and visceral surface drain into the phrenic lymph nodes. Efferent nodes drain into the thoracic duct while a few drain into the left gastric, parasternal and lymphatics of the anterior abdominal wall (Dalley and Moore, 1999).

2.4.4 HISTOLOGY OF THE LIVER

Sections through the liver show polygonal areas that constitute the hepatic lobules. In some species, the lobules are distinctly demarcated by connective tissue septa but in the human liver, the connective tissue is scanty and lobules often appear to merge with one another. In transverse sections, each lobule appears to be made up of cords of liver cells that are separated by sinusoids (Singh, 2002). The liver cells are arranged in the form of plates that branch and anastomose with one another to form a network and the spaces within the network are occupied by sinusoids. Along the periphery of each lobule, there are angular intervals filled by connective tissue and these intervals are called portal canals. Each canal forms a connective tissue network permeating the entire liver substance (Singh, 2002). Each canal contains a branch of the portal vein, a branch of the hepatic artery and an interlobular bile duct. These three structures collectively form the portal triad. Blood mainly from the terminal branch of the portal vein enters the sinusoids at the periphery of the lobule and passes towards its centre. The sinusoids open into a central vein which occupies the centre of the lobule (Singh, 2002).

The vessels in a portal triad usually give branches to parts of three adjoining lobules. The area of the liver tissue (i.e comprising of three hepatic lobules) supplied by one branch of the portal vein is regarded as the functional unit of the liver and is referred to as the portal lobule. On the other hand, a portal acinus consists of the area of liver tissue supplied by one hepatic arteriole running along the line of junction of two hepatic lobules. Two central veins lie at the ends of the acinus (Singh, 2002). The liver is covered by a connective capsule (Glisson's capsule) which extends into the liver substance through the portal canals where it surrounds the portal triads. Sinusoids are surrounded by reticular fibers while the connective tissues do not intervene between

adjoining liver cells. The sinusoids are lined by an endothelium in which there are numerous pores (fenestrae). Interspersed amongst the endothelial cells are hepatic macrophages (Kupffer cells). The surface of the Liver cell is separated from the endothelial lining of the sinusoid by a narrow perisinusoidal space (of Disse). Microvilli, present on the liver cells extend into this space, as a result of these factors, hepatocytes are brought into very intimate relationship with the circulating blood. Some fat cells may also be seen in the space of Disse (Singh, 2002).

2.4.5 FUNCTIONS OF THE LIVER

According to Singh (2002), the functions of the liver include:

- The Liver acts as an exocrine gland for the secretion of bile.
- It plays a prominent role in the metabolism of carbohydrate, proteins and fats.
- It acts as a store for various substances including glucose (glycogen).
- It plays a protective role by detoxifying substances including drugs and alcohol.
- During fetal life, the Liver serves as a centre for heamatopoiesis.
- It functions in the storage of blood.
- It functions in the secretion of plasma proteins (albumin, fibrinogen and prothrombin) and coagulation factors (V, VII and IX) (Kierszenbaum, 2007).
- It functions in the synthesis of bile salt, cholesterol, triglycerides, breakdown of fat soluble vitamins and destruction of erythrocytes (Kierszenbaum, 2007).

2.5 THE TESTIS

The testes are paired organs usually covered with pubic hair contained in the scrotum. The testis is an oval organ possessing a thick covering of fibrous tissue, the tunica

albuginea. The front and sides of the testis lie free in a serous space formed by the overlying tunica vaginalis, a remnant of fetal processus vaginalis. Testis, Epididymis and Tunica Vaginalis lie in the scrotum surrounded by thin membranes, adherent to each other, which are downward prolongations of the coverings of the spermatic cord. The right and left testes are separated by the median scrotal septum. Average testicular dimensions are 5cm (Length), 2.5cm (breadth), and 3cm (anteroposterior diameter). The appendix testis is a minute sessile cyst attached to the upper pole of the testis within the tunica vaginalis. It is a remnant of the paramesonephric duct (Sinnatamby, 1999).

2.5.1 HISTOLOGY OF THE TESTIS

Each testis is surrounded by a thick capsule of dense connective tissue, the tunica albuginea. The tunica albuginea is thickened on the posterior surface of the testis to form the mediastinum testis, from which fibrous septa penetrate the gland, dividing it into about 250 pyramidal compartments called testicular lobules. These septa are incomplete so there is frequent intercommunication between the lobules. Each lobule is occupied by one to four seminiferous tubules enmeshed in a web of loose connective that is rich in blood and lymphatic vessels, nerves and interstitial cells known as Leydig cells. The seminiferous tubules are lined with a complex stratified epithelium called the germinal or seminiferous epithelium. They produce male reproductive cells, the spermatozoa whereas the interstitial cells secrete testicular androgens. The seminiferous epithelium consists of supporting cells sertoli cells and cells that constitute the spermatogenic lineage (Junqueira and Carneiro, 2005).

Within the seminiferous tubules,

- Germ cells develop into spermatogonia, spermatocytes, spermatids and spermatozoon through the process of spermatogenesis. The gametes contain DNA for fertilization of an ovum (Ross and Paulina, 2006).
- Sertoli cells - the true epithelium of the seminiferous epithelium, critical for the support of germ cell development into spermatozoa. Sertoli cells secrete inhibin (Skinner *et al*, 1989).
- Peritubular myoid cells surround the seminiferous tubules (Gilbert, 2000).

Between tubules (interstitial cells)

- Leydig cells - cells localized between seminiferous tubules that produce and secrete testosterone and other androgens important for sexual development and puberty, secondary sexual characteristics like facial hair, sexual behavior and libido, supporting spermatogenesis and erectile function. Testosterone also controls testicular volume.
- Also present are:
    - Immature Leydig cells
    - Interstitial macrophages and epithelial cells.

2.5.2 BLOOD SUPPLY, NERVE SUPPLYAND LYMPHATICS OF THE TESTIS

- The paired testicular arteries arise directly from the abdominal aorta and descend through the inguinal canal. The Testis also has collateral supply from the cremasteric artery and artery to the ductus deferens (Dalley and Moore, 1999).
- The testis is supplied by sympathetic nerves, most of the connector cells lie in the T10 segment of the spinal cord (Sinnatamby, 1999).

- Lymphatic drainage of the testis follows the testicular arteries back to the paraaortic nodes (Singh, 2002).

2.5.3 FUNCTIONS OF THE TESTIS

Like the Ovaries (to which they are homologous), Testes are components of both the reproductive system (being Gonads) and the endocrine system (being endocrine glands). The respective functions of the testes are:

- Producing Spermatozoa
- Producing male sex hormones of which testosterone is the best known.

Both functions of the testes are under the control of gonadotropic hormones produced by the anterior pituitary

- Luteinizing Hormone (LH)
- Follicle Stimulating Hormone (FSH) (Sinnatamby, 1999).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

- 20 Adult male wistar rats
- Formalin (BDH chemicals, England)
- Laboratory sodium chloride (BDH chemicals, England)
- Table salt (Royal salt, Nigeria)
- Hematoxylin and eosin (BDH chemicals, England)
- Cages, gloves and dissecting sets
- Intravenous lines and glasswares

3.2 EXPERIMENTAL ANIMALS

A total of 20 Adult Male Wistar Rats weighing between 180g-220g were used for this study. The Animals were purchased from the pharmacology department, ABU Zaria and were kept in the Animal house of Anatomy Department ABU Zaria. The Animals were maintained on normal rat feed and water throughout the 2 weeks acclimatization period. At the end of which the Animals were randomly selected into 4groups of 5 Animals each in separate cages.

3.3 EXPERIMENTAL PROTOCOL

The animals were anaesthetized with chloroform in a box for 5minutes each, then, the blood was flushed out of the body with normal saline using an intravenous line taking an approximate time of 8minutes. Each animal was then perfused with the fixative

corresponding to its group for an approximate time of 10minutes. Then, the abdomino-thoracic cavity and scrotum were incised to harvest the Livers and Testes respectively and were fixed immediately for 12 hours, 24 hours, 48 hours, and 168 hours (one week) in groups of five animals each using the following fixatives groups:

- Group A (control group): Fixative was standard formal saline composed of formalin 100ml, distilled water 900ml and 9g sodium chloride prepared using the laboratory sodium chloride.
- Group B: Fixative was formal saline composed of formalin 100ml, distilled water 900ml and 9g sodium chloride prepared using the table salt.
- Group C: Fixative was saturated solution of sodium chloride prepared using laboratory sodium chloride.
- Group D: Fixative was saturated solution of sodium chloride prepared using the table salt.

3.4 EXPERIMENTAL DESIGN

Groups	Group A Formal Saline (with LS)		Group B Formal Saline (with TS)		Group C Saturated Nacl solution (from LS)		Group D Saturated Nacl solution (from TS)	
Duration								
	L	T	L	T	L	T	L	T
12 hours								
24 hours								
48 hours								
168 hours								

LS-STANDARD LABORATORY SALT, TS- TABLE SALT, L- LIVER AND T-TESTIS

3.5 EXPERIMENTAL TISSUE PROCESSING AND STAININGS

At the point of harvest, the liver from each animal was cut into pieces of about 3x5mm and then immersed in the corresponding fixative (approximately 50ml) (Speilberg *et al.*, 1993). The Testes were left whole in the corresponding fixatives (approximately 100ml) for 12 hours before cutting into smaller pieces to harden them for clean and uniform cut. Following Fixation for each of the Fixation periods 12 hours, 24 hours, 48 hours and 168 hours, samples were dehydrated via a graded alcohol series from 70% to absolute hourly, cleared in Xylene, infiltrated with molten paraffin wax using a Shandon Southern Duplex Processor (Shandon Southern Products Ltd, England), and manually embedded in Paraffin Wax using L-shaped moulds.

The blocks of tissues (liver and testis) were then trimmed and sectioned at 5microns using a rotary microtome. The ribbons of sections were floated out on a water bath and

then picked up on slides by immersing the slide lightly smeared with adhesive (albumen) vertically in water bath to three quarters of its length and manoeuvring the section into contact with the slide. On lifting the slide vertically from the water, the section was flattened onto the slide and labeling was transfered accordingly. The sections (Liver and Testis) were correctly positioned on the slides, drained in the vertical position and dried overnight in the oven.

Then, sections were dewaxed in xylene and rehydrated using grades of alcohol from absolute to 70%. They were then stained regressively in Harris hematoxylin for 10 minutes, differentiated in 1% acid alcohol for 10 seconds and blued in running water for 5 minutes. They were then counterstained in eosin Y for 10 minutes and washed in running tap water for 5 minutes. Sections were then dehydrated, cleared and mounted in DPX (dibutyl phthalate xylene) and coverslipped with glass.

3.6 HISTOLOGICAL EXAMINATIONS

This was performed using modified methods of Speilberg *et al*., (1993) and Gupta and Krishna, (2011). The H&E slides were coded, ensuring unknown identity until the examination was completed. Each slide was then evaluated individually by three sets of independent examiners. The principal criteria for their evaluation using a 20 point ranking scale (Excellent- 16.00 and above, Very good- 13.00-15.99, Good- 10.00-12.99, Fair- 8.00-9.99, poor 6.00-7.99 and Bad- 5.99 and below) were as follows:

- Tissue recognition and General tissue Appearance: Tissues must be correctly recognized as the labelled tissue and an overall assessment of the Tissue at a glance with respect to the examiners knowledge of the Tissue in question is graded. (2 points).

- Morphological appearance of the Nucleus and Cytoplasm: The cytoplasm should be clear and well delineated and the nucleus should be smooth, round or oval, centrally located and well outlined with no shrinkage or swelling. (5 points).
- Good Staining: The cytoplasm and connective Tissue should be distinctly eosinophilic, homogenous and well outlined, while the Nuclei should be smooth with an even distribution of chromatin and Basophilic. (3 points).
- Morphological appearance of the connective tissue including vasculature and sinusoids: These should all be readily recognizable with distinct endothelia and no swelling. (5 points).
- Absence of the features which indicated cell death (pyknosis, blebs, sloughing etc). (3 points).
- General Judgement: An overall judgement of the tissue architecture with respect to the sharpness of the cellular outline and arrangement of various cellular elements in relation to each other. (2 points).

3.7 HISTOMORPHOMETRY

This was performed using a software (Digimizer image analysis v4.1, Med Calc Software, Belgium). The software was installed into a system via its installation Compact Disc and test ran. Then the photomicrographs of the tissue sections (Testis) taken with a digital camera, (Amscope MA 500) were loaded onto the software. To measure a variable, the outline, shape or points to be measured depending on the variable to be measured was traced with the pointer and the software automatically highlighted similar shape, outline or points similar to the traced one on the micrograph and gave the mean value. The variables measured were:

- Area of Seminiferous Tubules.
- Diameter of Seminiferous Tubules.
- Height of Germinal Epithelium.

3.8 STATISTICAL ANALYSIS

Data obtained were analysed by ANOVA followed by Tuckey *post Hoc* Test. Values were considered statistically significant at $P< 0.05$. SPSS v17.0 for Windows was used for the analysis

CHAPTER FOUR

4.0 RESULTS

4.1 HISTOLOGICAL EVALUATIONS

4.1.1 AT 12 HOURS OF FIXATION

All sections were free of pathological changes for the Liver Tissue (plates 1, 2, 3 and 4), Hepatocytes, nuclei and connective tissue were well preserved. 10% formal saline with table salt (group B) had the highest mean fixative rank which was significantly higher than the control; standard 10% Formal saline (group A) ($P<0.05$). The mean fixative rank obtained from Saturated sodium chloride solution from laboratory salt (group C) was also significantly higher ($P<0.05$) than the control while that of group D (saturated sodium chloride solution from table salt) although higher, was not significantly so (Table 4.1). Taking the standard fixation period to be 24hrs only saturated sodium chloride solution from laboratory salt was significantly higher at 12hrs for both the Liver and Testes Tissues (Table 4.1and 4.2). In contrast to the Liver sections, standard 10% formal saline (control) gave an excellent preservation of the Testes, the integrity of the seminiferous Tubules and interstitium were well preserved, the cells of the germinal epithelium were well arranged in concentric layers (plate 5) and had the highest mean fixative rank which was significantly higher than the other groups B (plate 6) ($P<0.05$), C and D ($P<0.05$). More so in saturated sodium chloride solution groups C and D (which showed poorly preserved testes tissues, germinal epithelium could be seen detaching from the basement membrane (Plates 7 and 8) than in 10% Formal Saline with Table Salt, group B (Table 4.2).

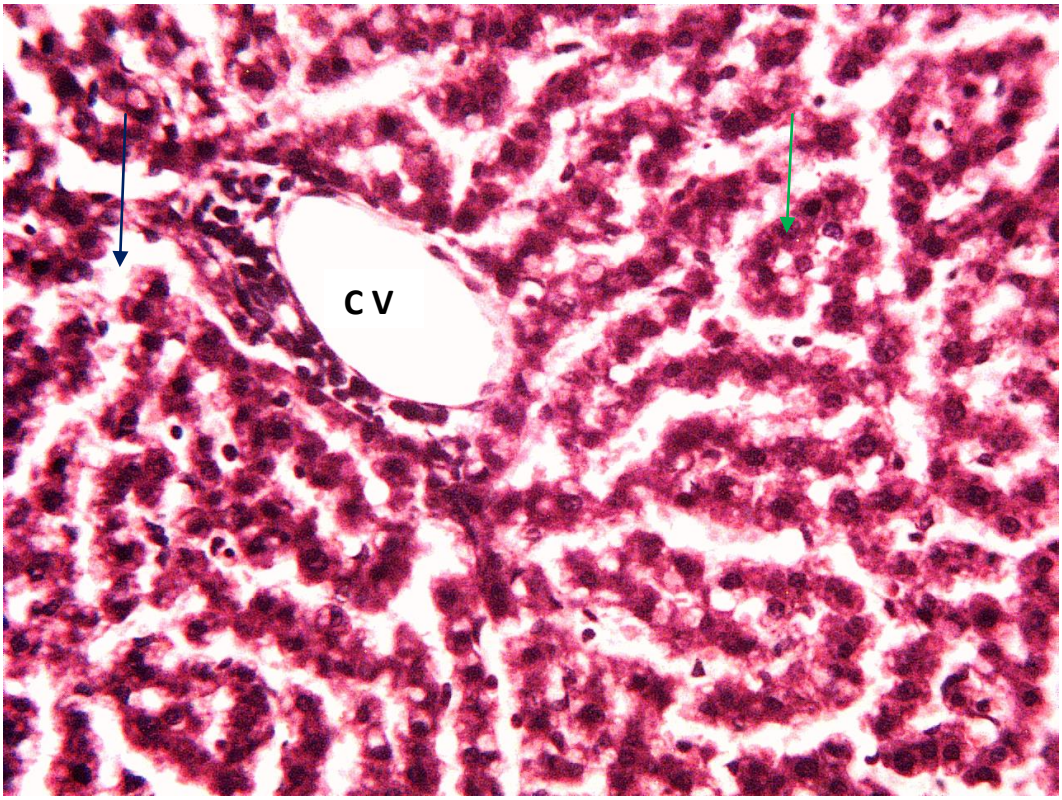


PLATE 1: Light microscopic section of the liver of wistar rats fixed in standard formal saline for 12 hours. Central Vein (CV), sinusoids (blue arrow) and Hepatocytes (green arrow) (H&E X250).

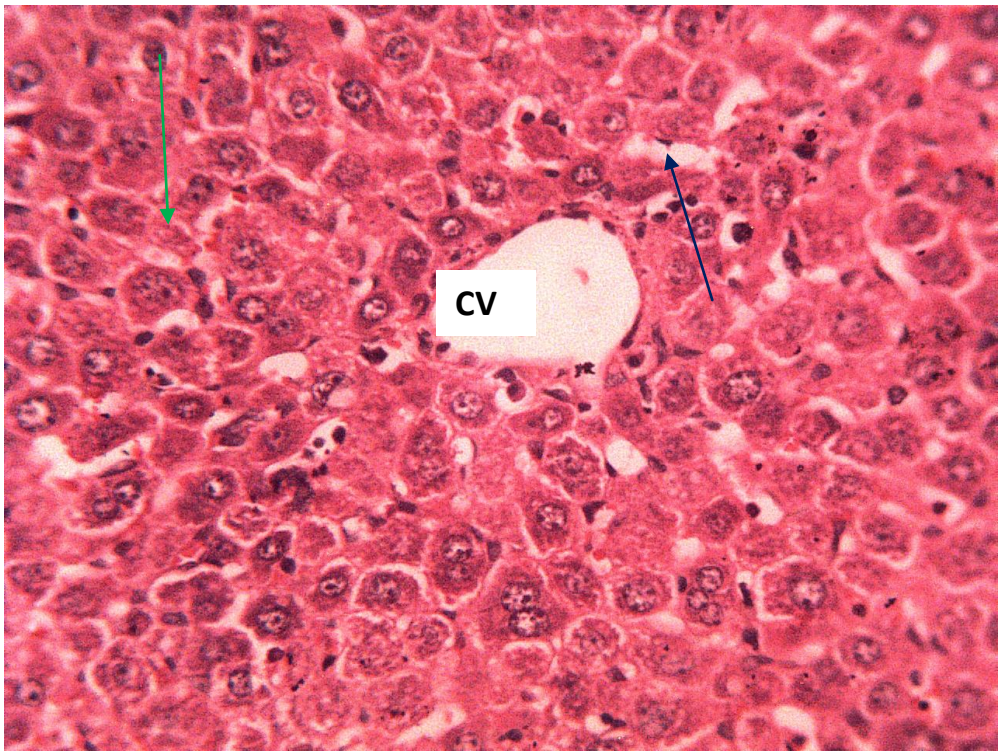


PLATE 2 Light microscopic section of the liver of wistar rat fixed in formal saline (prepared with table salt) for 12 hours. Central Vein (CV), sinusoids (blue arrow) and Hepatocytes (green arrow) (H&E x 400).

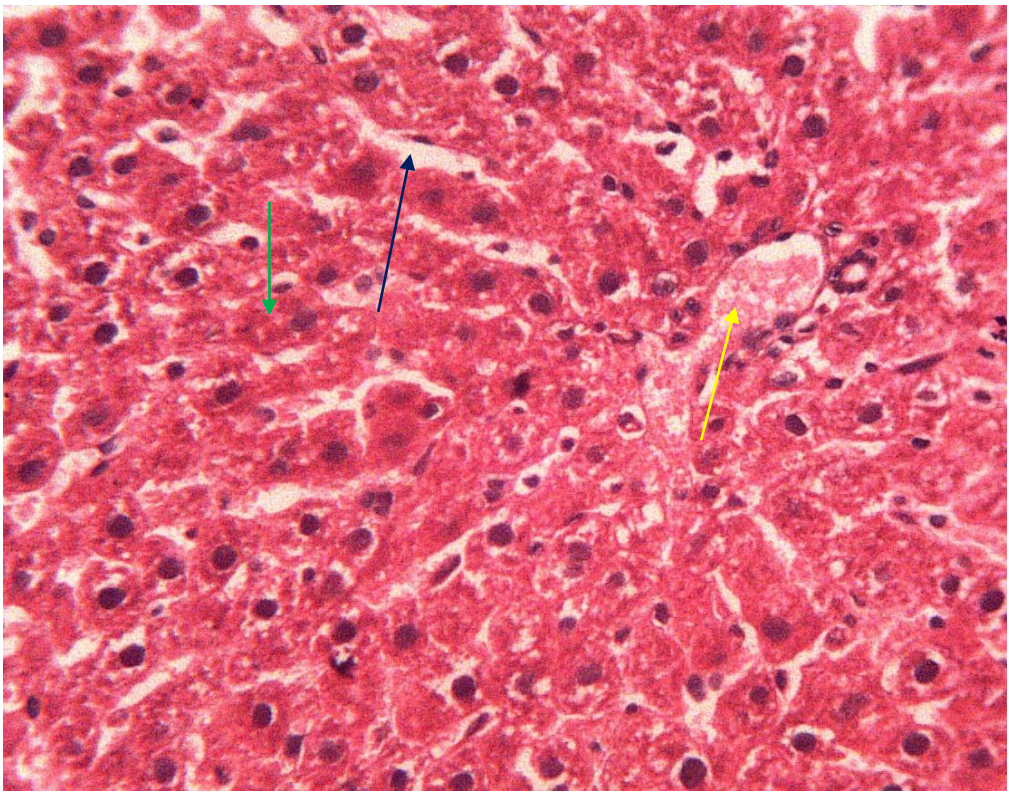


PLATE 3 Light microscopic section of the liver of wistar rats fixed in saturated sodium chloride solution (prepared with laboratory salt) for 12 hours. Central vein (yellow arrow), Sinusoids (blue arrow) and Hepatocytes (green arrow) (H&E x 250).

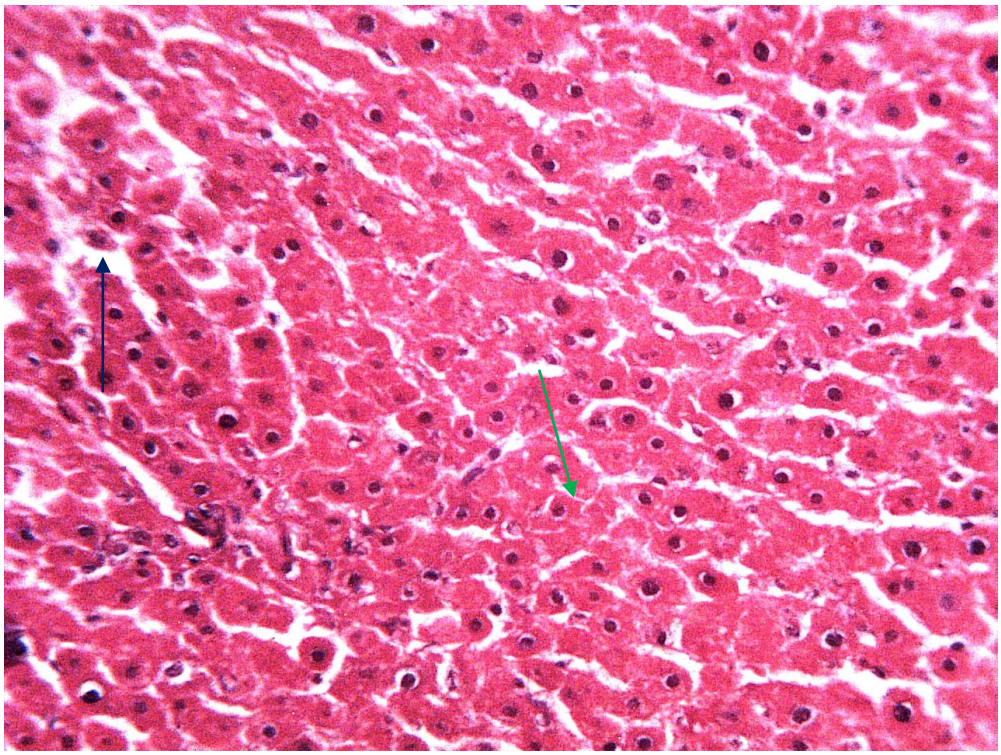


PLATE 4: Light microscopic section of the liver of wistar rats fixed in saturated sodium chloride solution (prepared with Table salt) for 12 hours. Sinusoids (blue arrow) and Hepatocytes (green arrow) (H&E x 250)

Table 4.1: MEAN PERFORMANCE OF THE FIXATIVES ACROSS THE FIXATIVE PERIODS FOR THE LIVER TISSUES

FIXATIVE GROUP / FIXATIONPERIOD	FORMAL SALINE LS (GRP A) MEAN±SEM	FORMAL SALINE TS (GRP B) MEAN±SEM	SAT. NaCl SLTN FRM SLS (GRP C) MEAN±SEM	SAT. NaCl SLTN FRM TS (GRP D) MEAN±SEM
12HOURS	12.67±0.33	17.67±0.33**	17.33±0.33** b	14.00±0.58
24HOURS	13.33±0.88	16.67±0.33*	13.67±0.33	13.67±0.88
48HOURS	13.67±0.33	16.33±0.88	13.67±0.33	13.33±1.53
168HOURS	13.00±1.15	10.33±1.15a	10.33±0.33a	13.33±0.58
TOTAL	13.17±0.37	15.25±1.26	13.75±1.07	13.58±0.25

SLS-Standard laboratory salt, TS-Table Salt, SAT-Saturated, SLTN- Solution, and GRP-Group

**** p value < 0.001, * p value < 0.05 for fixative groups and [a] P-value < 0.05, [b] P-value < 0.001 for the fixative periods**

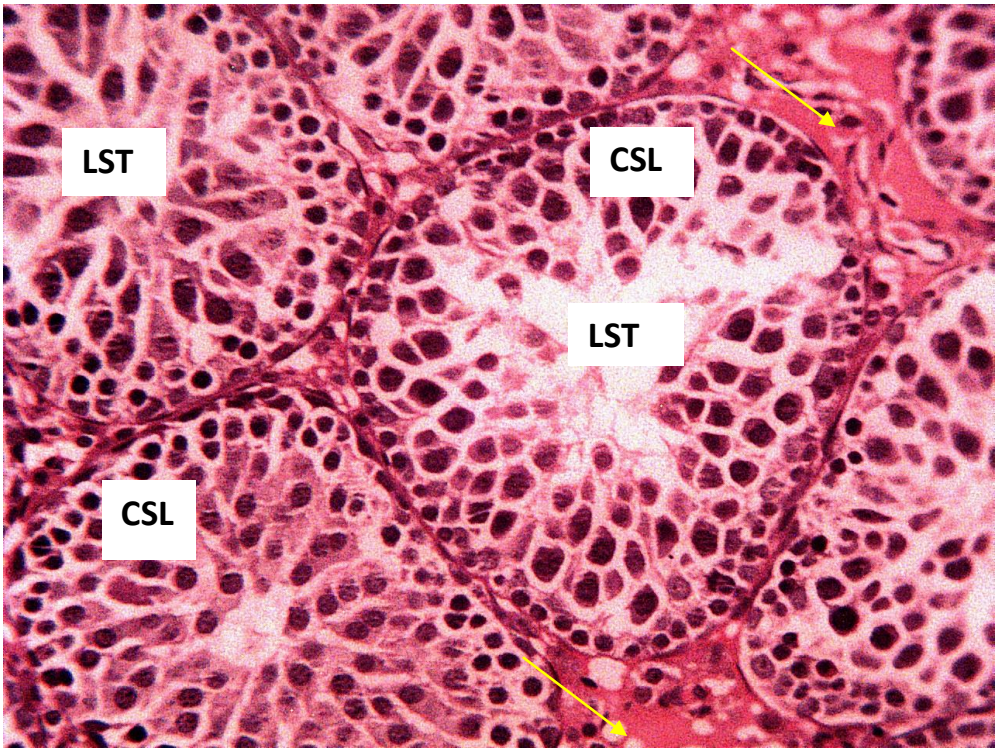


PLATE 5: Light microscopic section of the testis of wistar rats fixed in standard formal saline for 12 hours. Lumen of Seminiferous Tubules (LST), Cells of Spermatogenic Lineage (CSL), and Interstitium containing Leydig cells (arrows), (H&E X250).

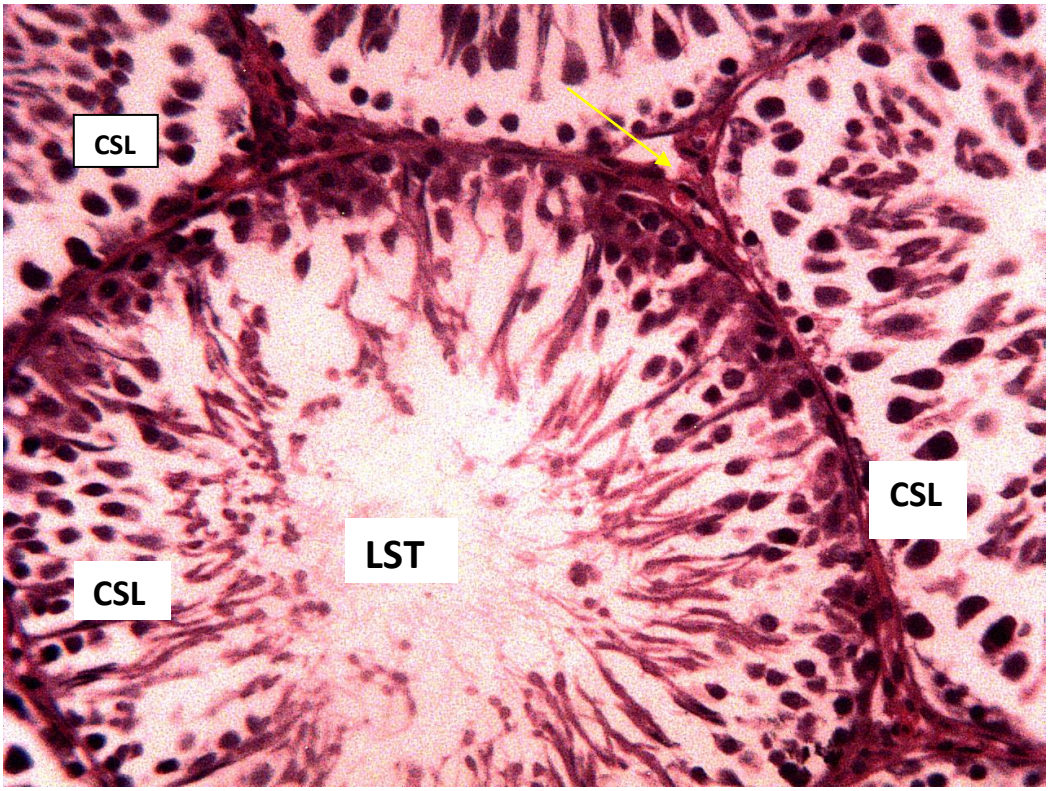


PLATE 6 Light microscopic section of the testis of wistar rats fixed in formal saline (prepared with table salt) for 12 hours. Lumen of Seminiferous Tubules (LST), Cells of Spermatogenic Lineage (CSL) and Intestitium containing Leydig cells (arrow) (H&E X400).

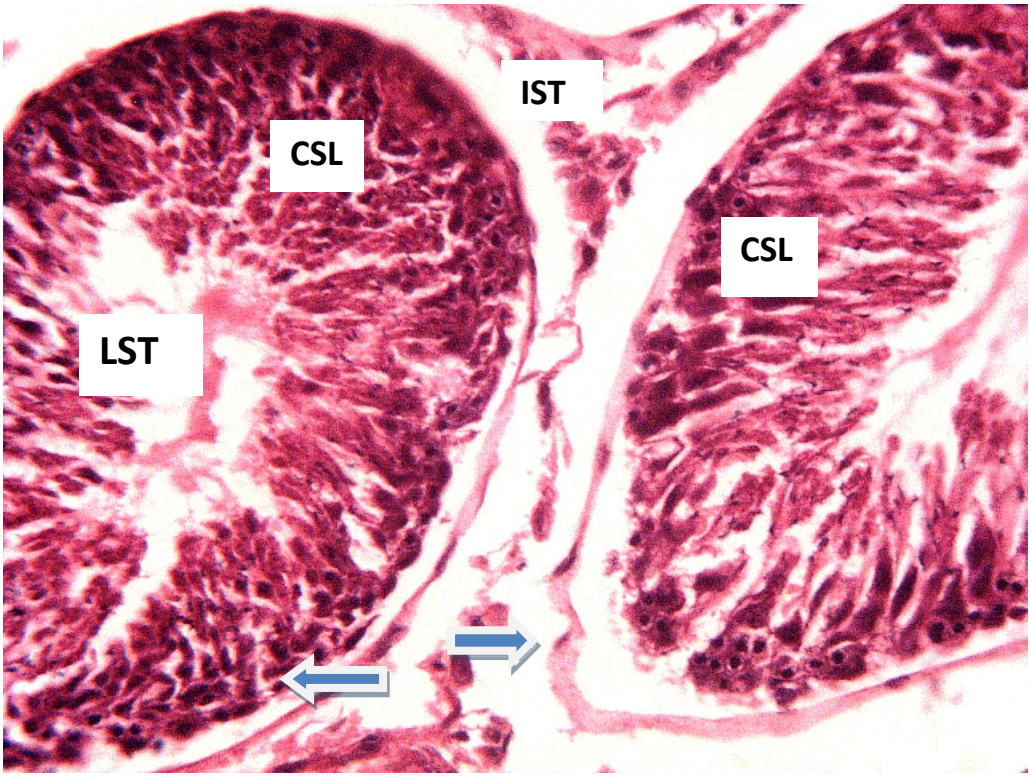


PLATE 7 Light microscopic section of the testis of wistar rats fixed in saturated sodium chloride solution (prepared with laboratory salt) for 12 hours. Lumen of Seminiferous Tubules (LST), Cells of Spermatogenic Lineage (CSL) and Interstituim (IST). Germinal epithelium detaching from the Basement Membrane (Arrows). (H&E X250)

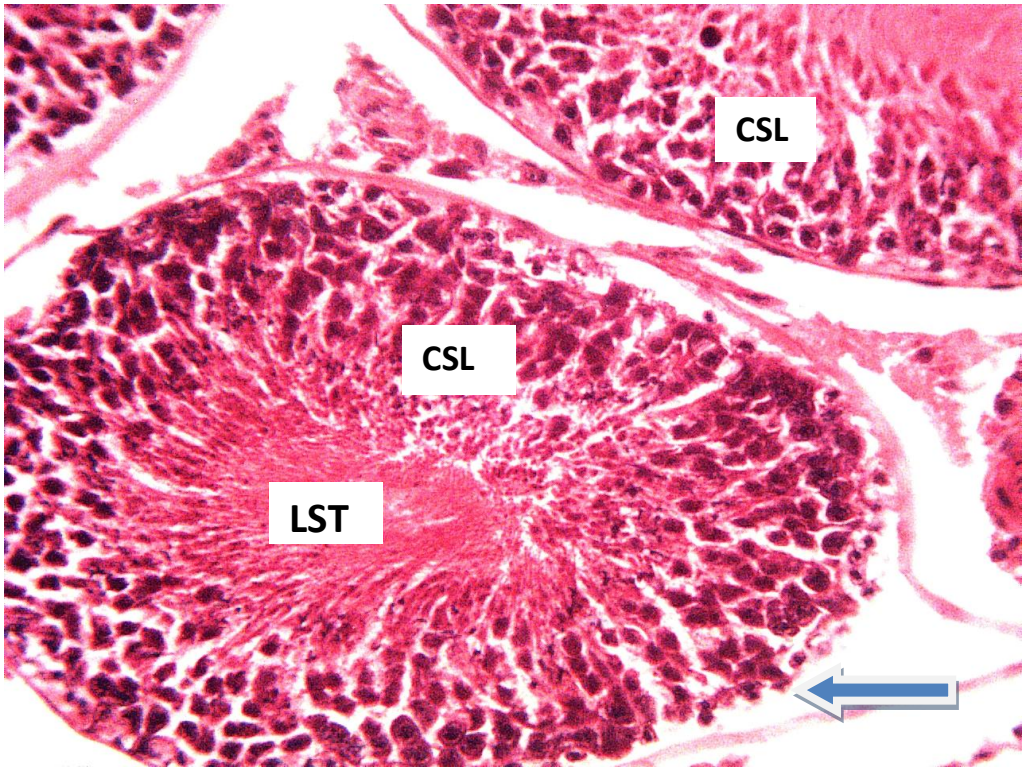


PLATE 8 Light microscopic section of the testis of wistar rats fixed in saturated sodium chloride solution (prepared with Table salt) for 12 hours. Lumen of Seminiferous Tubules (LST), Germinal epithelium detaching from basement membrane (Arrow), Cells of Spermatogenic Lineage (CSL), (H&E X250).

Table 4.2: MEAN PERFORMANCE OF THE FIXATIVES ACROSS THE FIXATIVE PERIODS FOR THE TESTES TISSUES

FIXATIVE GROUP / FIXATIONPERIOD	FORMAL SALINE LS (GRP A) MEAN±SEM	FORMAL SALINE TS (GRP B) MEAN±SEM	SAT. NaCl SLTN FRM LS (GRP C) MEAN±SEM	SAT. NaCl SLTN FRM TS (GRP D) MEAN±SEM
12HOURS	17.33±0.67	14.67±0.33*	6.33±0.33**a	8.33±0.67**
24HOURS	17.67±0.88	12.67±0.88*	4.33±0.88**	7.33±0.88**
48HOURS	11.00±1.15b	10.67±0.58a	8.67±0.00b	8.67±0.88
168HOURS	12.00±0.67b	10.00±1.15	4.00±0.58*	4.67±0.88*
TOTAL	14.50±2.57	12.00±1.29	5.83±0.93*	6.57±0.77*

SLS-Standard laboratory salt, TS-Table Salt, SAT-Saturated, SLTN- Solution, and GRP-Group

**** p value < 0.001, * p value < 0.05 for fixative groups and [a] P-value < 0.05, [b] P-value < 0.001 for the fixative periods**

4.1.2 AT 24 HOURS OF FIXATION

All the fixative groups A-D gave good preservation of the Liver Tissue (plates 9-12). Although, 10% Formal Saline with table salt, (group B) had the highest mean fixative rank and was significantly higher than the control (group A ($P<0.05$)), both saturated sodium chloride solution groups C and D were not significantly different from the control (Table 4.1). The testis was well preserved by the formal saline groups A and B (plates 13 and 14) but the control group A was significantly higher in the mean fixative rank than formal saline with table salt group B ($P<0.05$). more so in saturated sodium chloride solution groups C and D (both from laboratory and Table Salt) ($P<0.05$) which showed poor testicular tissue preservation with shrinkage of the seminiferous tubules and germinal epithelium detachment especially in group C (plates 15 and 16). (Table 4.2)

4.1.3 AT 48 HOURS OF FIXATION

No significant difference was found in the mean fixative rank obtained for the Liver sections across the fixative groups B-D from the control group A, although 10% formal saline with Table Salt had the highest mean fixative rank (Table 4.1). All sections showed a good preservation of the Liver architecture including connective Tissue and Sinusoids (Plates 17-20). There was no significance difference across the fixative groups for this fixative period for the Liver Tissues taking 24hrs to be the standard fixative period. Similarly, no significance was found in the Testes sections across all the Fixative groups from control group A (plate 21) although, it had the highest mean fixative rank (Table 4.2). Saturated sodium chloride solution groups C and D had a fair performance with less shrinkage and good connective Tissue (plates 23 and 24)

although there was delumenization of the seminiferous Tubules and detachment of the germinal epithelium from the basement membrane.

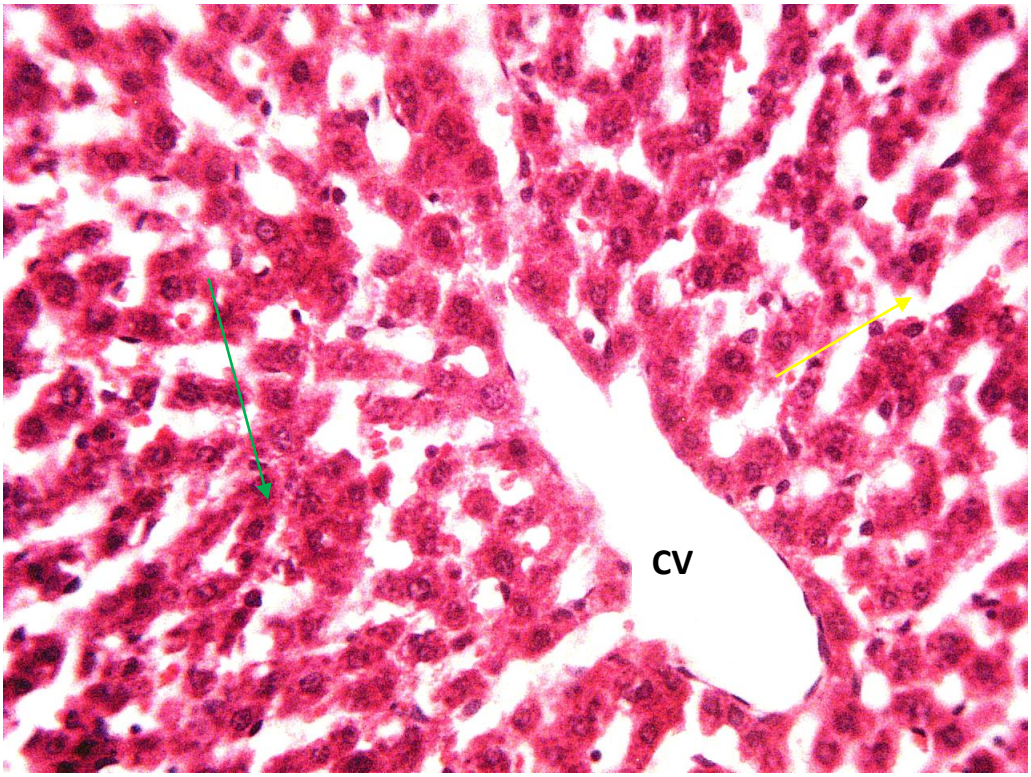


PLATE 9 Light microscopic section of the liver of wistar rats fixed in standard formal saline for 24 hours. Central vein (CV), sinusoids (yellow arrow) and hepatocytes (green arrow) (H&E x 250).

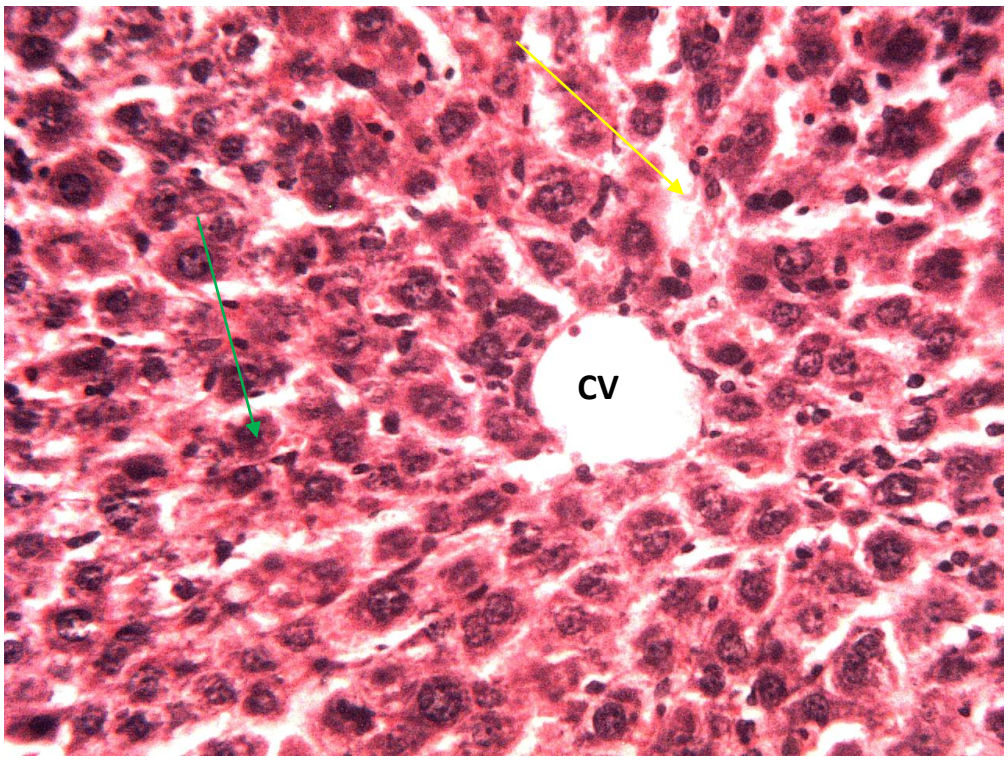


PLATE 10 Light microscopic section of the liver of wistar rats fixed in formal saline (prepared with table salt) for 24 hours. Central vein (CV), sinusoids (yellow arrow) and hepatocytes (green arrow) (H&E x 250).

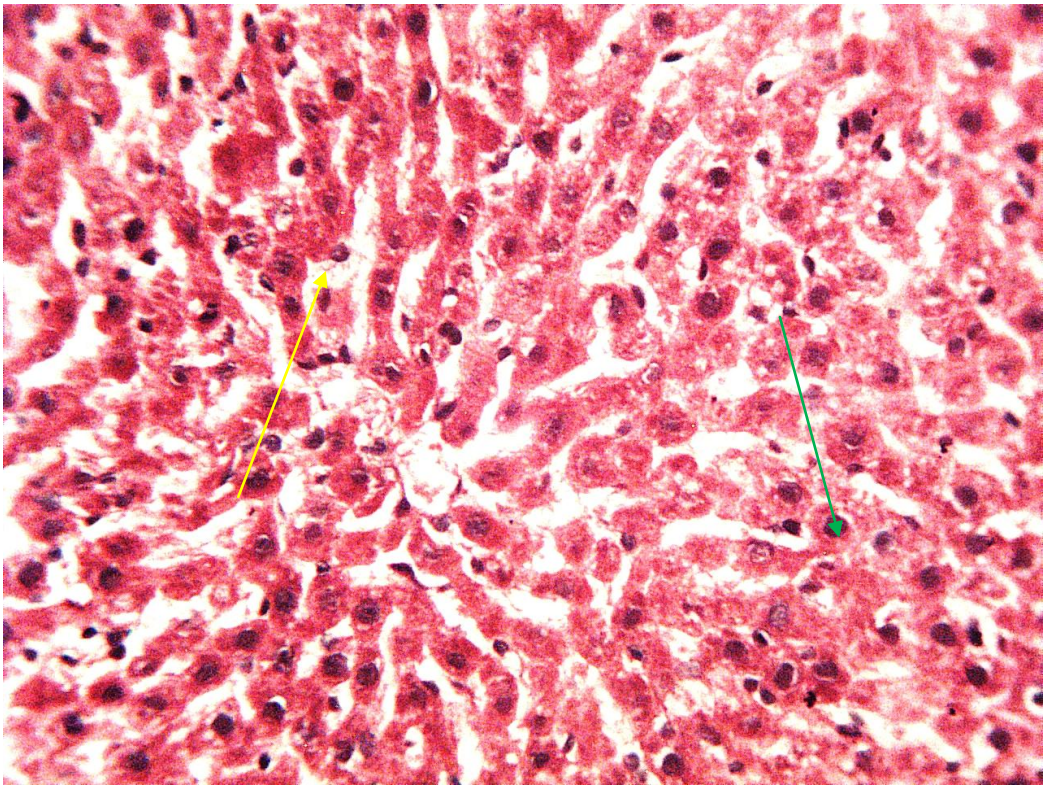


PLATE 11 Light microscopic section of the liver of wistar rats fixed in saturated sodium chloride solution (prepared with laboratory salt) for 24 hours. Sinusoids (yellow arrow) and hepatocytes (green arrow)) (H&E x 250).

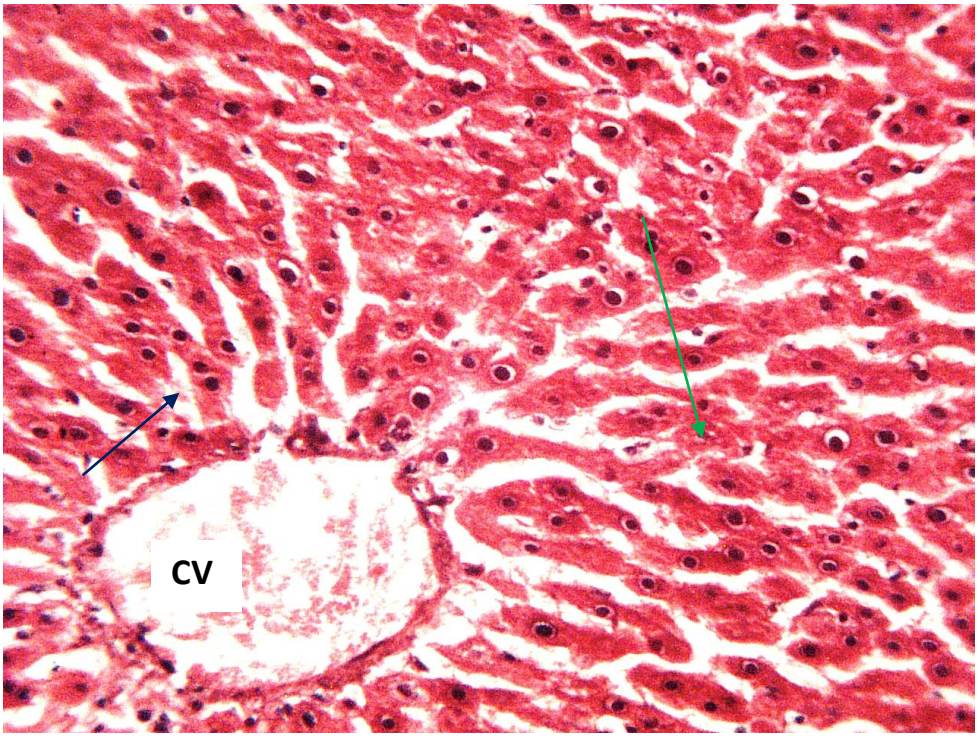


PLATE 12 Light microscopic section of the liver of wistar rats fixed in saturated sodium chloride solution (prepared with Table salt) for 24 hours. Central vein (CV), sinusoids (blue arrow) and hepatocytes (green arrow) (H&E x 250).

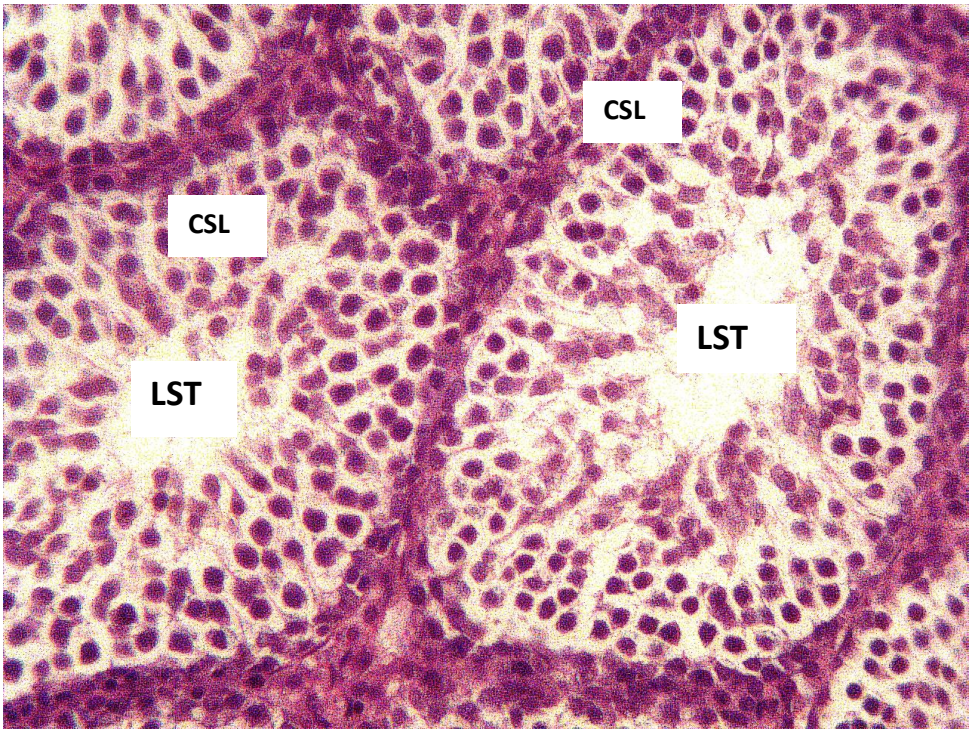


PLATE 13 Light microscopic section of the testis of wistar rats fixed in standard formal saline for 24 hours. Lumen of Seminiferous Tubules (LST) and Cells of Spermatogenic Lineage (CSL), (H&E X250).

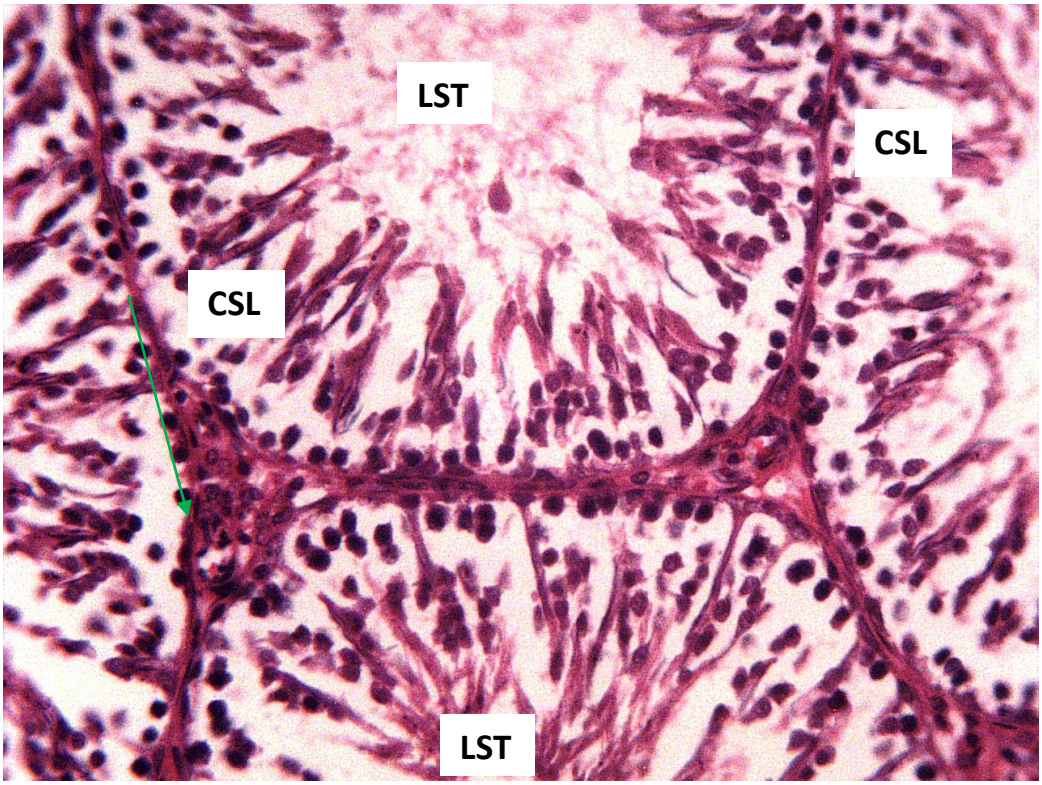


PLATE 14 Light microscopic section of the testis of wistar rats fixed in formal saline (prepared with table salt) for 24 hours. Lumen of Seminiferous Tubules (LST), Cells of Spermatogenic Lineage (CSL), and Interstitium with Leydig cells (green arrow), (H&E X400).

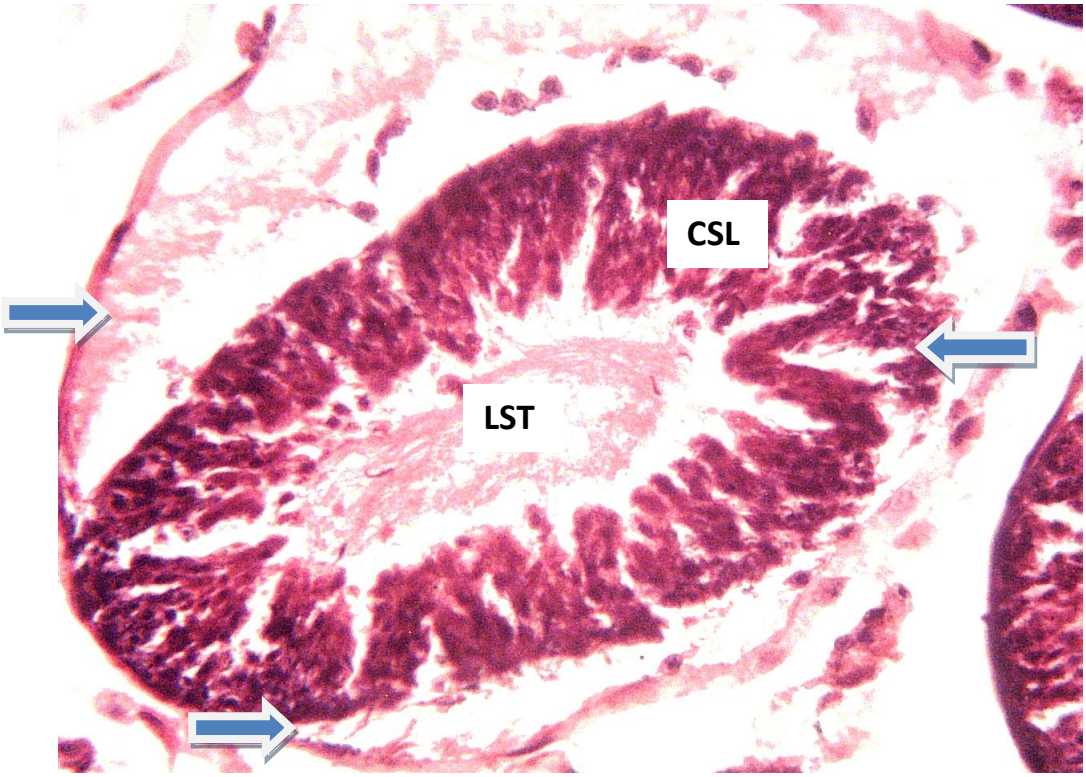


PLATE 15 **Light microscopic section of the testis of wistar rats fixed in saturated sodium chloride solution (prepared with laboratory salt) for 24 hours. Cells of Spermatogenic Lineage (CSL) Lumen of Seminiferous Tubules (LST) shrinkage and detachment of the germinal epithelium from basement membrane (Arrows) (H&E X250).**

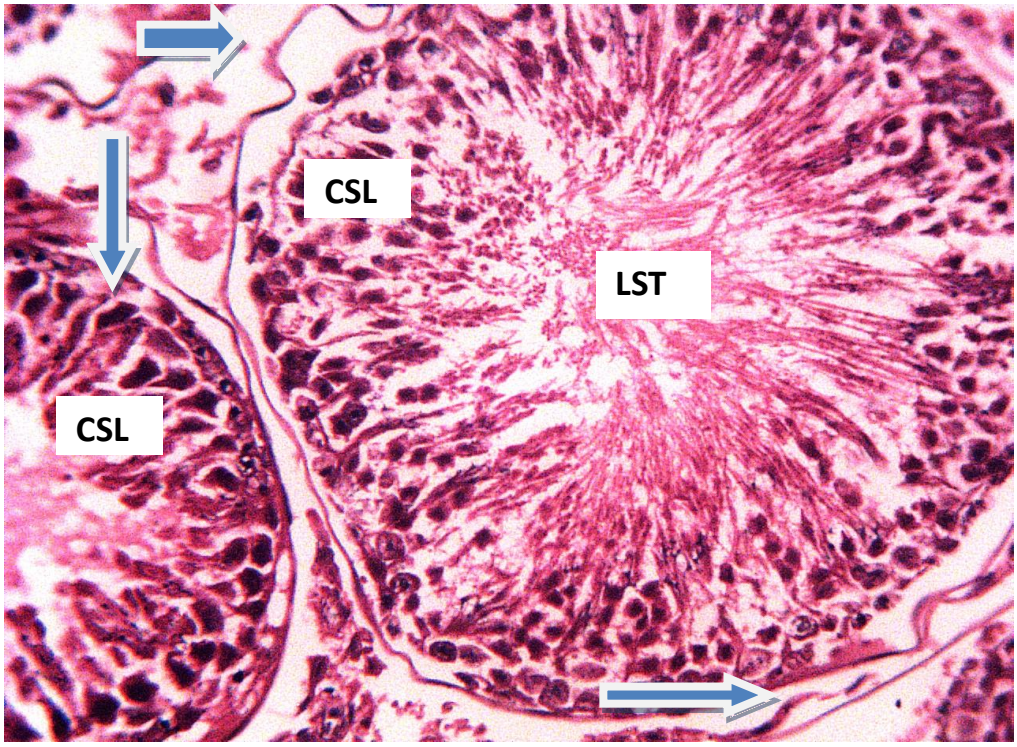


PLATE 16 **Light microscopic section of the testis of wistar rats fixed in saturated sodium chloride solution (prepared with Table salt) for 24 hours. Lumen of Seminiferous Tubules (LST), Cells of Spermatogenic Lineage (CSL) and detachment of germinal epithelium from the basement membrane (Arrows) (H&E X250).**

4.1.4 AT 168 HOURS OF FIXATION

For the Liver Sections, 10% Formal Saline (group A) the control has the highest mean fixative rank with good preservation of the Liver architecture although no significance was found from the other groups B (Formal saline with Table salt), C (saturated sodium chloride solution from the laboratory salt) and D (saturated sodium chloride solution from Table salt) (Table 4.1) (Plates 25-28). Taking 24hrs to be the standard fixative period, formal saline with table salt and saturated sodium chloride from laboratory salt were significantly lower than the standard (Table 4.1) For the Testes Sections, the control group A (plate 29) had the highest mean fixative rank with good testicular tissue preservation which was significantly different (Table 4.2) from the saturated sodium chloride solution groups C and D ($P<0.05$) that showed badly preserved testicular tissue with collapse of the seminiferous tubules (plates 31 and 32). The control group was not significantly different from group B (10% formal saline with table salt) which only showed fewer cells in the germinal epithelium (Plate 30). Only the control group was significantly lower than the standard fixation period of 24hrs at this fixation period (Table 4.2).

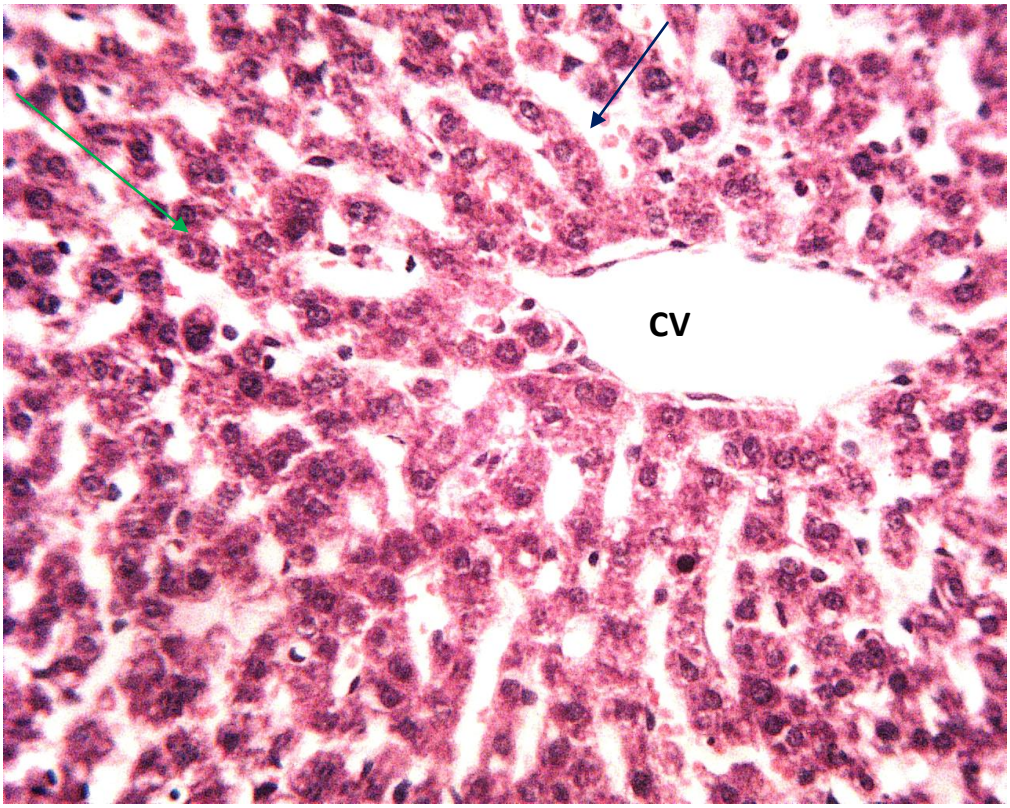


PLATE 17 Light microscopic section of the liver of wistar rats fixed in standard formal saline for 48hours Central vein (CV), sinusoids (blue arrow) and hepatocytes (green arrow) (H&E x 250)

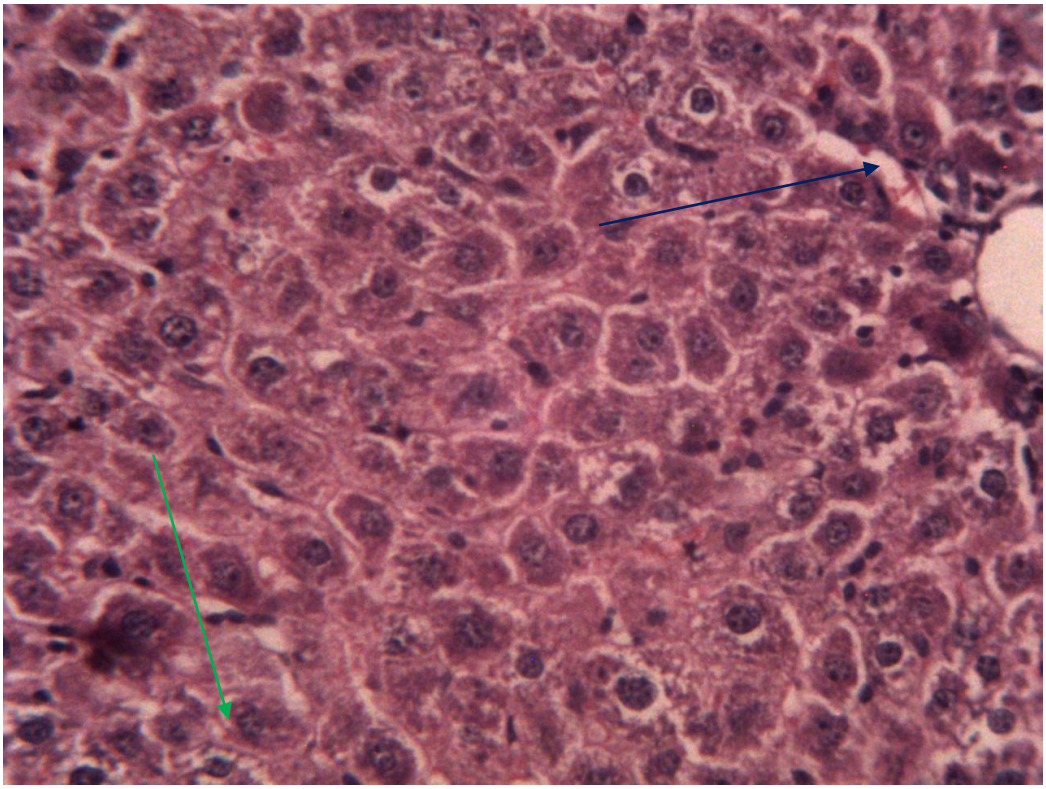


PLATE 18 Light microscopic section of the liver of wistar rats fixed in formal saline (prepared with table salt) for 48 hours. Sinusoids (blue arrow) and hepatocytes (green arrow) (H&E x 250).

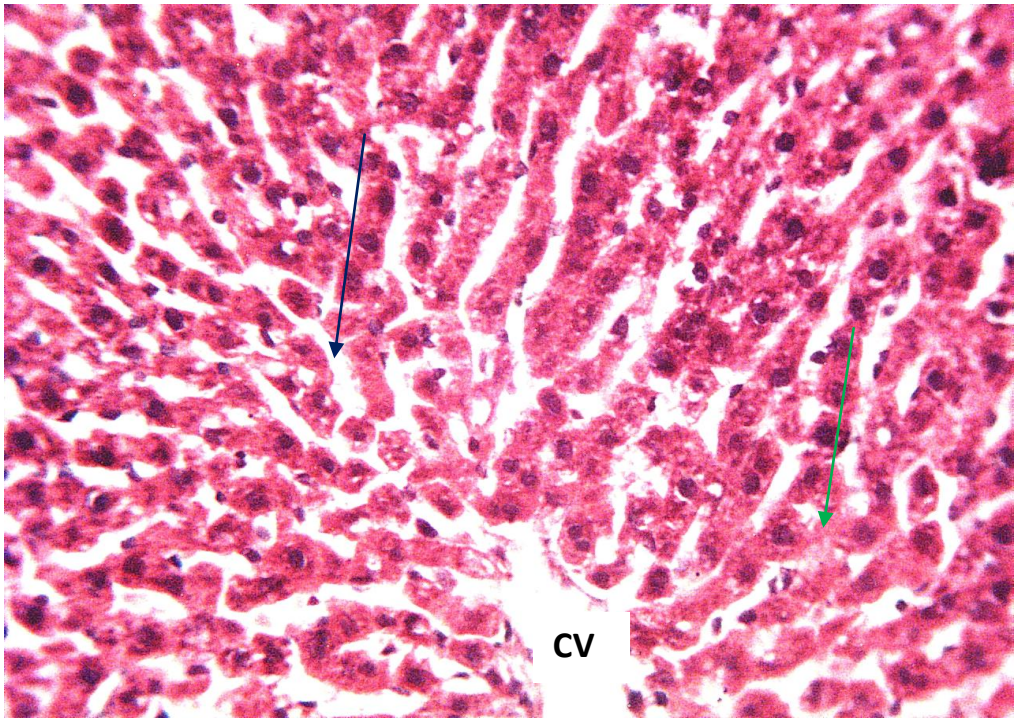


PLATE 19 **Light microscopic section of the liver of wistar rats fixed in saturated sodium chloride solution (prepared with laboratory salt) for 48 hours. Central vein (CV), sinusoids (blue arrow) and hepatocytes (green arrow) (H&E x 250).**

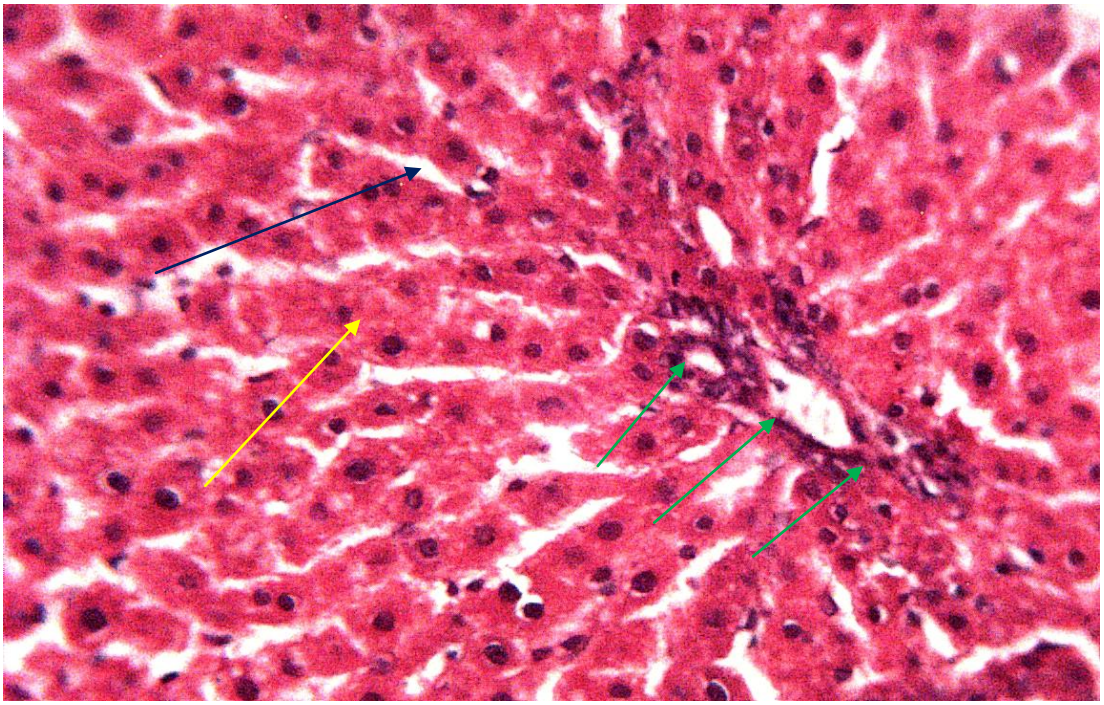


PLATE 20 **Light microscopic section of the liver of wistar rats fixed in saturated sodium chloride solution (prepared with Table salt) for 48 hours. An interlobular bile duct, branch of the Portal vein (PV) and branch of hepatic artery (green arrows from left to right portal triad), sinusoids (blue arrows), and hepatocytes (yellow arrow) (H&E x 250).**

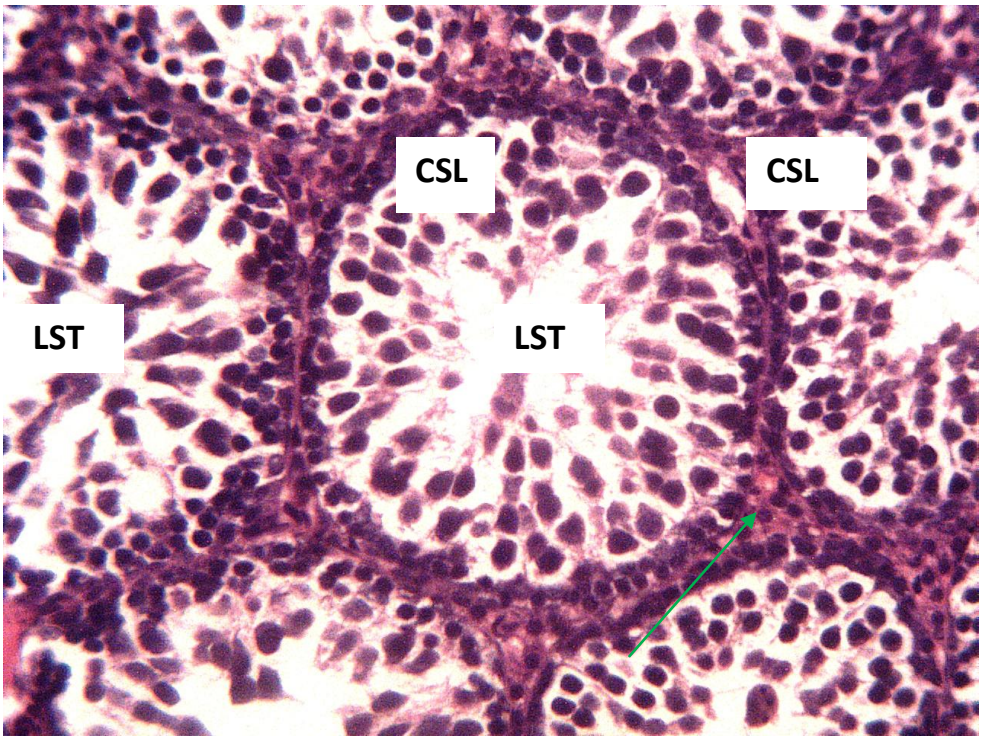


PLATE 21 Light microscopic section of the testis of wistar rats fixed in standard formal saline for 48 hours. Lumen of Seminiferous Tubules (LST), Cells of Spermatogenic Lineage (CSL) Interstitium (arrow) (H&E X250).

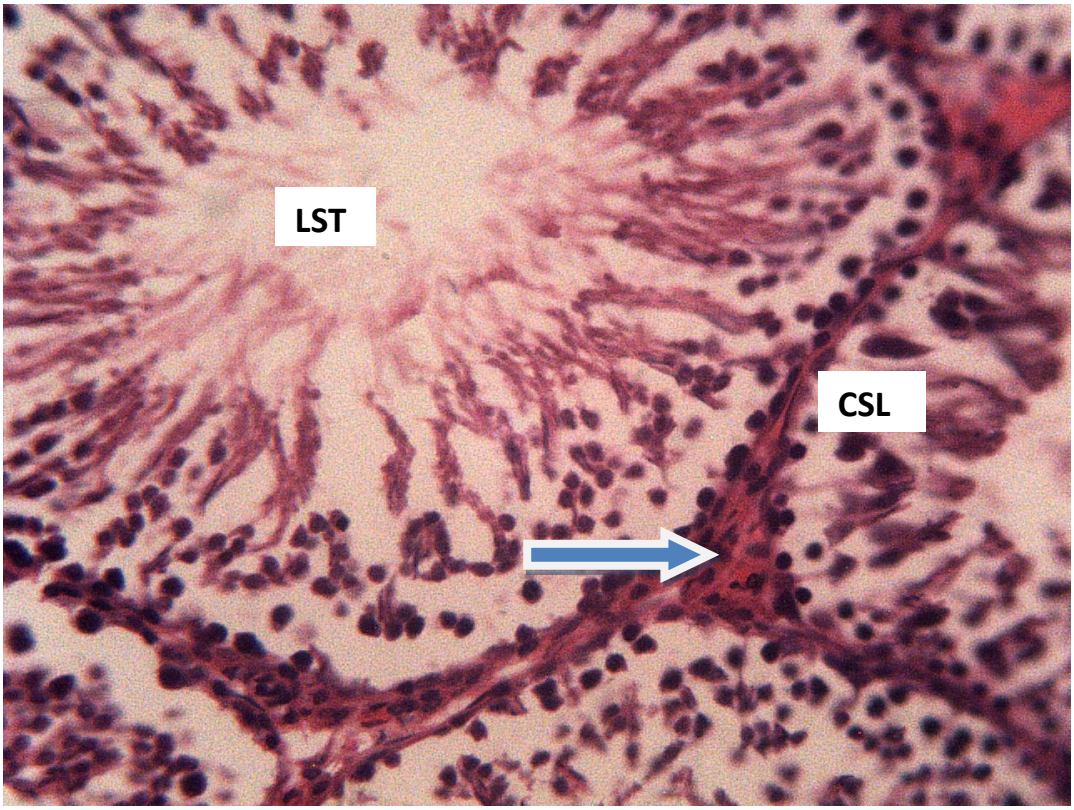


PLATE 22 Light microscopic section of the testis of wistar rats fixed in formal saline (prepared with table salt) for 48hour. Cells of Spermatogenic Lineage (CSL), Lumen of the Seminiferous Tubules (LST) and interstitium containing Leydig cells (Arrow), (H&E X400).

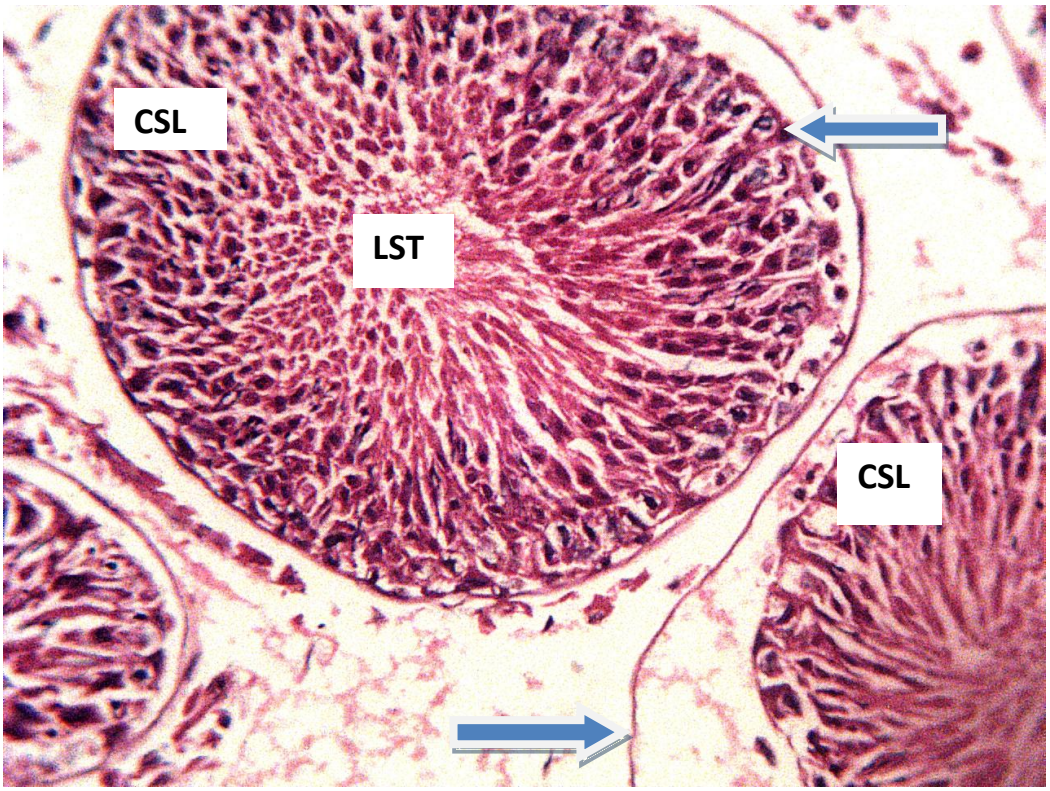


PLATE 23 **Light microscopic section of the testis of wistar rats fixed in saturated sodium chloride solution (prepared with laboratory salt) for 48hours. Lumen of Seminiferous Tubules (LST), Cells of Spermatogenic Lineage (CSL), depleted interstitium (IST) and shrinkage of the seminiferous tubules (Arrows) (H&E X250).**

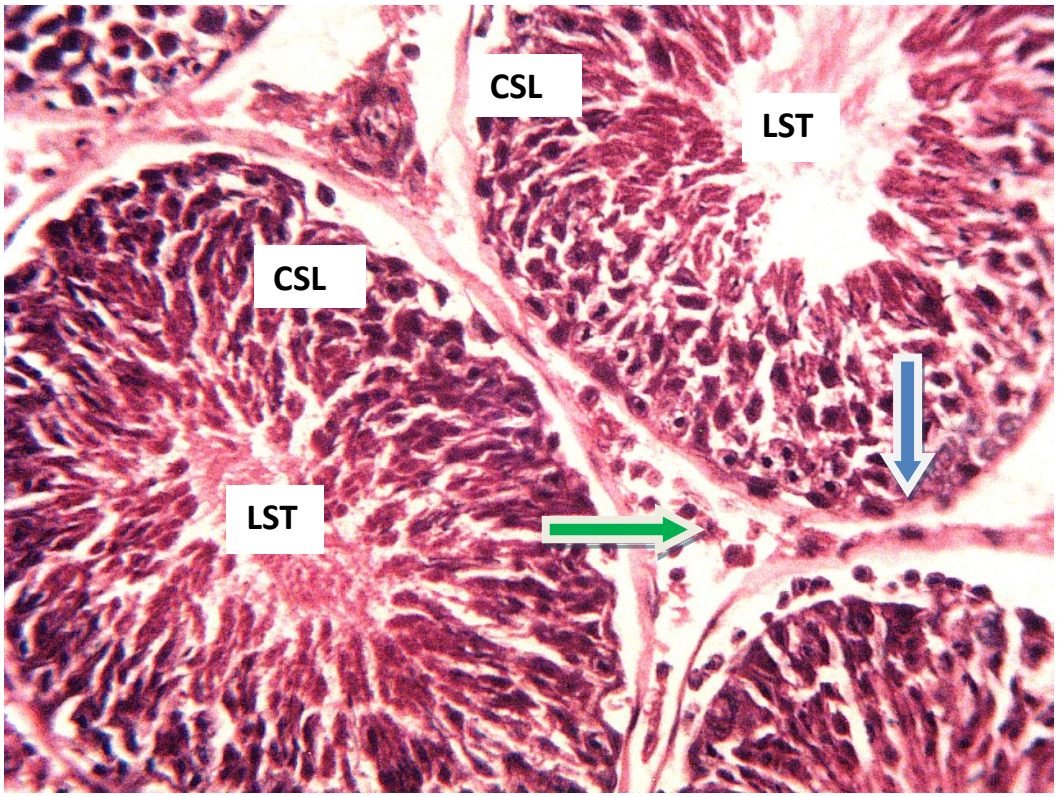


PLATE 24 **Light microscopic section of the testis of wistar rats fixed in saturated sodium chloride solution (prepared with Table salt) for 48 hours. Lumen of Seminiferous Tubules (LST), Cells of Spermatogenic Lineage (CSL), Interstitium (green arrow) and shrinkage of the seminiferous tubules (blue arrow) (H&E X250).**

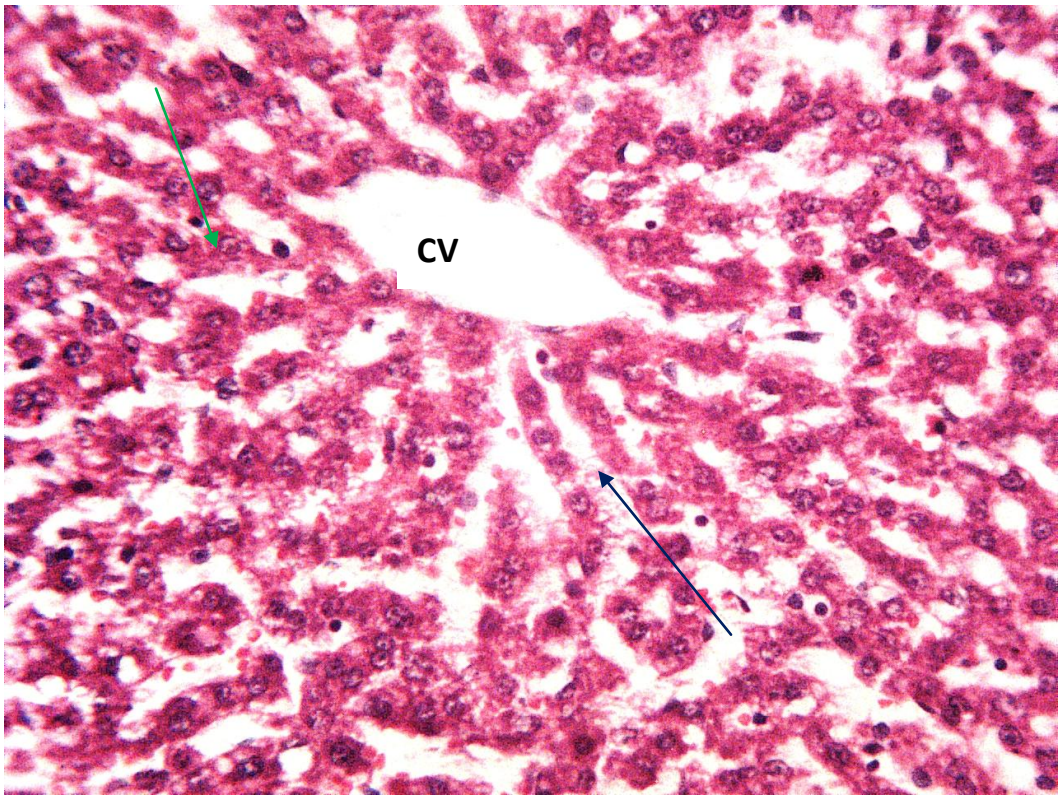


PLATE 25 Light microscopic section of the liver of wistar rats fixed in standard formal saline for 168hours. Central vein (CV), sinusoids (blue arrow) and hepatocytes (green arrow) (H&E x 250).

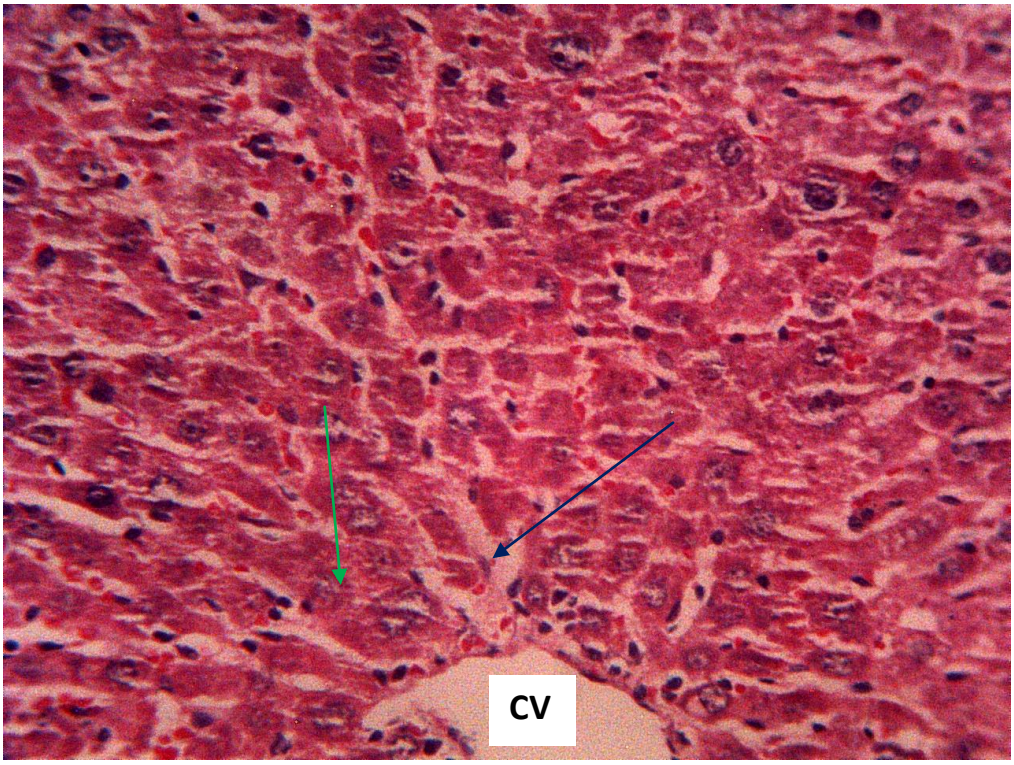


PLATE 26 Light microscopic section of the liver of wistar rats fixed in formal saline (prepared with table salt) for 168 hours. Central vein (CV), sinusoids (blue arrow) and hepatocytes (green arrow) (H&E x 250).

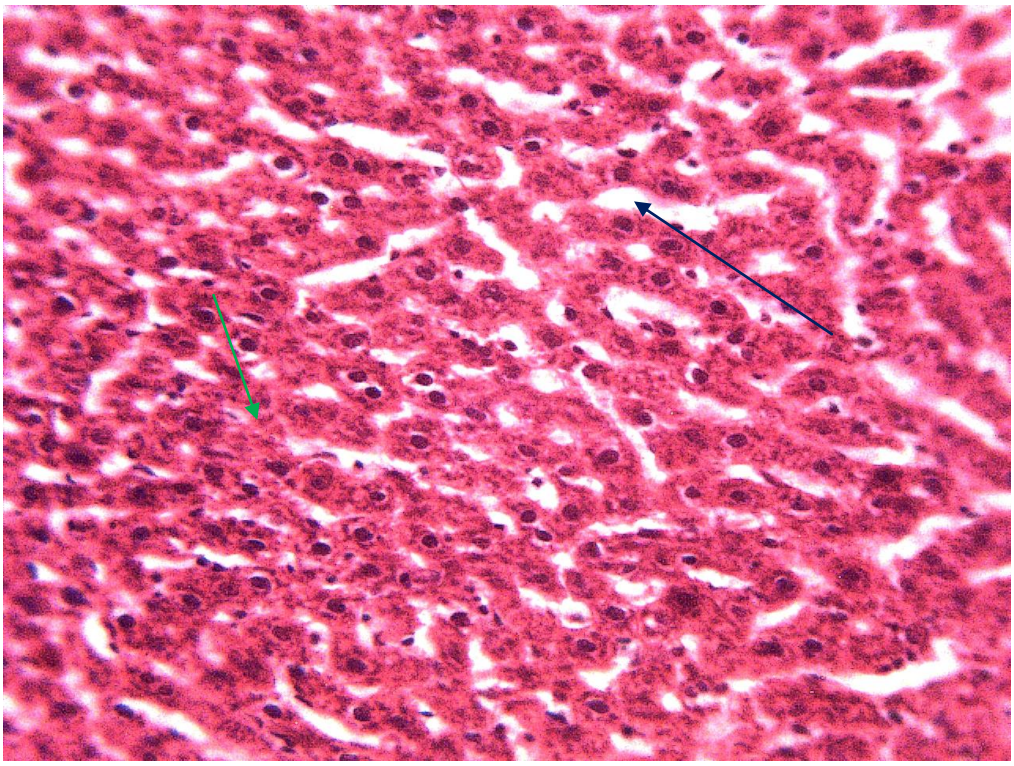


PLATE 27 Light microscopic section of the liver of wistar rats fixed in saturated sodium chloride solution (prepared with laboratory salt) for 168hours. Sinusoids (blue arrow) and hepatocytes (green arrow) (H&E x 250).

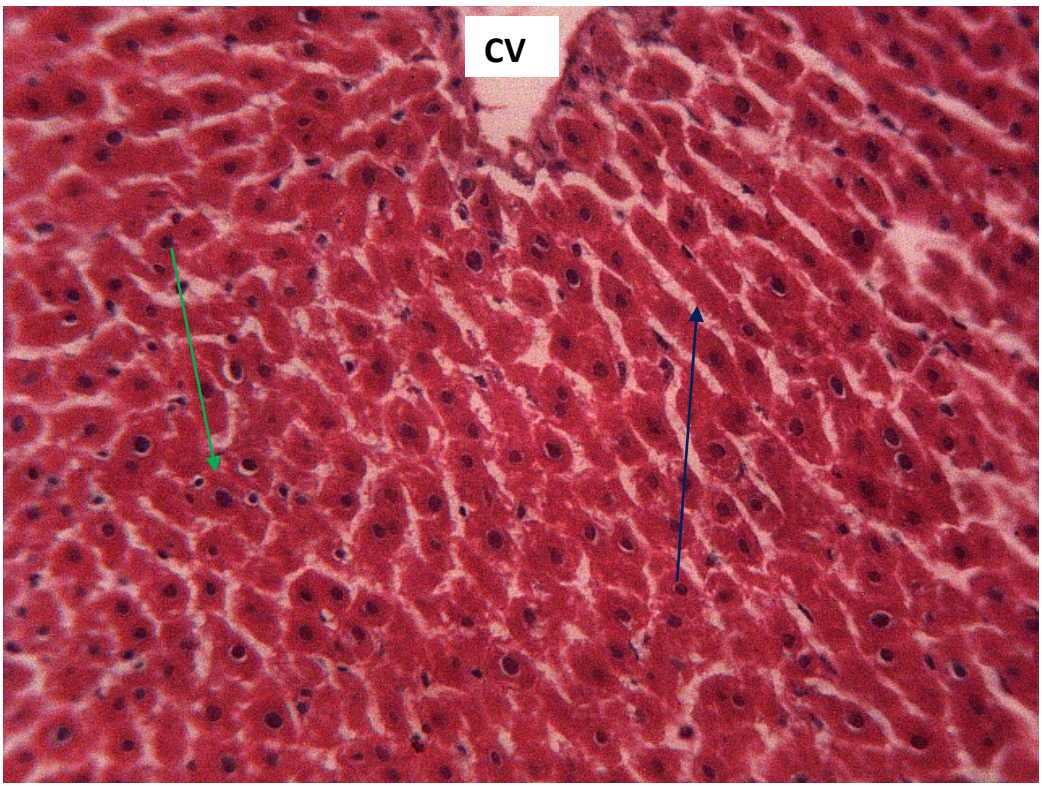


PLATE 28 **Light microscopic section of the liver of wistar rats fixed in saturated sodium chloride solution (prepared with Table salt) for 168 hours. Central vein (CV), sinusoids (blue arrow) and Hepatocytes (green arrow) (H&E x 250).**

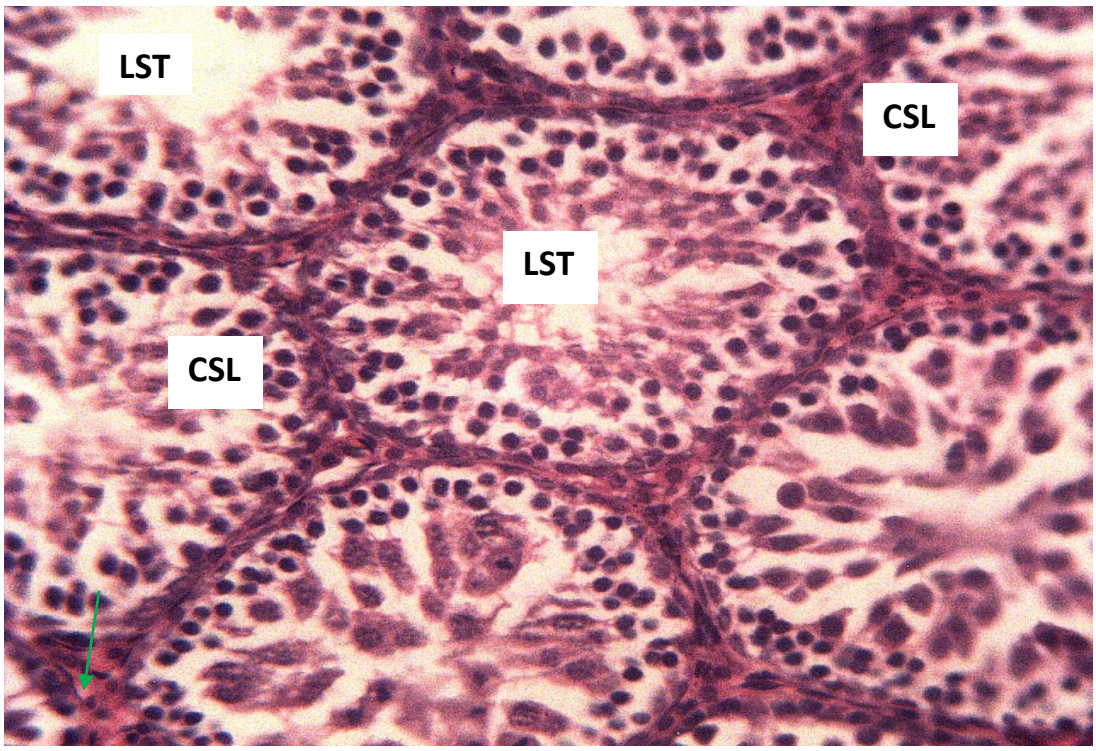


PLATE 29 Light microscopic section of the testis of wistar rats fixed in standard formal saline for 168 hours. Lumen of Seminiferous Tubules (LST) and Cells of Spermatogenic Lineage (CSL) Interstitium (arrow) (H&E X250).

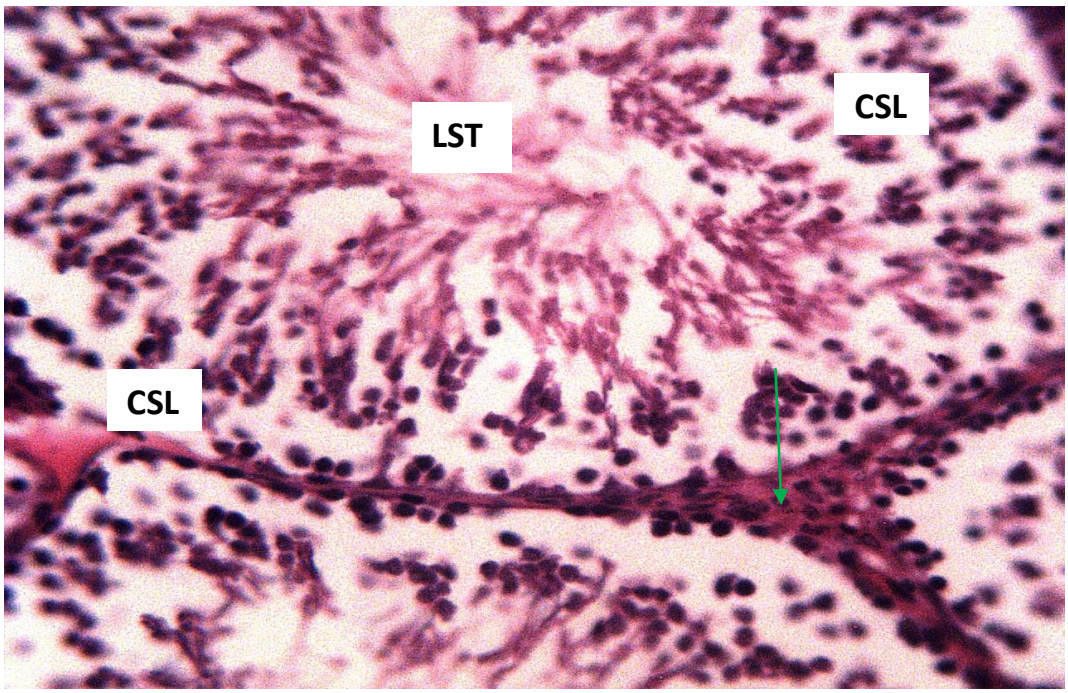


PLATE 30 Light microscopic section of the Liver of wistar rats fixed in formal saline (prepared with table salt) for 168 hours. Lumen of Seminiferous Tubules (LST) and Cells of Spermatogenic Lineage (CSL) Interstitium (arrow) (H&E X400).

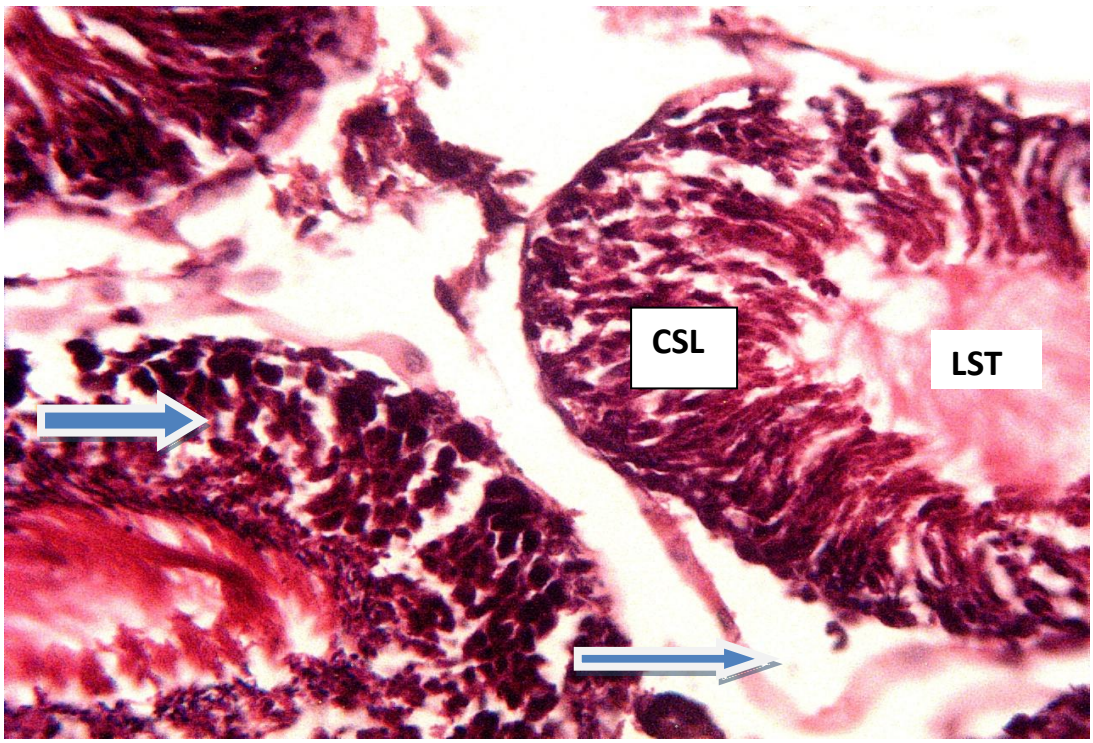


PLATE 31 **Light microscopic section of the testis of wistar rats fixed in saturated sodium chloride solution (prepared with laboratory salt) for 168 hours. Lumen of Seminiferous Tubules (LST), cells of Spermatogenic Lineage (CSL), shrinkage and collapse of the seminiferous tubules (Arrows) (H&E X250)**

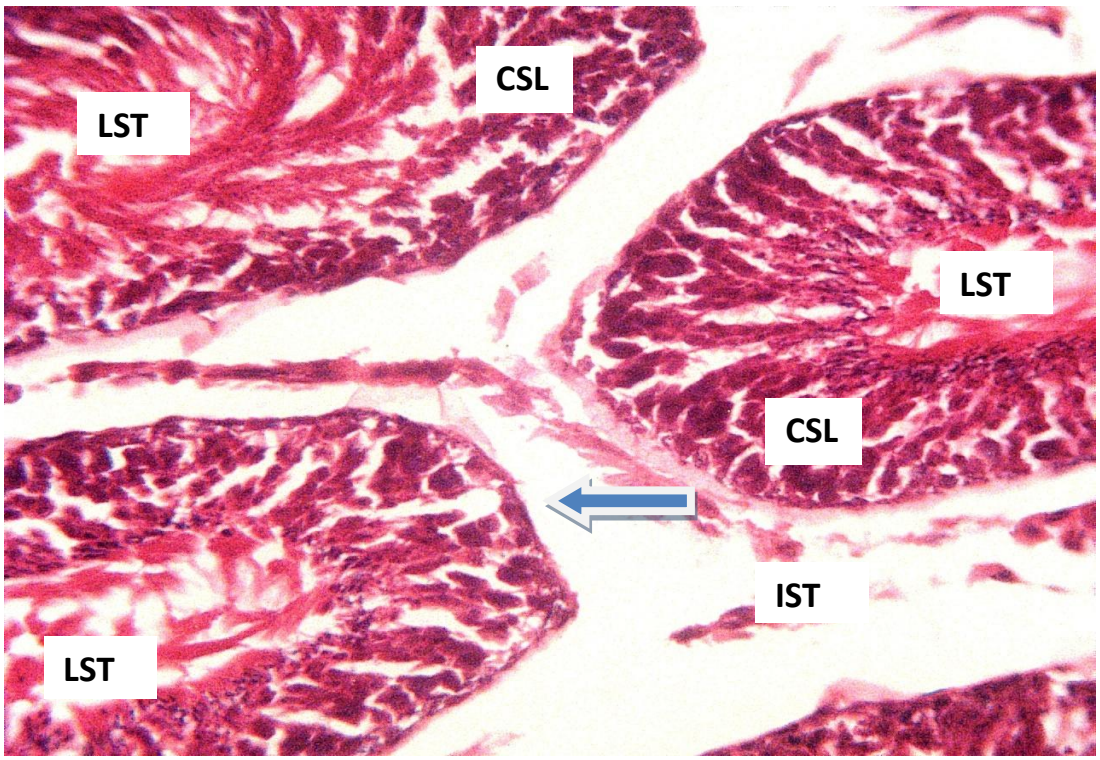


PLATE 32 **Light microscopic section of the testis of wistar rats fixed in saturated sodium chloride solution (prepared with Table salt) for 168 hours. Lumen of Seminiferous Tubules (LST), Cells of Spermatogenic Lineage (CSL), and depleted interstitium (IST) with shrinkage (Arrow) (H&E X250).**

4.2 HISTOMORPHOMETRIC EVALUATIONS

4.2.1 AT 12 HOURS OF FIXATION

For the Area of seminiferous tubules measured, the fixatives group B (formal saline with table salt), group C (saturated sodium chloride solution from laboratory salt) and group D (saturated sodium chloride solution from table salt) were significantly higher than the control group ($P<0.05$) and they were significantly lower for the height of germinal epithelium measured ($P<0.05$). Only the diameter of the tubules of testes fixed in 10% formal saline with table salt (group B) was significantly higher than that of the control group A (standard formal saline) ($P<0.05$) (Tables 4.3, 4.4 and 4.5). Taking 24hrs as the standard fixation period, 12hrs was significantly higher for the standard formal saline group ($P<0.05$) and for saturated sodium chloride solution from laboratory salt for the area of seminiferous tubules measured. ($P<0.05$) (Table 4.3). For the diameter of seminiferous tubules measured, only standard formal saline had 12hrs significantly higher than the standard fixation period of 24hrs. No significance was found in the height of germinal epithelia measured.

4.2.2 AT 24 HOURS OF FIXATION

All the fixative groups B (formal saline with table salt) ($P<0.05$), C (saturated sodium chloride solution from laboratory salt) ($P<0.05$) and D (saturated sodium chloride solution from table salt) ($P<0.05$) were significantly higher than the control group A (standard formal saline) for the Area and Diameter of the Tubules measured. The height of germinal epithelium of tubules of testes fixed in saturated sodium chloride solution groups C and D were significantly lower than that of the control group A (standard formal saline) ($P<0.05$) which was not different from that of group B (formal

saline with table salt) (Tables 4.3, 4.4 and 4.5).

TABLE 4.3 AREAS OF SEMINIFEROUS TUBULES MEASURED ACROSS FIXATIVE GROUPS AND PERIODS

FIXATIVE GROUP / FIXATIONPERIOD	FORMALSALINE SLS (GRP A) MEAN±SEM µm^2	FORMALSALINE TS (GRP B) MEAN±SEM µm^2	SAT. NaCl SLTN FRM LS (GRP C) MEAN±SEM µm^2	SAT. NaClSLTN FRM TS (GRP D) MEAN±SEM µm^2
12HOURS	652.50±35.04^{a}	1064.15±12.96**	1063.39±77.78^{*b}	860.21±71.23*
24HOURS	478.03±23.07	1278.59±82.62**	659.26±23.71*	847.50±61.46**
48HOURS	361.82±22.30^{b}	1227.85±93.16**	624.85±82.09*	644.97±30.81^{*a}
168HOURS	534.26±18.25	831.76±3.21^{**b}	723.87±47.95**	445.42±42.91^{b}

SLS -Standard laboratory salt, TS-Table Salt, SAT-Saturated, SLTN- Solution, and GRP-Group. ** P-value < 0.001, * P-value < 0.05 for fixative groups and ^{a} P-value < 0.05, ^{b} P-value < 0.001 forthe fixative periods

TABLE 4.4 DIAMETERS OF SEMINIFEROUS TUBULES MEASURED ACROSS FIXATIVE GROUPS AND PERIODS

FIXATIVE GROUP	FORMALSALINE	FORMALSALINE	SAT. NaClSLTN	SAT. NaCl SLTN
FIXATIONPERIOD	SLS (GRP A)	TS (GRP B)	FRM SLS (GRP C)	FRM TS (GRP D)
	MEAN±SEM (µm)	MEAN±SEM (µm)	MEAN±SEM (µm)	MEAN±SEM (µm)
12HOURS	292.46±8.22[b]	374.66±12.60[**]	304.25±12.04	290.91±8.57
24HOURS	225.48±7.47	335.59±25.24[**]	277.45±16.42[*]	318.83±15.92[**]
48HOURS	186.86±8.37[b]	362.33±32.39[**]	254.26±15.88[*]	276.93±10.15[*a]
168HOURS	256.71±9.48[a]	243.20±3.46[a]	236.35±7.79[a]	180.95±16.80[** b]

SLS -Standard laboratory salt, TS-Table Salt, SAT-Saturated, SLTN- Solution, and GRP-Group. ** P-value < 0.001, * P-value < 0.05 for fixative groups and [a] P-value < 0.05, [b] P-value < 0.001 for the fixative periods

TABLE 4.5 HEIGHTS OF GERMINAL EPITHELIA OF THE SEMINIFEROUS TUBULES MEASURED ACROSS FIXATIVE GROUPS AND PERIODS

FIXATIVE GROUP / FIXATIONPERIOD	FORMALSALINE SLS (GRP A) MEAN±SEM (µm)	FORMALSALINE TS (GRP B) MEAN±SEM (µm)	SAT. NaClSLTN FRM SLS (GRP C) MEAN±SEM (µm)	SAT. NaCl SLTN FRM TS (GRP D) MEAN±SEM (µm)
12HOURS	88.36±6.45	52.28±6.48**	39.61±3.51**	38.48±4.92**
24HOURS	87.67±8.86	69.45±8.61	44.48±3.50*	46.00±3.69*
48HOURS	54.53±5.53[b]	60.47±3.04	50.29±2.12	43.88±3.32
168HOURS	74.24±6.90	45.46±4.71*[a]	42.42±4.07**	30.55±1.26**[a]

SLS -Standard laboratory salt, TS-Table Salt, SAT-Saturated, SLTN- Solution, and GRP-Group. ** P-value < 0.001, *P-value< 0.05 for fixative groups and [a] P-value < 0.05, [b] P-value < 0.001 for the fixative period

4.2.3 AT 48 HOURS OF FIXATION

The Area and Diameter of Tubules of Testes fixed in fixative groups B (formal saline with table salt) ($P<0.05$), C (saturated sodium chloride solution from laboratory salt) ($P<0.05$) and D (saturated sodium chloride solution from table salt) ($P<0.05$) were significantly higher than the control group A (Standard formal saline). However, the heights of their germinal epithelia were not significantly different from that of the control group A (Tables 4.3, 4.4 and 4.5). For the area and diameter of seminiferous tubules measured, standard formal saline and saturated sodium chloride solution from table salt were significantly lower than the standard fixation period of 24hours. ($P<0.05$) (Table4.3 and 4.4) while only standard formal saline was significantly lower for the height of germinal epithelia measured (table 4.5)

4.2.4 AT 168 HOURS OF FIXATION

Only the Diameter of Tubules of Testes fixed in saturated sodium chloride solution from table salt (group D) ($P<0.05$) was significantly lower than that of the control group A (standard formal saline) and only their Areas were not significantly different from the control group A whereas, other groups B (formal saline with table salt) ($P<0.05$) and C (saturated sodium chloride solution from laboratory salt) ($P<0.05$) were significantly higher. All the groups B-D were significantly lower than the control group A for the height of the germinal epithelium measured ($P<0.05$) (Tables 4.3, 4.4 and 4.5). formal saline with table salt and saturated sodium chloride solution from table salt were significantly lower than the standard fixation period of 24 hours in the area of seminiferous tubules measured while all the fixative groups were significantly lower at 168 hours of fixation than the standard fixation period of 24 hours except the control which was significantly higher in the diameter of seminiferous tubules measured (table

4.4). For the height of germinal epithelia measured, only formal saline with table salt and saturated sodium chloride solution from table salt were significantly lower than the standard fixation period of 24 hours ($p<0.05$) (table 4.5)

CHAPTER FIVE

5.0 DISCUSSION

A systematic study of fixatives began in the latter half of the 19th century, however, Much attention was focused on developing fixatives that would preserve cells and tissue constituents in as close a life-like state as possible while allowing them to undergo further preparative procedures without change (Leong *et al*, 1996) which Formaldehyde was close to doing but not without creating other health problems for the user. A method to overcome the problems of Formaldehyde is to use an alternative fixative that is not only suited for the preservation of tissues but is also cheap, available and non-carcinogenic.

In this study, Formal saline prepared with table salt gave an excellent preservation of the Liver Tissue at 12 hours of fixation through 24 hours to 48 hours performing even better than the control group of standard formal saline which gave a very good performance and only better than the modified formal saline at 168 hours of fixation noting that the standard fixation period is 24 hours. The drop in the performance of the formal saline prepared with table salt by 168 hours of fixation could probably be attributed to the presence of other compounds (such as iodine, potassium ferrocyanide) present in the table salt which could have an interfering effect on the fixation process but it is comparable with standard formal saline at 12 hours, 24 hours and 48 hours of fixation and these two fixatives were not significantly different from each other. Testicular tissue was also well preserved by these 2 fixatives for 12 hours and 24 hours although more so by the standard formal saline (control) than the modified formal saline. The drop in the performance of standard formal saline by 48 hours through 168

hours of fixation could be one of the reasons why formal saline may not be the ideal fixative for testes preservation considering it differs in composition from the liver tissue.

The excellent fixation properties of formaldehyde and formaldehyde- based fixatives are well-known and have been documented and are still being researched. Formaldehyde fix tissues by crosslinking proteins particularly between the lysine residues and these cross-linkages do not harm the structures of the proteins greatly (Williams *et al*, 1999). However previous workers have indicated that exposure to formaldehyde has serious effects and is highly suspected to be a human carcinogen (OSHA, 2002 & Al saraj *et al*., 2003) while others (Prent and Lyon, 1997; Titford and Horenstein, 2005; Moelans *et al*., 2011; Benedetta *et al*., 2012) are still of the opinion that formaldehyde or formaldehyde based fixatives remains the "Gold standard" for fixation in histopathology and medical research after comparing it with other newer commercial fixation systems such as RCL2, PAX Gene, Finefix etc. The World Health Organization in June 2011 concluded that formaldehyde is a known human carcinogen.

Saturated sodium chloride solution (from laboratory salt) demonstrated a very good preservation of the Liver sections at 12 hours (even better than standard Formal saline), 24 hours and 48 hours, but there was a drop by 168 hours unlike the saturated sodium chloride solution from Table salt that demonstrated a very good preservation of the Liver tissues all through the fixation periods, more similar to standard formal saline (control) in performance by uniformity. The slight difference observed in the performances of these two solutions could probably be attributed to the presence of other compounds (such as iodine, potassium ferrocyanide) contained in table salt which

may have a stabilizing effect on the fixation process. The result obtained from this study is in accordance with those obtained by Al saraj, (2010) that suggests that saturated sodium chloride solution is comparable to formaldehyde in the preservation of Liver tissues of adult male wistar rats and may be classified as an unknown mechanism fixative (like picric acid, mercuric chloride etc). Other substances such as honey (Ozkan *et al*., 2012), commercial formalin substitutes (Zanini *et al*., 2012), Glyoxal (Yak-Nam *et al*., 2011), universal molecular fixative UMFIX (Vladimir *et al*., 2003) have all been demonstrated to have good morphological preservation and comparable to formaldehyde in results.

The relative poor performances of the saturated sodium chloride solutions (both with standard laboratory salt and table salt) as fixatives for testicular tissue in contrast to their performances in the Liver tissues are probably accounted for by the fact that testicular tissue differs in structure and composition from the liver tissue and is also very soft and delicate and most likely requires finely standardized fixative as Saturated sodium chloride solution is hypertonic and its effects could be too tough on the delicate testicular tissue. There is sparse literature on the subject of saturated sodium chloride solution being used as fixative for testicular tissue. Formal saline is isotonic and performed significantly better than the saturated sodium chloride solution for testes fixation probably because the hypertonicity of the saturated sodium chloride solutions made the delicate testicular tissue prone to shrinkage and rapid degeneration as observed in the results obtained from this study.

The significant increase in the diameter and area of seminiferous tubules measured in the groups B (formal saline with table salt), C (saturated sodium chloride solution from laboratory salt) and D (saturated sodium chloride solution from table salt) could be due

to the differences in the sizes of the seminiferous tubules measured. As 24 hours is the standard fixation period, the general increase in the area and diameter recorded at 12 hours across the fixative groups maybe as a result of ongoing or incomplete fixation processes as fixatives are known to cause considerable shrinkage that has variable effects on the sizes of tissue fixed at their best, standard formal saline inclusive. Beyond the standard period of fixation, there was a general decrease in the area and diameter measured across the fixative groups which is in line with the variable shrinking effects of fixatives on tissues beyond their optimum period of action. The area and diameter of seminiferous tubules measured in the control group were in range with those measured by Akinloye *et a*l., 2002; Saalu *et al*., 2009; Toman *et al*., 2011; 2012) but not in line with those of (Asoquo *et al*., 2007 and Awobajo *et al*., 2010).

The significant differences in the height of germinal epithelium observed across the fixative periods in all the fixative groups B (formal saline with table salt), C (saturated sodium chloride solution from laboratory salt) and D (saturated sodium chloride solution from table salt) could probably be explained by the differences noticed in the stages of spermatogenesis in which the tubules were arrested in as observed in plates (5, 6, 7 & 8). The values obtained for the control group A (standard formal saline) were in range with those of (Mehraein and Negahdar, 2010; Toman *et al*., 2011; 2012).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 SUMMARY AND CONCLUSION

Fixation is a series of chemical events not fully understood making attempts to explain the observed differences in this study difficult. The aim of this study was to comparatively evaluate the effect of Table Salt in 10% formal saline and saturated sodium chloride solution as tissue fixatives with the ultimate goal of getting a cheaper and non-carcinogenic alternative to formaldehyde.

This study was able to show that

i. Standard formal saline can fix Liver tissue of adult male Wistar rats for routine demonstration by H&E for 12 hours, 24 hours, 48 hours and 168 hours.
ii. Formal saline (prepared with table salt) can fix liver tissue of adult male Wistar rats for routine demonstration by H&E for 12 hours, 24 hours, 48 hours and 168 hours.
iii. Saturated sodium chloride solution (both from laboratory and table salt) can fix liver tissues of adult male Wistar rats for routine demonstration by H&E for 12 hours, 24 hours and 48hours.
iv. Standard formal saline and Formal saline (prepared with table salt) can fix testicular tissue of adult male Wistar rats for routine demonstration by H&E for 12 hours and 24 hours.
v. Saturated sodium chloride solution (both from laboratory and table salt) cannot fix testicular tissues of adult male Wistar rats for routine demonstration by H&E.

vi. Standard formal saline was not significantly better in the fixation of liver and testicular tissues of adult male wistar rats than the modified formal saline (prepared with table salt).

vii. Formal saline performed significantly better than saturated sodium chloride solution in the fixation of testicular tissue of adult male wistar rats.

In conclusion, the saline composed in formal saline could be replaced with table salt without having any significant effect on the fixation of the liver and testis tissues of adult male Wistar rats. Also, sodium chloride can be used as a fixative for liver tissues of adult male Wistar rats for a maximum period of 48 hours in the form of a saturated solution for routine histological procedures as it is cheaper, readily available and non-carcinogenic until such a time that its mechanism of action will be understood fully and the procedure finessed to perfection. It has a moderate penetration time and the procedure is simple and easy to use with very little or no risk to the user.

A completely objective method of evaluating and comparing fixatives does not exist. The results obtained in a comparison of fixatives will always be dependent on individual preferences by the observers (Speilberg *et al.*, 1993). Several efforts were made to minimize possible sources of error in this comparative study. Parallel samples of Liver and Testes from all the Rats were fixed in all fixative groups, and the slides were coded. Furthermore, the independent observations of several observers of the same slides were combined to produce the final results. Judgments were quantified and made comparable by means of a grading scale and finally, data were treated statistically to minimize the risk of drawing conclusions based on the law of chance.

This research work serves as a baseline study for the use of saturated sodium chloride as a fixative in Nigeria and as a confirmation to the claim made by Alsaraj 2010 that saturated sodium chloride has some fixative properties.

6.2 RECOMMENDATIONS

Although saturated sodium chloride solution has demonstrated its potentials as a fixative, the following are suggested for it to be standardized and recognized:-

I. The mechanism of action of sodium chloride as a fixative need to be studied and documented.

II. The effects of sodium chloride fixation on various organs of the body are required as significant differences are being recorded.

III. More studies on various sodium chloride solutions are also required to get its optimum concentration for best fixation.

IV. Saturated sodium chloride solution should be used as the routine fixative for liver tissues in the histology laboratory of the department.

V. The Saline in the 10% formal saline prepared and used in the histology laboratory of the department could be substituted with table salt.

REFERENCES

Akinloye, A. K Abatan, M.O, Alaka O. O And 'Oke, B. O (2002) "Histomorphometric And Histopathological Studies On The Effect*of Calotropis Procera* (Giant Milkweed) On The Male Reproductive Organs Of Wistar Rats" *African Journal of Biomedical. Research (2002): Vol 5; 57 – 61*

Al-Hubaity A and AL-Saraj A (2003). The use of saturated table salt solution as an embalming agent. Z *Journal of Medical Sciences*. vol;7 (2):37-40.

Al-Saraj A, AL-Hubaity A. (2003) "Histological and histopathological changes of lung of rats during different periods of formaldehyde exposure" *Iraqi Journal of Veterinary Sciencess.; 17(2):111-21.*

Al-Saraj,A (2010). "Use of saturated sodium chloride solution as a tissue fixative" *Iraqi Journal of Veterinary Sciences, Vol. 24, No. 1, (53-58).*

Asuquo O. R., A. O. Igiri1, O. O. Olawoyin, E. U. Eyong (2007) "Correlation of Histological And Histometric Changes In Rats Testes Treated With Chloroquine Phosphate" *Nigerian Journal Of Physiological Sciences 22 (1-2): 135-139* ©Physiological Society Of Nigeria

ATSDR (1999) Agency for Toxic Substances and Disease Registry. Toxicological Profile for Formaldehyde. US department of Health and Human Services. Atlanta, US.

Awobajo, F. O.; Raji, Y. & Akinloye, A. K. (2010). "Histomorphometric Changes in the Testes and Epididymis of Wistar Strain Albino Rats Following Fourteen Days Oral Administration of Therapeutic Doses of Some Antibiotics" *International. Journal of Morphology, 28(4):1281-1287, 2010.*

Baan R, Grosse Y, Straf K, Secreton B, El ghassassi F, Bouvard V, Benbrahim-Talla L, Guha N, Freeman C, Galichet L, Cogliano V (2009) WHO IARC monographs working group: A review of human carcinogens- part F: chemical Agents and related occupations. *Lancet oncology* 10: 1143-1144

Benedetta Belloni, Chiara Lambertini, Paolo Nuciforo, Jay Phillips, Eric Bruening, Stephane Wong, Reinhard Dummer (2012) "Will PAXgene substitute formalin? A morphological and molecular comparative study using a new fixative system" *Journal of Clinical Pathology* vol0:1–12. doi:10.1136/jclinpath-2012-200983 JCP Online First, published on November 3, 2012 as 10.1136/jclinpath-2012-200983

BDH Chemicals Ltd poole England product Number 30123 and 33160.

Buesa RJ (2008): "Histology without Formalin." *Annals of Diagnostic Pathology*, 12: 387-396

Carson, Freida L; Christa Hladik (2009). *Histotechnology: A Self-Instructional Text* (3 ed.). Hong Kong: *American Society for Clinical Pathology Press. ISBN 9780891895817*

Casanova-Schmitz M, Raymond MD, Heck H d'A. (1984b) "Oxidation of formaldehyde and acetaldehyde by NAD^+-dependent dehydrogenases in rat nasal mucosal homogenates" *Biochemistry and Pharmacology 33:1137-1142*.

Casanova-Schmitz M, Starr TB, Heck Hd'A. 1984a. "Differentiation between metabolic incorporation and covalent binding in the labeling of macromolecules in the rat nasal mucosa and bone marrow by inhaled [^{14}C]- and [^{3}H] formaldehyde" *Toxicology and Applied Pharmacology 76:26-44.*

CCOHS (2004) Canadian Centre for Occupational Health and Safety. Formaldehyde gas, Chemical information.

Cogliano VJ, Grosse Y, Baan RA, Straf K< Secretan MB, El ghissassi F (2005): "meeting reports; summary of IARC monographs on formaldehyde, 2-butoxyethanol and tert-butoxy-2-propanol." *Environmental Health perspectives* 113: 1205-1208

Cotran, Ramzi S.; Kumar, Vinay; Fausto, Nelson; Nelso Fausto; Robbins, Stanley L.; Abbas, Abul K. (2005). *Robbins and Cotran pathologic basis of disease* (7th ed.). St. Louis, MO: Elsevier Saunders. p. 878. ISBN 0-7216-0187-1.

Cristina Zanini, Elisa Gerbaudo, Elisabetta Ercole, Anna Vendramin and Marco Forni (2012), "Evaluation of two commercial and three home-made fixatives for the substitution of formalin: a formaldehyde–free laboratory is possible" *Environmental Health* 11:59 doi:10.1186/1476-069X-11-59 http://www.ehjournal.net/content/11/1/59

Cross CE, van der Vliet A, O'Neill CA, Louie S and Halliwell B (1994). "Oxidants, antioxidants, and respiratory tract lining fluids" *Environmental Health Perspectives and Suppliments* 102 10: 185-91.

Dalley, A.F., Moore K.L. (1999). "Clinically Oriented Anatomy". 5th Edition. Lippincott Williams and Wilkins.

Dapson R.W. (1993) Fixation for the 1990s: a review of needs and accomplishments. *Biotechnology and Histochemistry 68:75-82*.

Eitoum I., Fredenburgh J., Myers R.B., Grizzle W. (200lb) "Introduction to the theory and practice of fixation of tissues." *Journal of Histotechnology* 24:173-190.

Eitoum L-E., FredenburghJ., Grizzle W.E. (2001a)" Advanced concepts in fixation: effects of fixation on immunohistochemistry and histochemistry, reversibility of fixation and recovery of proteins, nucleic acids, and other molecules from fixed and processed tissues, special methods of fixation". *Journal of Histotechnology* 24:201-210.

Feldman Susan R.. (2005) Sodium chloride. *Kirk-Othmer Encyclopedia of Chemical Technology*. John Wiley & Sons, Inc. Published online. doi:10.1002/0471238961.1915040902051820.a01.pub2

Finnish Environment Institute (1/9/2007). "Migration of alternative de-icing chemicals in aquifers (MIDAS)". Press release

Franks SJ (2005). "A mathematical model for the absorption and metabolism of formaldehyde vapour by humans" *Toxicology and Applied Pharmacology 206(3): 309-320.*

Gewartowska M and Olszewski WL (2007). Preservation of molecular structure of arterial grafts in pulverized sodium chloride: an experimental study Transplant Procedure. vol;39(9):2917-9.

Gilbert F Scott (2000) Online textbook: "Developmental Biology" 6th ed. published by Sinauer Associates, Inc. of Sunderland (MA).

Golden Robert, (2005) "Formaldehyde: Overview of Current Issues and Challenges for the Future," the International Nonwovens Technical Conference, Sept.19-22.

Grammer I. C (1990) "Clinical and immunologic evaluation of 37 workers exposed to gaseous formaldehyde" *Journal of allergy and clinical immunology*, **86**: 177–181.

Gupta Tulika and Gauba Krishna (2011) "cadaveric tissue Histology: A viable Alternative" *Journal of clinical and diagnostic research*, vol 5(8):1505-1509. www.jcdr.net

Heck HD, White EL, Casanova-Schmitz M. (1982). "Determination of formaldehyde in biological tissues by gas chromatography/mass spectrometry" *Biomedical Mass Spectrometry* 9(8): 347-353.

HSDB. (2009). *Hazardous Substances Data Bank.* National Library of Medicine. http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB and search on CAS number. Last accessed: 5/18/09.

IARC (2006) Formaldehyde. In *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Formaldehyde, 2-Butoxyethanol and 1*-tert-*Butoxypropan-2-ol*, vol. 88, Lyon, France: International Agency for Research on Cancer. pp. 39-325.

IARC (2012) Formaldehyde. In IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Volume 100F. A review of Human carcinogens :chemical agents and related occupations. Formaldehyde. http://momgraph.iarcfr/ENG/monographs/vol/100F/mono100f-29.pdf

IPCS (1989) International Programme on Chemical Safety . Formaldehyde. Environmental Health Criteria 89. WHO. Geneva. *Environmental Health Criteria No. 89: Formaldehyde.* International Programme on Chemical Safety. http://www.inchem.org/documents/ehc/ehc/ehc89.htm.

Junqueira Luiz Carlos and Caneiro Jose (2005). "Basic Histology, text and atlas" (11th edition) McGraw Hill Companies 2003; pp 418-419.

Kierszenbaum L. Abraham (2007) *"Histology and cell biology an introduction to pathology*" (2nd edition) Mosby Elsevier Ltd Philadelphia. pp502.

Kmieć Z (2001). "Cooperation of liver cells in health and disease". *Advances in Anatomy Embryology and Cell Biology* 161: III–XIII, 1–151. PMID 11729749.

Kuhlmann, W.D. (2009) "Fixatives" Division of Radiooncology, Deutsche Krebsforschungszentrum, 69120 Heidelberg, Germany 28.07.

Leong ASY, James CL, Thomas AC. (1996): "Handbook of Surgical Pathology". New York, Churchill Livingstone., pp viii, 321.

Lykidis D, Van Noordan S, Armstrong A, Spencer-Dene B, Li J, Zhang Z, Stamp GW (2007) "Novel Zinc-based fixative for high quality DNA, RNA and protein analysis." *Nucleic Acid res*, 35: e85

Maibach, H (1983). "Formaldehyde: effects on animal and human skin". *In*: Gibson, J.E. ed. *Formaldehyde toxicity*. Washington, DC, Hemisphere

Mariani-constantini R, Awadelkarim KD, Barberis M (2011) "Building sustainable capacity for disease diagnosis in sub-saharan Africa: case studies of cooperation in diagnostic pathology." In new knowledge in a new Era of globalization. Edited by Pachura P, Ryeka Croatia. Im technology : 243-266

Markel H (1987). ""When it rains it pours": endemic goiter, iodized salt, and David Murray Cowie, MD". *American journal of public health 77 (2): 219-29. PMID 3541654.*

Mclaughlin, J.K (1994). "Formaldehyde and cancer: a critical review". *International archives of occupational and environmental health*, 66: 295–301

Mehraein Fereshteh, and Feraidoon Negahdar. (2010) "Morphometric Evaluation of Seminiferous Tubules in Aged Mice Testes after Melatonin Administration" *Cell Journal (Yakhteh), Vol 13, No 1, Spring 2011, Pages: 1-4*

Moelans Cathy B., PhD, Natalie ter Hoeve, Jan-Willem van Ginkel, Fiebo J. ten Kate, MD, PhD and Paul J. van Diest, MD, PhD (2011), "Formaldehyde Substitute Fixative Analysis of Macroscopy, Morphologic Analysis, and Immunohistochemical Analysis" *American Journal of Clinical Pathology, 136, 548-556.doi: 10.1309*/AJCPHH1B0COCBGOM

Nordman H, Keskinen H, and Tupparainen M. (1985). "Formaldehyde asthma - Rare or overlooked". *Journal of Allergy and Clinical Immunology. 75(1 pt 1):91-99.*

NTP (2010). *Report on Carcinogens Background Document for Formaldehyde.* NationalToxicologyProgram.http://ntp.niehs.nih.gov/ntp/roc/twelfth/2009/November/Formaldehyde_BD_Final.pdf.

Olszewski WL, Moscicka M, Zolich D, and Machowski Z. (2007) Human skin preserved in anhydric sodium chloride for months can be successfully transplanted. *Annals of Transplant.* vol: 9(4):37-9.

Olszewski WL, Zolich D, and Tripathi MF (2004) :Sodium chloride fixation of tissues under field conditions in tropical countries. *Journal of Immunological Methods.* vol; 284(1-2):39-44.

OSHA (2002) Occupational Safety and Health Administration: Formaldehyde fact sheet. U.S. Department of Labor.

Ozkan N, Salva E, Cakalağaoğlu F, Tüzüner B. (2012) "Honey as a substitute for formalin?" *Biotechnic and Histochemistry (Biological stain commission). Feb;87(2):148-53. doi: 10.3109/10520295.2011.590155*. Epub 2011 Aug 23.

Prent P and Lyon H. (1997) "Commercial formalin substitutes for histopathology". *Biotechnic and Histochemistry; 72 (5):273-82.*

Puchtler H, and Meloan SN (1985): On the chemistry of formaldehyde fixation and its effects on immunohistochemical reactions. *Histochemistry* vol; 82:201-204.

Ross Michael H. and Wojciech Paulina, (2006) "Histology, A Text and Atlas" Lippincott Williams & Wilkins, 5th edition.

Royal Salt Limited, Kirikiri Lighter Terminal, Phase 1, Tincan Island, Apapa, Lagos, Nigeria. NAFDAC No. 01-7605.

Saalu L. C., L. A. Enye and A. A. Osinubi (2009) "An assessment of the histomorphometric evidences of doxorubicin-induced testicular cytotoxicity in Wistar rats", *International Journal of Medicine and Medical Sciences Vol. 1(9), pp. 370-374*

Santivito A, Shilliro T, Castellano S, Cervella P, Bigatti MP, Gilli G, Bono R, Delpero M, (2011) "combined analysis of chromosomal aberrations an glutathione-s-transferase M1and T1 polymorphisms in pathologists occupationally exposed to formaldehyde." *Archives of toxicology*, 85: 1295-1302

Shaham J, Bomstein Y, Gurvich R, Raskovsky M, Kaufman Z (2003) "DNA-Protein crosslinks and p53 protein expression in relation to occupational exposure to formaldehyde" *Occupational and environmental medicine*, 60: 403-409

Shaham J.A., Bomstein.Y, Meltzer.A., Kaufman Z, Palma,E. and Ribak J. (1996) "DNA-protein crosslinks, a biomarker of exposure to formaldehyde—*in vitro* and *in vivo* studies". *Carcinogenesis*, 17, 121—125.

Shara MA, Dickson PH, Bagchi D, (1992). "Excretion of formaldehyde, malondialdehyde, acetaldehyde and acetone in the urine of rats in response to 2,3,7,8-tetrachlorodibenzo-p-dioxin, paraquat, endrin and carbon tetrachloride". *Journal of Chromatography 576:221-233*.

Singh, I. (2002). "Textbook of Human Histology with colour atlas" 4th edition., JAYPEE Publishers.

Sinnatamby S. Chummy (1999) "Lasts Anatomy, regional and applied (10th edition)" Churchill Livingstone; Elsevier Limited (rev.ed) 2004. Pp 222-223

Skinner M, McLachlan R, Bremner W (1989). "Stimulation of Sertoli cell inhibin secretion by the testicular paracrine factor PModS". *Molecular and Celularl Endocrinology* 66 (2): 239–49. doi:10.1016/0303-7207(89)90036-1. PMID 2515083.

Speilberg L, Evensen O, Bratberg B and Skjerve E (1993) " Evaluation of five different immersion fixatives for light microscopic studies of liver tissue in Atlantic salmon *Salmo salar" Diseases of. Aquatic Organisms. 17: 47-55.*

Stayner.L., Ward.E. and Petsonk.E.L. (1994) Formaldehyde. In ZenzX.(ed.), "Occupational Medicine: Principles and Practical Applications." Mosby Year Book, St Louis, MO, pp. 675-679.

Tbakhi A, Totos G, Hauser-kronberger C, Pettay J, Baunoch D, Hacker GW, Tubbs RR (1998) "Fixation conditions for DNA and RNA in situ hybridization: a reassessment of molecular morphology dogma." *American journal of pathology* 152: 35-41

Thompson CM, Sonawane B, Grafstrom RC. (2009). "The ontogeny, distribution, and regulation of alcohol dehydrogenase 3: implications for pulmonary physiology". *Drug Metabolism Disposal* 37(8): 1565-1571.

Thrasher JD, Broughton A, and Madison R. (1990). "Immune activation and autoantibodies in humans with long-term inhalation exposure to formaldehyde". *Archives of Environmental Health 45:217-223.*

Titford_ME and Horenstein_MG. (2005), "Histomorphologic assessment of formalin substitute fixatives for diagnostic surgical pathology". *Archives of Pathology and Laboratory Medicine. Apr;129 (4):502-6.*

Toman Róbert, Mária Adamkovičová, Peter Massányi, Michal Cabaj, Norbert Lukáč, Monika Martiniaková, Radoslav Omelka (2012) "Cadmium And Diazinon-Induced Changes In The Rat Testis Structure After A Peroral Administration In Drinking Water", *Journal of Microbiology, Biotechnology and Food Sciences: 2 (2) 564-575*

Toman Robert, Mária Adamkovičová, Svätoslav Hluchý, Michal Cabaj, Jozef Golian. (2011) "Quantitative Analysis of the Rat Testes after an Acute Cadmium and Diazinon Administration". *Animal Science and Biotechnologies , 44 (2)*

Vladimir Vincek, Mehdi Nassiri, Mehrdad Nadji and Azorides R Morales (2003), "A Tissue Fixative that Protects Macromolecules (DNA, RNA, and Protein) and Histomorphology in Clinical Samples". *Laboratory Investigation 2003, 83:1427–1435*

WHO, (2011). *Formaldehyde in Drinking-water. Background Document for Development of WHO Guidelines for Drinking-water Quality.* World Health Organization. http://www.who.int/water_sanitation_health/dwq/chemicals/formaldehyde130605.pdf.

Williams C, Ponten F, Moberg C, Soderkvist P, Uhlen M, Ponten J, Sitbon G, Lundeberg J (1999) "A high frequency of sequence alterations is due to formalin fixation of archival specimens". *American Journal of Pathology.;155:1467-1*

Yak-Nam Wang, Ph.D. Kara Lee, B.S. Shruti Pai, Ph.D. William R. Ledoux, Ph.D. (2011) "Histomorphometric Comparison after Fixation with Formaldehyde or Glyoxal". *Information on Healthcare, Oct, Vol. 86, No. 5, pgs. 359–365*.

Zanini Cristina, Elisa Gerbaudo, Elisabetta Ercole, Anna Vendramin and Marco Forni (2012), "Evaluation of two commercial and three home-made fixatives for the substitution of formalin: a formaldehyde–free laboratory is possible" *Environmental Health* 11:59 doi:10.1186/1476-069X-11-59 http://www.ehjournal.net/content/11/1/59.