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RESEARCH ARTICLE

COMPARATIVE EVALUATION OF ALTERNATE EMERGENCY FIXATIVES

Dr. Rumaisa Pandit, Dr. Sanjeet Singh, Dr. Paramjit Singh, Dr. Nishant Singh and Dr. Kanika Sharma

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Abstract

Fixation is the cornerstone of histopathology.In order to preserve the morphology of the tissue for further details its essential that all the components are preserved in a life like state. Formalin is the universal fixative but on account of its carcinogenic potential needs to be replaced by the alternate fixatives which are easily available.This study aims to assess and compare the efficacy of alternative fixatives like local anesthetic solution, normal saline , distilled water and life buoy hand sanitizer by using H & E stained sections. Total sample size of 100 sections was obtained from 10 paraffin embedded tissue blocks obtained from Indian goat tongue each fixed in LA solution ,Normal Saline, Distilled water, Hand sanitizer and in Formalin for 12 hour and 24 hour and named as group A and group B respectively. The sections were evaluated on five parameters and then subjected to statistical analysis.Kruskal wallis test, Mann – Whitney U test and Kappa Statistic were used .Significant difference was noted in the four alternate fixatives(< 0.05).On the basis of the results obtained , Local Anesthetic solution was found to be used as an alternate emergency fixative.

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Introduction:-

Proper tissue fixation is essential to ensure the highest level of specimen evaluation. Fixation is the critical step in processing tissues.[1]

Fixation is a complex series of chemical events that differs for the different groups of chemical substances found in the tissues.Baker classified the fixatives as coagulants and non coagulants .[2]

The primary aim of fixation is to preserve the tissues in a life like state, prevent bacterial putrefaction, prevent autolysis and increase the refractive index of the tissue.[3]

The objective of fixation is to preserve and harden the cell and tissue constituents in as close as a life like state as possible and to allow these to undergo further preparative procedures without change. Fixation is the first step and the foundation in a sequence of events that culminate in the final examination of a tissue section.[4]

Alexander M.Butlerov first discovered formaldehyde in 1859, but it was used as a tissue fixative by Ferdinand Blum.[5]

Formaldehyde is a gas. It's small molecules (HCHO – of which CHO is the aldehyde group) dissolve rapidly in water with which they combine chemically to form methylene hydrate, HO $–CH_2$ –OH.[6]

Corresponding Author:- Dr. Rumaisa Pandit

$H_2O + n\{HCHO\} \rightarrow HOCH_2OH$

Formaldehyde →Formaldehyde Polymer.

The formalin fixed tissue stained with hematoxylin and eosin is considered as the gold standard due to it's ease of availability and cost effectiveness.In spite of the advantages the International Agency for Research on Cancer (IARC) emphasized it as Group A carcinogen .[5]

Janet and Richard Dapson (2005) have called formaldehyde as 'Hazardous Material in the Histopathology Laboratory'. The main organization that influences the assessment of reagents as carcinogenic is the World Health Organization's (WHO) International Agency for Research on Cancer (IARC) based in France .[7]

The U.S Occupational Safety and Health Administration (OSHA) stated that employers must reduce worker exposure to formaldehyde at or below permissible exposure limits and the TWA (time weighted average) should be less than or equal to 0.75 ppm .The 15 – minute short term exposure limit (STEL) is 2 ppm .[8]

Guidelines for ambient formaldehyde levels in living spaces have been set in several countries in the range of 0.05 - 0.4 ppm, with a preference to 0.1 ppm.[3]

4% Formaldehyde as 10% formalin is no doubt the most widely accepted fixative. But in spite of it's extensive use in the laboratory it is not routinely kept in the clinics owing to it's several undesirable effects which include it's carcinogenic and irritating potential .[9]

In order to make a formalin free clinic various agents which are readily available in clinics are used like the local anesthetic solution, normal saline, distilled water and the hand sanitizer .[9]

Lignocaine is one of the most commonly used amide local anesthetic agents in dentistry, with a 50-year history of effectiveness and safety in providing regional anesthesia for dental therapies. Lignocaine as a local anesthetic was introduced into practice in the 1950s and, because of its excellent efficacy and safety, has become the prototypic dental local anesthetic. Besides having excellent anesthetic efficacy, lignocaine has limited allergenicity.[10]

Normal saline is the solution of 0.09% of NaCl , or 9.0g NaCl per litre of water. In most of the clinics it's used as a fixative in histopathological procedures .[11]

0.9% Saline / Sodium Chloride was first described by Dr.Hartold Jacob Hamburger in the 1890's .Having a similar freezing point to human serum and causing no visible erythrocyte lysis,the solution was named by him as 'indifferent fluid '. Saline today remains one of the most frequently used solutions for resuscitation of acutely ill patients with a variety of medical problems .[12]

Distilled water is steam from boiling water that's been cooled and returned to its liquid state. Distilled water is a type of purified water.[13]

Ethyl Alcohol / Ethanol is widely used in all kinds of products with direct exposure to the human skin (e.g. medicinal products like hand disinfectants in occupationalsettings, cosmetics like hairsprays or mouthwashes, pharmaceutical preparations, and many household products). [14]

Safety concerns on the toxic and carcinogenic effects of formalin exposure have drawn increasing attention to the search for alternative low risk fixatives for processing tissue specimens in laboratories. This study is designed to establish the efficacy of tissue fixation with other regularly found agents in the dental clinic i.e. local anesthetic solution, normal saline,distilled water and ethyl alcohol (hand sanitizer) in terms of nuclear and cytoplasmic staining characteristics and it's applicability as alternate fixative in private clinics .

Aim

To assess and compare the efficacy of alternative fixatives like Local Anesthetic solution (Lignocaine with Adrenaline 2%), 0.9% Normal Saline, Distilled water and Life buoy Hand sanitizer (95% ethyl alcohol) by using H & E stained sections .

Objectives:-

1. To stain and evaluate the H & E stained tissue sections using Local Anesthetic solution with 2% Adrenaline as a fixative.
2. To stain and evaluate the H & E stained tissue sections using 0.9% Normal Saline as a fixative.
3. To stain and evaluate the H & E stained tissue sections using (Lifebuoy) Hand Sanitizer with 95% alcohol as a fixative .
4. To stain and evaluate the H & E stained tissue sections using Distilled water as a fixative.
5. To evaluate and compare the efficacy of the alternate fixatives based on time duration of 12 hours and 24 hours.
6. To stain and evaluate the H & E stained tissue section using 10% Neutral Buffered Formalin as control group.
7. To evaluate the best emergency fixative.

Materials and Methods:-

Source of Data

The goat tongue was obtained from a local slaughter house from Modinagar, Ghaziabad and then the ten blocks were obtained from it and fixed with Local anesthetic solution, Normal saline, Distilled water, Life buoy hand sanitizer and Formalin for a period of 12 hours and 24 hours.

Sample Size

The total sample size of 100 sections was obtained from 10 paraffin embedded tissue blocks each fixed in Local Anesthetic solution, Normal Saline, Distilled water, Hand sanitizer and in Formalin (12 hour and 24 hour and grouped as group A and group B respectively).

Study group are as follows:

Group A - 50 histological sections were stained with conventional Hand E ,using Local Anesthetic solution, Normal saline, Hand sanitizer, Distilled water and Formalin as fixative for a 12 hour period .

Group B -50 histological sections were stained with conventional H and E ,using Local Anesthetic solution, Normal saline, Hand sanitizer, Distilled water and Formalin as fixative for a 24 hour period.

Group A (the tissue kept in the respective fixative for 12 hours)

A_1 -10 histological sections were stained with conventional Hematoxylin and Eosin using the Local anesthetic solution as a fixative agent.

A_2-10 histological sections were stained with conventional Hematoxylin and Eosin using the Normal saline as a fixative agent.

A_3 -10 histological sections were stained with conventional Hematoxylin and Eosin using Distilled water as a fixative agent.

A_4- 10 histological sections were stained with conventional Hematoxylin and Eosin using the Hand sanitizer as a fixative agent.

A_5-10 histological sections were stained with conventional Hematoxylin and Eosin using Formalin as a fixative agent.

GROUP B : (the tissues are kept in the respective fixative for 24 hours)

B_1-10 histological sections were stained with conventional Hematoxylin and Eosin using Local anesthetic solution as a fixative agent.

B_2-10 histological sections were stained with conventional Hematoxylin and Eosin using the Normal saline as a fixative agent.

B_3- 10 histological sections were stained with conventional Hematoxylin and Eosin using Distilled water as a fixative agent.

B_4- 10 histological sections were stained with conventional Hematoxylin and Eosin using the Hand sanitizer as a fixative agent.

B_5- 10 histological sections were stained with conventional Hematoxylin and Eosin using Formalin as a fixative agent.

Reagents Used –

1. 2% Lignocaine with Adrenaline injection (1: 200000) / German remedies
2. 0.9% w/v Sodium Chloride (Normal saline)
3. Distilled water
4. Hand sanitizer (Life buoy-95% Ethyl Alcohol)
5. Formalin (37- 41%)

The mounted sections of the two groups were evaluated for the staining quality, cellular outline, nuclear details, ease of sectioning ,tissue folds and the overall morphology by two different independent observers by grading them using the four point grading system i.e.
Grade 1 –Poor
Grade 2 –Satisfactory
Grade 3 – Good
Grade 4 –Excellent

H and E stained sections were examined under the conventional microscope for further evaluation including the nuclear morphology, cytoplasmic morphology and the overall morphology.

Evaluation –
50 slides from group A were reviewed for the different parameters including the staining quality, the cellular outline, the nuclear details, the ease of sectioning, tissue folds and the overall morphology.

50 slides from group B were reviewed for the different parameters including the staining quality, the cellular outline, the nuclear details, the ease of sectioning, tissue folds and the overall morphology.

Slides were assessed for the following parameters

Ease of Sectioning	Difficulty encountered / Or No Difficulty encountered
Staining Quality	Uniformity Intensity Vacuolization
Cellular Outline	Distinct Or / Blurred or Indistinct
Nuclear Details	Distinct chromatin condensation Prominent nuclear membrane Crisp staining of the nucleus Or Indistinct .
Artifacts	Tissue folding / Present or Absent
Overall morphology	Sufficient for Diagnosis / Or Insufficient for Diagnosis.

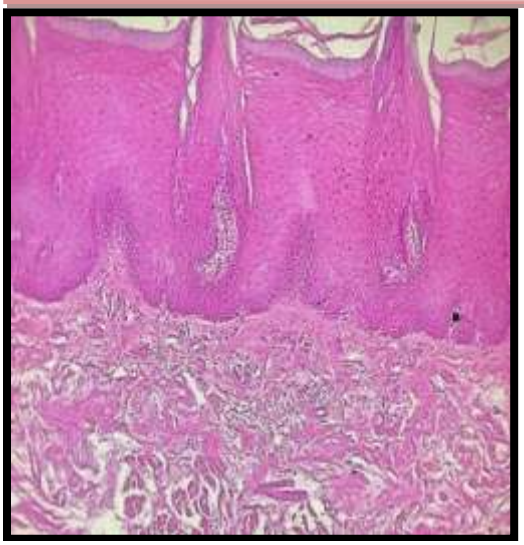
Scoring Criteria :
Each histomorphologic criteria will be rated on a scale of 1- 4:

a.	Poor	1
b.	Satisfactory	2
c.	Good	3
d.	Excellent	4

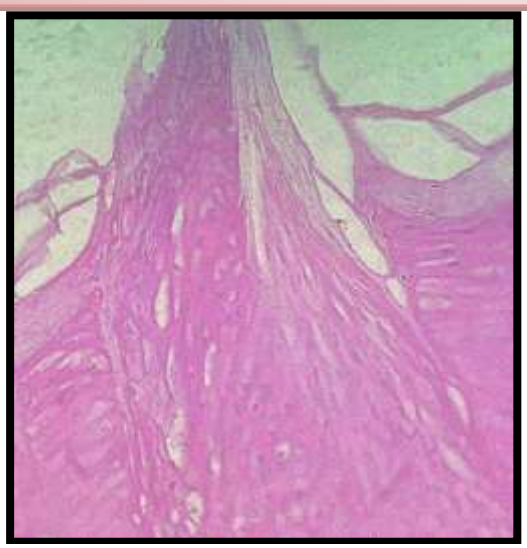
Depending upon the scores obtained ,total scores are taken to group the tissues into the following:

a.	Poor	1-10
b.	Satisfactory	11-19
c.	Good	20-24
d.	Excellent	>24

PHOTOMICROGRAPHS SHOWING H & E STAINING WITH LOCAL ANESTHETIC SOLUTION AS A FIXATIVE IN 12 AND 24 HOURS

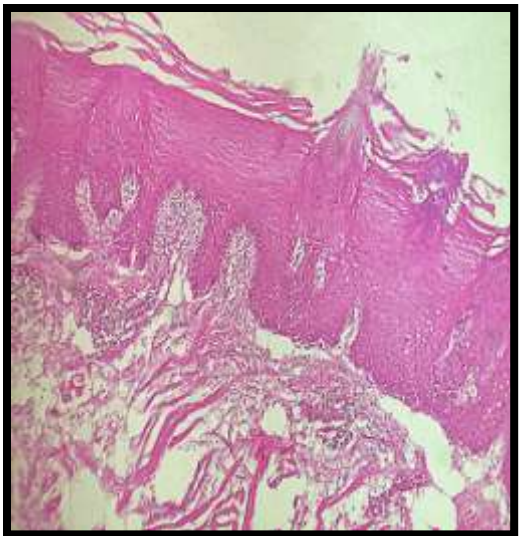


(a) 10 X

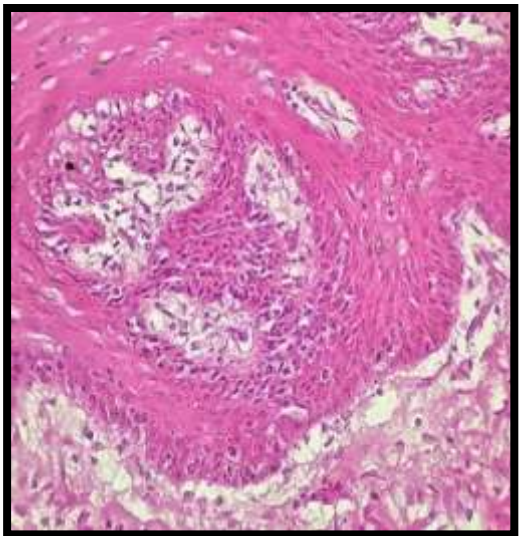


(b) 40 X

Photomicrograph 1:Showing H &E stained sections under magnification (a) 10 X & (b) 40 X for 12 hours duration using Local Anesthetic solution as a fixative .



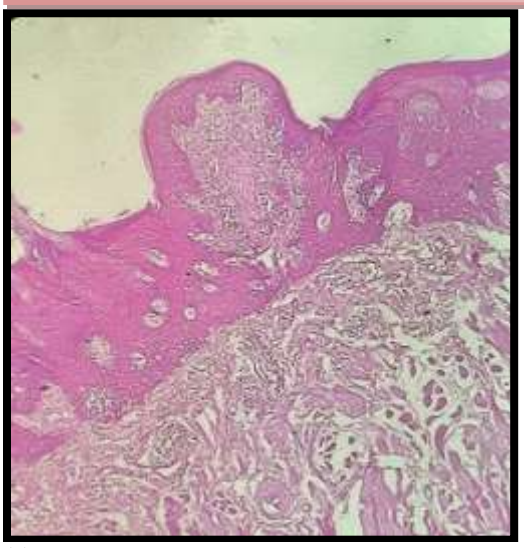
(c) 10 X



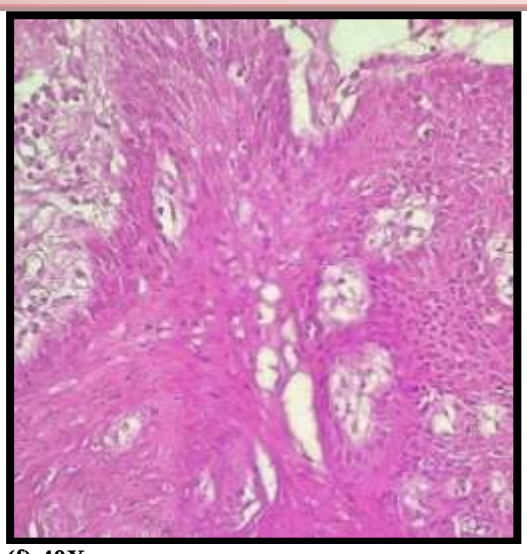
(d) 40X

Photomicrograph 2: Showing H & E sections under magnification (c) 10 X & (d) 40 X for 24 hours duration using Local Anesthetic solution as a fixative .

PHOTOMICROGRAPHS SHOWING H& E STAINING WITH FORMALIN AS A FIXATIVE IN 12 AND 24 HOURS

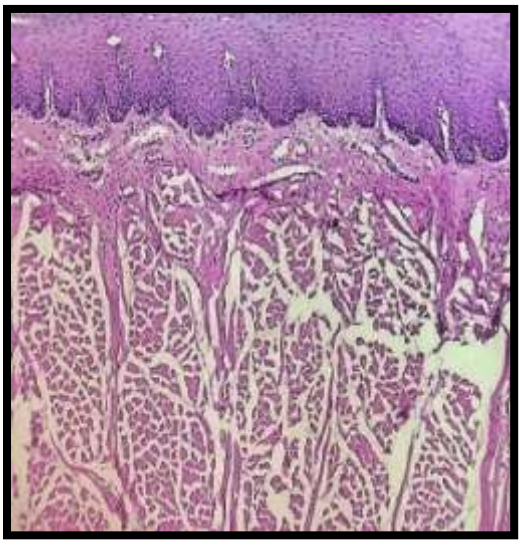


(e) 10X

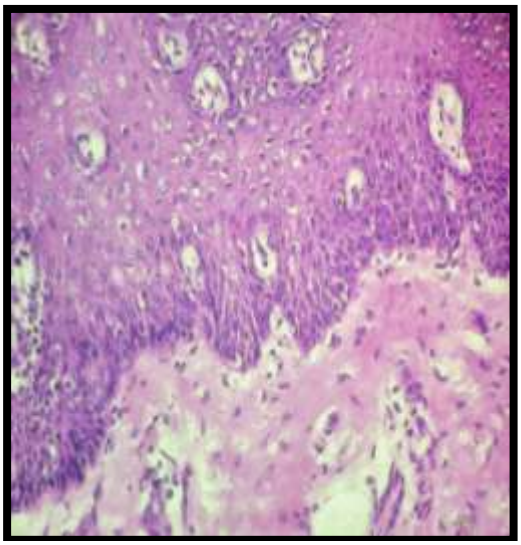


(f) 40X

Photomicrograph 3:Showing H &E stained sections under magnification (e) 10 X & (f) 40 X for 12 hours duration using Formalin as a fixative



(g) 10X



(h) 40X

Photomicrograph 4 :Showing H & E stained sections under magnification 10 X (g) & 40 X(h) for 24 hours duration using Formalin

Results And Observations:-

BAR DIAGRAM

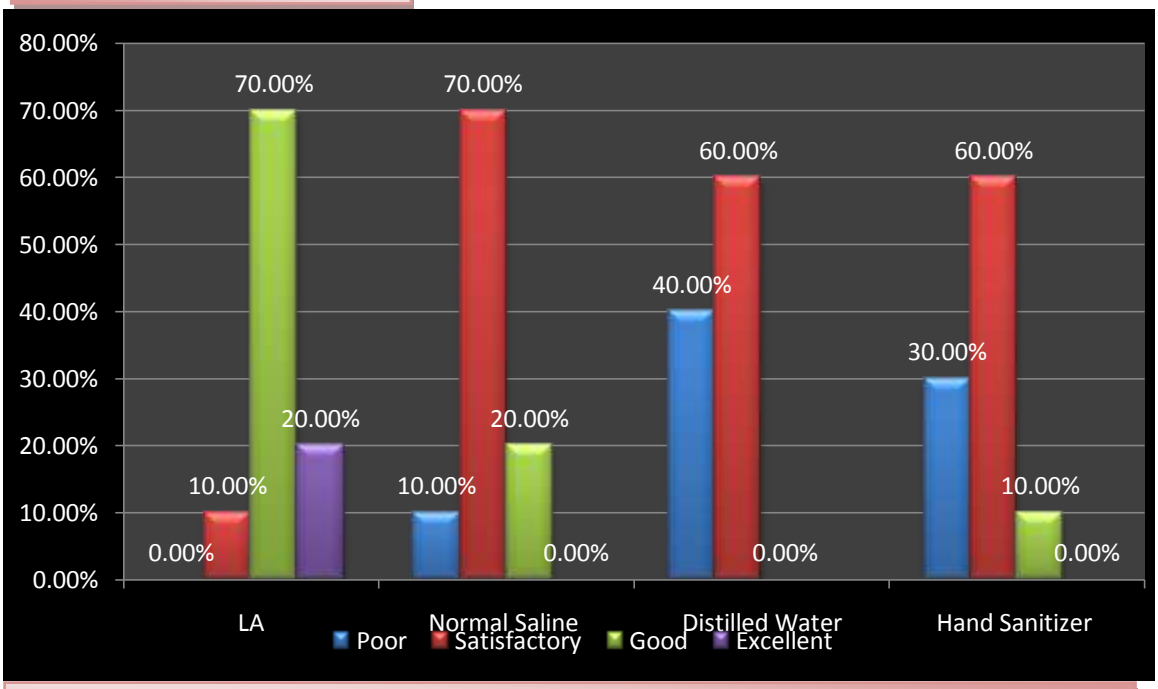


Figure 1 :- Showing the comparison of the Overall Morphology (12 hours)

BAR DIAGRAM

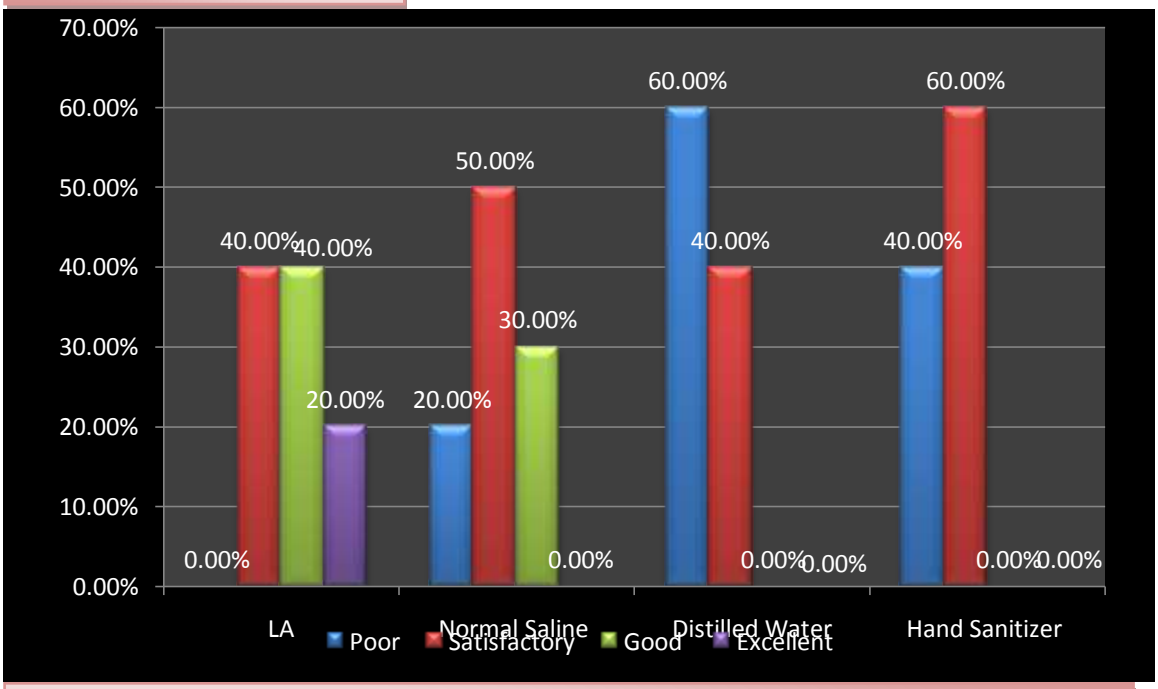


Figure 2 : -Showing the comparison of the Overall Morphology (24 hours)

BAR DIAGRAM

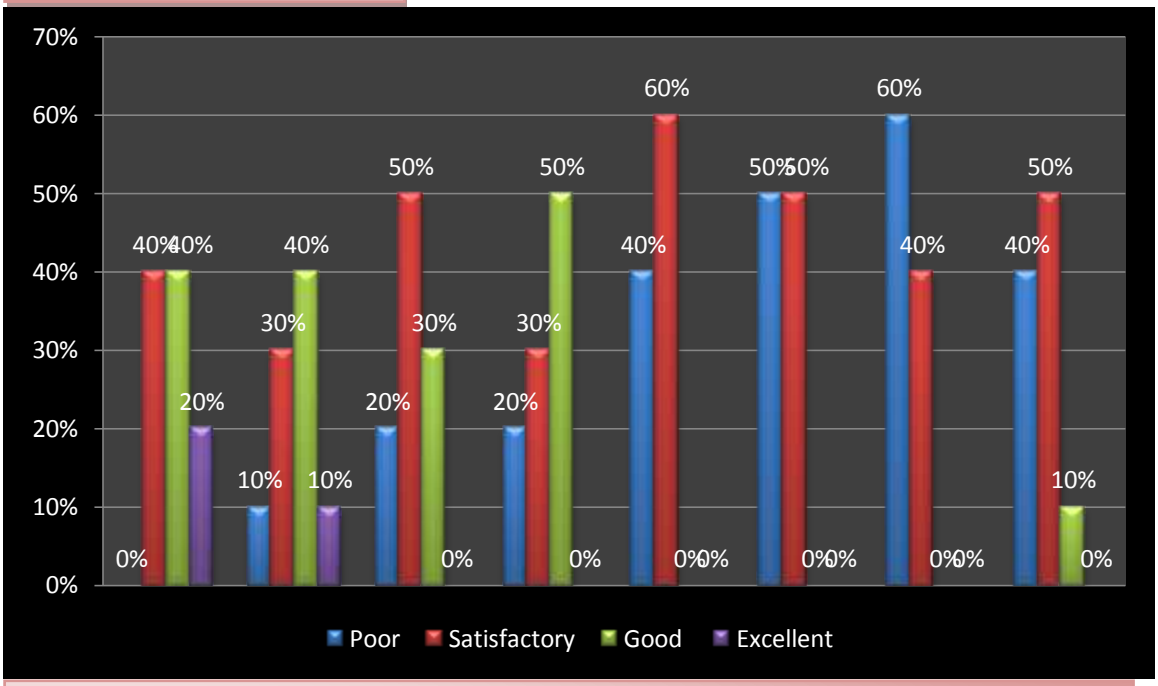


Figure 3 : -Showing the Inter Observer Variability (12 hours)

BAR DIAGRAM

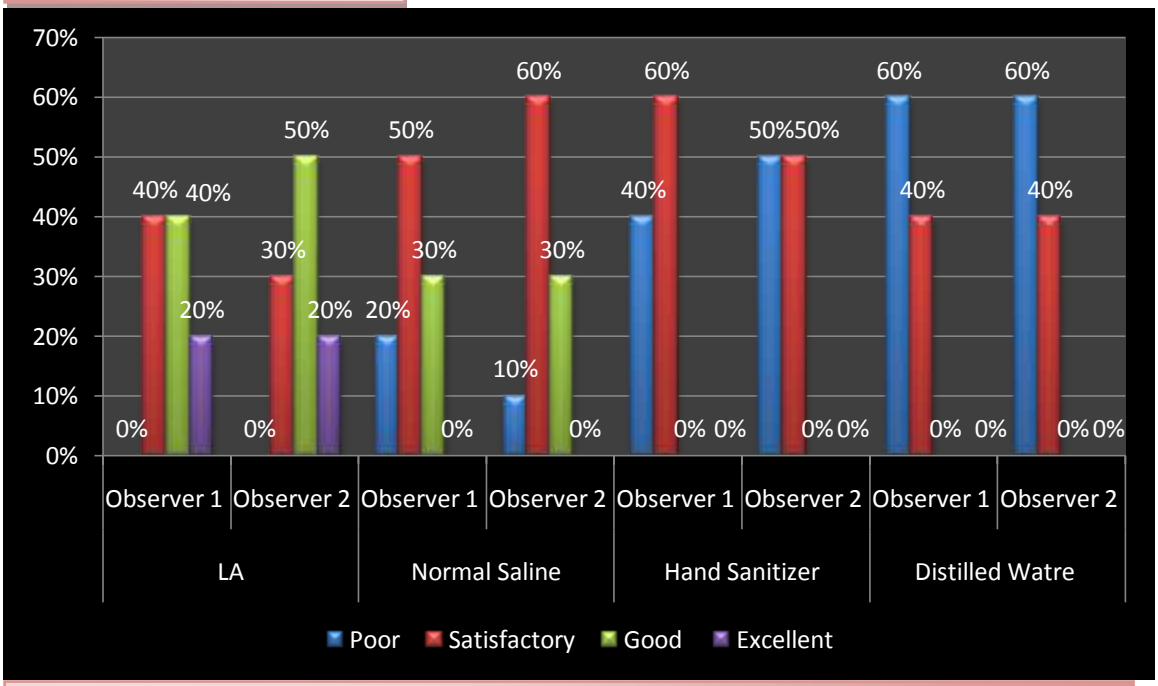


Figure 4 : - Showing the Inter Observer Variability (24 Hours)

Discussion:-

Foundation of all good microscopic preparations depends on the treatment of tissues as soon as it is removed from the body. The tissue should be immediately fixed in an appropriate fixative solution. If the tissue is unfixed or dried out, the valuable details will be missed.[54]

Bacterial contamination and the enzymes released cause tissue autolysis as a result of which the labile substances are lost.[55]

The major objective of fixation is to maintain clear and consistent morphological features and to minimize the loss of cellular components.[2]

In certain situations, there might be unavailability of formalin and valuable tissue specimens are discarded. In such situations the need arises to find an alternate solution that can be used for preserving tissues.[54]

Thus the concept behind the present study was to assess and compare the efficacy of alternative fixatives like the local anesthetic solution, normal saline, distilled water and hand sanitizer for 12 hour and 24 hour period by using H & E sections.

In this study the sample consisted of 100 tissue specimens of the goat tongue divided into two groups namely **Group A** and **Group B** (each having 50 specimens). Ten tissue samples each, were kept in respective fixatives i.e. local anesthetic solution, normal saline, distilled water and hand sanitizer for 12 and 24 hours.

The mechanisms by which fixatives act may be broadly classified into dehydrants, heat effects, cross linkers and effects of acids and combinations of these. Agents that combine with proteins are called additives and those that precipitate proteins are called coagulants. At this time it is accepted that no one fixative fulfils all of the aims of the cell or tissue preservation i.e. the prevention of autolysis and preservation of physical and chemical properties of the cells.[32]

A study was conducted by **G.L.Nicolson (1976)** which focussed on the interaction of the local anesthetics with cell membrane, membrane associated cytoskeletal organization and it's effects on the morphology of the cells.They concluded that the local anesthetics interacts with the membrane lipids and produces variety of effects which included altered osmotic fragility, inhibition of cell spreading, movement adhesion and fusion. It might raise intracellular calcium concentrations to levels greater than 10 M so as to induce microtubule depolymerisation. Local Anesthetics are considered to interact with membranes both by hydrophobic and electrostatic interactions in proximity with the anionic groups of acidic phospholipids which might be the possible explanation of it's mechanism of action.[9]

The mechanism of preservation of the molecular structure of cells by sodium chloride has not been described in the literature. It might be a sodium specific, chloride anion and hyperosmolarity effect. Hypertonic saline inhibits N – formyl –methionyl–leucyl–phenylalanine (fMLP) stimulated increases in intracellular calcium and shedding of receptors. Osmotic dehydration of the cells and the intercellular matrix also plays it's role. Sodium chloride penetrates the whole specimen and does not crystallize.[29]

The alcohol when used as a fixative causes protein denaturation through the removal of water from the free carboxyl, hydroxyl, amino, amido and imino groups of proteins which results in protein coagulation and tissue shrinkage.[41] Ethanol is considered to be a coagulant that denatures proteins. It replaces water in the tissue environment disrupting hydrophobic and hydrogen bonding thus exposing the internal hydrophobic groups of proteins and altering their tertiary structure and their solubility in water. Fixation commences at a concentration of 50-60%for ethanol. Ethanol is used to preserve glycogen but causes distortion of nuclear and cytoplasmic detail.[56]

Distilled water fails to form the cross links between proteins resulting in poor fixation and artefactual changes. The specimen undergoes autolysis rendering the tissues progressively undecipherable histologically.[27]

In an aqueous solution formaldehyde forms methylene hydrate, a methylene glycol as the first step in fixation. Methylene hydrate reacts with several side chains of proteins to form reactive hydroxymethyl side groups which happens to be the primary and characteristic reaction. The side chains of the peptides that are most reactive with

methylene hydrate and have the highest affinity for formaldehyde include lysine, cysteine, histidine, arginine, tyrosine and reactive hydroxyl groups of serine and threonine.[2]

Kasetty S. et al (2018) compared the efficacy of local anesthetic solution, distilled water and normal saline as emergency fixatives. The sections were evaluated for staining quality and subjected to statistical analysis.There was a significant difference **($p < 0.05$)** in the efficacy of all the three emergency fixatives and on the basis of the results obtained local anesthetic solution could be used as an emergency fixative. This is in accordance with the findings of our study.[9]

In a study by **Dhengar Y.S (2016)** in which the natural substitutes like sugar and jaggery syrup were compared with that of formalin and distilled water using H & E stain. The lowest mean value was obtained for tissue section fixed with distilled water.It showed significant cellular swelling and poor staining with H & E (autolysis).This finding is in accordance with our study in which tissues kept in distilled water caused considerable difficulty to reach a diagnosis.The nuclei in the tissues from distilled water were found to be overstained with hematoxylin stain.[57]

A study was conducted by **Titford M.E. and Horenstein M.G. (2005)** on histomorphologic assessment of formalin substitute fixatives. The commercially available fixatives contained ethanol, sodium chloride and zinc salts as their major components. He concluded that the overall cellular outline score for formalin was significantly higher as compared to the substitutes. Tissues soaked in sodium chloride containing fixative showed slight tearing of the blocks where dense connective tissue was present and some tissues needed additional soaking. These findings are in accordance with our study by highlighting that formalin substitutes containing sodium chloride do not preserve cellular details as well as formalin.[28]

A study by **Singhal P. et al (2016)** for the evaluation of histomorphometric changes in tissue architecture was done. The microscopic details of tissues kept in different carrying media at different time intervals were assessed followed by standard fixation. According to this study Normal Saline is more suitable than the Local Anesthetic as a carrying media which is contrast with our study.[4]

A study by **Singhal P. et al (2016)** for the evaluation of histomorphometric changes in tissue architecture. The sectioning ability, staining intensity and microscopic details of tissues kept in different carrying media at different time intervals were assessed followed by standard fixation. According to this study Normal Saline is more efficient than Local Anesthetic as a carrying media which is contradictory to our findings.[4]

In a study by **Kiran Qidwai et al (2014)** it was put forth that if tissues are put in ethanol it causes cell shrinkage and will make the tissue brittle. This is in accordance with our study.[1]

In our study, vacuolization was evident in almost all the epithelial layers in the tissues obtained from Normal Saline. Our study is in accordance with the study carried out by **Sengupta S. et al (2013)** who put forth various artefacts produced by Normal Saline when used as a holding solution for biopsy tissues in transit. Various regressive alterations in the epithelium and connective tissue were noted after keeping the specimens in normal saline.[11]

A comparative study was done by **Chittemseti S. et al (2018)** to find the natural substitutes for formalin and jaggery and khandsari.It was found that with respect to the cellular outline, cytoplasmic staining, nuclear details, staining quality and overall morphology formalin gave the best results. This study is in accordance with our study.[5]

A comparative study conducted by **Rajanikanth M.et al (2015)** among formalin, LA, hydrogen peroxide, honey, coconut oil, rose water, coconut milk, milk, betadine, ice cold water, saline and spirit gave ideal results for 10% formalin which served as the positive control for their study.This study is in accordance with our study which gave similar results.[54]

The Inter observer Variability for **Group A** came out to be Non Significant in all the four alternate fixatives i.e. for Local Anesthetic solution p value = 0.657, for Normal Saline p value = 0.769, for the Hand sanitizer p value = 0.584 and for the distilled water p value =0.485.The Inter observer Variability for **Group B** came out to be Non Significant in all the four alternate fixatives i.e for Local Anesthetic solution p value =0.857, for Normal Saline p value = 0.816, for the Hand sanitizer p value =0.912 and for the distilled water p value =1.000 .

In our study it was concluded that the result of local anesthetic solution with adrenaline as an alternate fixative was statistically significant among the four alternative fixatives which included normal saline, hand sanitizer and distilled water.

The study was conducted considering the toxicit and non availability of formalin. An attempt was made to find the alternative fixatives apart from formalin which is best suited.

Studies comprising of larger sample sizes with addition of other substitutes alternative to formalin is recommended for confirmatory validity analysis.

Summary And Conclusion:-

Fixation is the initial and an important step in tissue fixation. Despite being the gold standard on account of it's effectiveness, low cost and consistent results, Formalin has it's own toxic effects. The possible routes of exposure to formaldehyde are ingestion, inhalation and dermal absorption. Repeated exposure to formaldehyde in occupational settings is a causative irritant of the mucous membrane of the eyes, nose, mouth and upper respiratory tract which has potential health hazards.Considering the serious adverse effects of Formalin various attempts have been made to replace this agent with safer alternatives.

This study was designed to establish whether the usage of local anesthetic solution, hand sanitizer, normal saline and distilled water as a fixative had any effect on the nuclear morphology, cytoplasmic morphology and the overall morphology.The aim of this study was to assess and compare the efficacy of alternative fixatives like local anesthetic solution, normal saline, distilled water and hand sanitizer by using the H & E stained sections.

Tissues fixed in local anesthetic solution showed comparable morphologic features when compared to the tissues fixed in formaldehyde. The nuclear morphology for the tissues fixed in Local Anesthetic solution could be diagnosed efficiently. The tissues in Normal Saline, Hand Sanitizer and Distilled Water posed problems pertaining to nuclear staining, staining quality, cellular outline, tissue artifacts and the ease of sectioning.

In conclusion the data suggests that the specimens in Local Anesthetic solution show best results among all alternative fixatives and can be used as alternate fixative in emergency condition .In spite of the extensive use of formalin in lab, it's not routinely kept in clinics owing to it's undesirable effects which include it's carcinogenic and irritating potential. The risk of formaldehyde as a chemical carcinogen must not be underestimated. The present study evaluated the efficacy of tissue fixation with other regularly found agents in the clinic that is the local anesthetic solution, normal saline, distilled water and hand sanitizer as alternate fixatives.

To date no ideal fixative has been found that is a fixative that perfectly preserves cellular morphology and yet does not modify the specimen composition. Because of this issue the selection of a particular fixative warrants multiple and careful considerations.

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