Artefacts Produced by Normal Saline When Used as a Holding Solution for Biopsy Tissues in Transit

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Abstract Oral surgeons, surgeons and dentists carrying out small biopsy procedures on an out-patient basis have been frequently sending poorly stored biopsy tissues to the Departments of Oral Pathology of various dental colleges, wherein the tissues were being stored in normal saline and then transferred to formalin. Such tissues often show abnormal cellular and architectural changes, which pose a challenge to diagnosis, especially when the histopathologist is not aware that the tissue was stored in normal saline for some time. We wanted to know the extent of artefactually induced cellular and architectural changes in tissues that were transported in normal saline and received by us, based on the duration for which they were held in normal saline. Normal oral soft tissue was subjected to storage in normal saline for periods varying from 1 to 36 h, before being fixed in 10 % formalin. Soft tissue specimens were

collected during routine surgical extractions and each was cut into six parts. One was transferred immediately to formalin as control and other bits kept in normal saline for intervals of 1, 3, 12, 24, and 36 h before fixing in formalin. A comparative photographic analysis was carried out to analyze the histopathological changes. The tissues kept in normal saline before fixation underwent various changes. In no way should normal saline be used, even for a short time, to store and transport excised tissue; it is imperative that such tissues be placed immediately in a proper fixative like 10 % formalin.

Keywords Fixation · Normal saline · Formalin

Introduction

The objective of fixation is to preserve cells and tissues in as close to a life-like state as possible. It allows them to undergo further preparative procedures without 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. Therefore, it is regarded the first step and the foundation in a sequence of events that culminates in the final examination of a tissue section [1].

Fixatives have the property of forming cross-links between proteins, thereby forming a gel. Soluble proteins are fixed to structural proteins and thus rendered insoluble. The whole structure gains mechanical strength which permits the subsequent manoeuvres to take place [2]. Majority of lipids are labile and contain phosphatidyl ethanolamine, which is fixed by aldehyde [2]. In tissue fixed in formaldehyde-calcium, a combined effect of the mildly

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acidic milieu and reaction with free amino acids of phospholipids may be presumed [3].

Formaldehyde initiates DNA denaturation at regions of double stranded DNA and created sites for various chemical interactions (addition, electrophilic, hydrolysis), however fixation of RNA occur by the formation of compound N-methylol followed by an electrophilic attack to form a methylene bridge between amino groups [4].

It is our observation that often operating surgeons, oral surgeons and dentists do not have the required fixatives available to them in their surgery, and a common practice followed is to initially keep the removed tissue in normal saline, before transferring it to a fixative later. Such a practice is more prevalent in semi-urban and rural areas due to lack of awareness and/or lack of ready availability of fixatives. This article proposes to not only study the different levels of cellular damage caused due to usage of normal saline as an intermediate transport medium for varying time periods before transferring tissues into a recommended fixative, but also highlights the fact that an erroneous histopathological diagnosis can be made based on the histopathologist not being informed that the tissue is question has been immersed in normal saline for even 1 h before being transferred to a fixative.

Material and Methods

We used normal oral mucosa, which was removed during surgical extraction of non-inflamed impacted third molars after taking informed consent from the patient. Five such specimens were collected and each specimen was cut into six parts. Each of the 6 parts was labeled as F₀, F₁, F₃, F₁₂, F₂₄ and F₃₆. One part (F₀) was immediately placed in 10 % neutral buffered formalin, as control; the other parts were transferred to 10 % neutral buffered formalin after being kept in commercially available normal saline for a period of 1, 3, 12, 24, 36 h respectively.

All the specimens were processed for routine histopathology and 4 micrometer thin sections were cut. Staining was done using Harris' Hematoxylin and Eosin. A photographic analysis between these tissues and their normally fixed control was carried out.

Results

All the tissue bits taken as control exhibited stratified squamous epithelium with stratum basale revealing low columnar to cuboidal cells and stratum spinosum with polyhedral cells. The desmosomal junctions were appreciable intercellularly and the integrity of cells was well-maintained.

Table 1 Regressive alterations in the epithelium after keeping the specimens in normal saline

Duration in normal saline (h)	Epithelial changes
1	Cell vacuolization in basal layer
3	Widespread vacuolization
12	Loss of cellular integrity
24	Non-vital cells without nuclei
36	Total lysis of cellular architecture

All the tissue bits which were kept in normal saline for any length of time, showed various regressive alterations in the epithelium and connective tissue, when compared to controls (Table 1).

Epithelial Changes

Tissue bit kept in normal saline for 1 h showed cell vacuolization in the basal cell layer, mimicking mild dysplasia or the basilar degeneration observed in lichen planus. After passage of 3 h, further degradation of tissue could be seen as widespread vacuolization within the spinous cells, producing a false appearance of leukoedema. At 12 h interval, there was loss of cellular integrity. Non-vital, anucleated cells could be seen in tissue that had been left for 24 h in normal saline. After 36 h of being left in the normal saline solution, total lysis of cellular architecture had taken place rendering the tissue non-diagnostic. (Table 1).

Connective Tissue Changes

The connective tissue showed less dramatic changes but it progressively changed its normal architecture. After holding for just 1 h in normal saline before transferring to formalin, the connective tissue fibres appeared to be less cohesive. Further separation of collagen fibres could be appreciated after 3 h; after 12 h or more, the connective tissue appeared hyaline and acellular, with the collagen fibres being completely lost later on. (Table 2).

Discussion

Tissue necrosis needs to be prevented for histological examination. Bacterial contamination leads to putrefaction of tissues, enzymes released from within the tissues cause autolysis, and the labile substances are destroyed. The prime aim of fixation is to prevent or arrest these changes [5].

Any substance that aims to preserve cells and tissues in as close to a life-like state as possible is called a fixative. It also preserves tissue substances and proteins. Therefore, it is regarded as the first in the series of tissue processing [6].

Table 2 Regressive alterations in the connective tissue after keeping the specimens in normal saline

Duration in normal saline (h)	Connective tissue changes
1	Reduced cohesiveness of collagen fibres
3	Visible separation of collagen fibres
12	Loss of cellular integrity; reduction in number of fibres
24	Hyalin-like acellular areas appear
36	Total lysis of cellular architecture

The standard solution used is 10 % neutral buffered formalin, where the purpose of the buffer is to prevent a drop in the pH that can lead to enhanced autolysis [7]. The pH of the fixative buffer dramatically influences the degree of cross-linking. Being amphoteric, amino acids are influenced by the pH. They are charged at a lower pH and uncharged at a higher pH. When using neutral buffered formalin, the pH is shifted to neutrality, which causes release of hydrogen ions from the charged amino acids. These uncharged groups contain reactive hydrogen that can react with formalin to form additional cross-links. Hence, the use of 10 % buffered formalin tends to produce more cross-links than non-buffered formalin [8]. Other types of fixatives used are coagulative fixatives that precipitate proteins by breaking hydrogen bonds in the absence of protein cross-linking, e.g. ethanol. Most proteins in the body contain hydrophilic and hydrophobic moieties. While using ethanol, water is being removed and this causes destabilization of the proteins by affecting the hydrogen bonding. Thus, the tertiary structure of protein is precipitated [8].

It must be noted that fixation by itself introduces a major artefact. The foremost concern with formaldehyde is the amount of distortion produced in the tissue in the form of shrinkage [9].

Al-Hubaity and Al-Saraj [10] had carried out work on saturated table salt and suggested that it could be used in place of formalin to preserve bodies [10]. Al-Saraj [1] worked on saturated sodium chloride solution and concluded that it can be used as a fixative in histopathological procedures. The results obtained were same as that obtained by using conventional formaldehyde. An added advantage of saturated sodium chloride was that it did not cause any distortion of the cell [11]. In contrast, our study used normal saline and found various architectural alterations in the tissues. Saturation is the point at which a solution of a substance can dissolve no more of that substance and additional amounts of it will appear as a separate phase. Normal saline is the solution of 0.09 % (w/v) of NaCl, about 300 mOsm/L or 9.0 g NaCl per liter of water. This suggests that the deleterious effects could be the result

of water present in normal saline as against the saturated sodium chloride solution.

Olszewski et al. [12] experimented on preserving human skin fragments in anhydric, pulverized sodium chloride for months before successfully transplanting them. 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 the N-formyl-methionyl-phenylalanine (f MLP) stimulated increase in intracellular calcium and shedding of receptors [12].

Gewartowska and Olszewski [13] suggested that dehydration in anhydric NaCl may be considered to be a novel approach to preservation. Osmotic dehydration by sodium could be a possible mechanism involved.

On the contrary, the present study failed to demonstrate the viability of using a normal solution of sodium chloride even as a holding solution for very short durations.

Titford and Horenstein [14] conducted a study on histomorphologic assessment of formalin substitute fixatives. The commercially available fixatives (Glyo-Fixx, Omnifix II, Histofix, Histochoice) 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. These findings support our study by highlighting that sodium chloride substitutes do not preserve cellular details as well as formalin. This could also be attributed to the habituation of pathologists to observing the changes induced by formalin [14].

Conflict of interest Nil

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