Spotlight on Koilocytes-Determination of Best Fixative for Koilocytes

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Abstract

Aim and objective: To evaluate the better choice for fixation of Koilocytes. **Materials and methods:** Tissue samples will be taken from 30 patients that have been diagnosed clinically and histopathologically as oral squamous cell carcinoma, for the purpose of the study during excisional biopsy. These tissue samples were taken and fixed in the different fixatives (formaldehyde, glutaraldehyde and osmium tetra oxide) according to their respective protocols and subjected to routine hematoxylin and eosin staining. The presence of koilocyte will then be evaluated in tissue sections that have been fixed in these mentioned fixatives**. Results:** It was found that the finest demonstration of koilocytes was possible with Osmium tetroxide,when compared with Formaldehyde & Gluteraldehyde. **Conclusion:** Osmium tetroxide was the best fixative for the demonstration of the koilocyte. It demonstrates koilocytes with a sharp and well defined perinuclear halo and distinct nuclei. There is minimum background staining. Gluteraldehyde fixes the tissue more rapidly as compared to Formaldehyde, but the features of koilocytes are not as clear as those seen in tissues fixed in Formaldehyde and Osmium tetroxide.

Key Words: Koilocytes, Fixation, Formaldehyde, Gluteraldehyde, Osmium tetroxide, OSCC

Introduction

Koilocytes are defined as squamous cells, predominantly superficial and intermediate cells, with a large, well-demarcated, clear perinuclear zone surrounded by a dense peripheral cytoplasmic rim. Basically koilocyte is a squamous epithelial cell that has undergone a number of structural changes, which occur as a result of infection of the cell by human papillomavirus as it is the most reliable morphological evidence of human papilloma virus (HPV) infection. In 1951 a Canadian cytologist **Ernest Ayre** described and demonstrated squamous epithelial cell with a 'perinuclear halo' in smears from the uterine cervix [1].These 'halo cells' were described as mononucleated or binucleated squamous cells with hyperchromatic nuclei and peri-nuclear clearing**. Koss** and **Durfee**, in 1956, renamed these cells as "koilocytes", from the Greek word "koilos" meaning "hollow cell" [1]. Later it was established that koilocytes are actually virus-infected squamous epithelial cells and the virus found in the nuclei of koilocytes were consistent with HPV. Koilocytosis or koilocytic atypia are terms used in histology and cytology to describe the presence of koilocytes in a specimen [1].

Oral squamous cell carcinomas (OSCCs) are reflected by multiphasic and multifactorial etiopathogenesis. Tobacco and alcohol are the most common risk factors for oral malignancy. Other factors, including DNA viruses, for example Human papilloma virus (HPV) have been found to play an important role in the initiation or development of these lesions [2,3]. HPV involvement in OSCC was first proposed in 1983 by Syrjanen et al. and then supported by others on basis of the fact that HPV shows epitheliotropism and has the ability to immortalize human oral keratinocytes in vitro [4]. The most recognized, pathognomic feature of human papillomavirus (HPV) infection is the attendance of halo or koilocytotic cells in the differentiated layers of the squamous epithelium. These koilocytes are squamous epithelial cells that grasp an acentric, hyperchromatic nucleus that is moved or displaced by a large perinuclear vacuole [5,6]. However, the formation of the cytoplasmic vacuole has remained unclear, particularly because both HPV-DNA replication and virion assembly occur exclusively within the nucleus. In clinical biopsies, koilocytosis is observed in both low risk and high-risk HPV infections; and had been demonstrated that the E5 and E6 proteins from both low risk- and high-risk HPVs cooperate to induce koilocyte formation in human cervical cells in vitro, using both stable and transient assays [5]. The presence of koilocytes in PAP smears and cervical biopsies remains fundamental to pathological diagnosis [5,7]. In HPV infections, koilocytosis is only observed in the uppermost, well-differentiated layers of stratified squamous epithelium and it is in these layers (and specifically in koilocytes) that the viral capsid proteins are expressed and assembled into infectious virions.

The foundation of all good tissue specimen preparations is complete fixation. The primary function of tissue fixation is to prevent putrefaction and autolysis. Faults in fixation cannot be remedied at any later stage, and the finished product can only be as good as its initial fixation [8]. Function of an ideal fixative is:

1. To set organs or parts of organs so that microanatomical arrangement of tissue elements will not be altered.
2. To set intracellular inclusion bodies so that the histocytologic and cytologic conditions of cells may be studied.
3. To arrest autolysis, putrefaction, and other changes.
4. To bring out differences in refractive index of tissues.
5. To render cell constituents insoluble and make them resistant to subsequent processes

Fixation of tissues is the most crucial step in the preparation of tissue for observation in the microscopy. Fixation consists of two steps:

a. *Cessation of normal life functions in the tissue (killing)*
b. *Stabilization of the structure of the tissue (preservation).*

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The goal of fixation is to preserve structure as faithfully as possible compared to the living state. Aldehydes are the most commonly used fixatives. They serve to stabilize the fine structural details of cells and tissues prior to examination by light or electron microscopy [8].

Research workers, technicians, pathologists and others who regularly use aldehyde fixatives frequently do not appreciate the nature and properties of these compounds or the reasons for choosing to fix a specimen in formaldehyde, glutaraldehyde or a mixture of the two. Misconceptions are widespread also about formalin and paraformaldehyde, the commercial products from which formaldehyde-containing solutions are made [9]. Formaldehyde has small molecules: HCHO, of which the -CHO is the aldehyde group that can combine with nitrogen atoms of proteins or with two such N atoms if they are very close together, forming a crosslink -CH2- called a methylene bridge [9]. Glutaraldehyde has fairly small molecules, each with two aldehyde groups, separated by a flexible chain of 3 methylene bridges. It is HCO-$(CH_2)_3$-CHO. The potential for cross-linking is obviously much greater because this can occur through both the -CHO groups and over variable distances. In aqueous solutions, glutaraldehyde is present largely as polymers of variable size. There is a free aldehyde group sticking out of the side of each unit of the polymer molecule, as well as one at each end. All these -CHO groups will combine with protein nitrogen with which they come into contact, so there is enormous potential for cross-linking, and that is just what you get [9]. Osmium tetroxide has an important, if limited, part in fixation for light microscopy as it is the only fixing agent known which completely and easily fixes fats. It is generally believed that fixation of cell membranes by osmium tetroxide (OsO_4) involves unsaturated lipids [10-12]. There is less certainty as to the involvement of protein [10,13,14], although the importance of OsO_4-protein interaction has long been recognized [15]. There is evidence that OSO_4 has a fixative or cross-linking function on tissue protein [16] and that protein-lipid cross-linking may occur [17,18].

Materials and Method

The study was carried out at *Sharad Pawar Dental College, Sawangi, Wardha* in the Department of Oral and Maxillofacial Pathology. **30** samples were selected who had been diagnosed clinically and histologically with OSCC. Patient's consent as well approval from university ethical committee was taken prior to the conduction of the study. These samples had been procured during the curative surgical procedure for frozen section. After which each tissue sample was cut in to 3 parts with very adjacent margins (so that each separate part will be evaluated for the same field under the microscope) and then kept in different fixatives as are *4% Formaldehyde, 2.5% Gluteraldehyde and 1% Osmium tetroxide* respectively, with their prescribed time period (i.e. *24 hours for formaldehyde; 6 hours for Gluteraldehyde; 2 hours for Osmium tetroxide*) as mentioned in literature. After which the fixed tissue were embedded in to paraffin wax and then cut in to sections and labelled. After completing the whole sets of tissue of 30 samples, the cut tissue section in the slides were stained together in normal Hematoxylin and Eosin stain and then evaluated by three observers for the appearances of koilocytes under light microscope.

Results

30 Samples which were sectioned in to 3 parts and blindly evaluated under light microscope, following results were obtained.When evaluated for the staining characteristics the **Formaldehyde fixed tissue** shows the nuclear detail and cytoplasmic details but not clearly demarcated *(Figure 1)*. The **Gluteraldehyde fixed tissue** shows the results for the same criteria (nuclear detail and cytoplasmic details) were found to be little obscure as compare to that of the previous *(Figure 2)*. The tissue fixed in **Osmium tetroxide** when evaluated gives the result far clearer and sharply delineated as compare to both formaldehyde and Gluteraldehyde *(Figure 3)*. *Thus i*t was found that the finest demonstration of koilocytes was possible with Osmium tetroxide, when compared with Formaldehyde & Gluteraldehyde.

Discussion

Fixation is the single most influential factor in the long sequence of steps between procurement of the specimen and cover-slipping the stained slide; nearly any other step can be reversed to ameliorate a problem [19]. Different fixatives produce their own morphological patterns. That is an objective fact that does not imply good or bad. Whether we like what we see is a subjective matter predominantly based on our individual training. Many chemicals act as fixatives in that

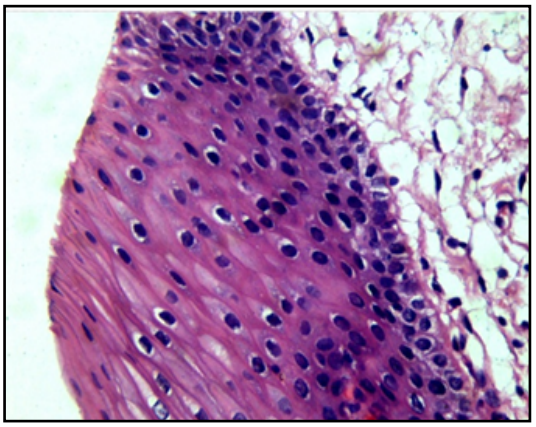

Figure 1. *Foramline fixed H&E stained tissue section in 40X magnification.*

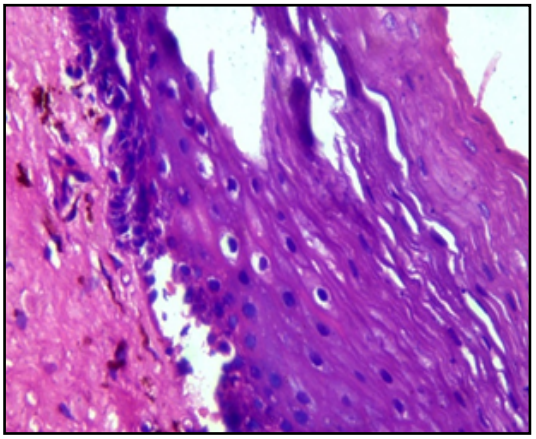

Figure 2. *Gluteraldehyde fixed H&E stained tissue section in 40X magnification.*

they denature macromolecules, but few produce "acceptable" results because each creates its own unique pattern of changes visible at the level of the light microscope.

Some observers give high preference to mercuric fixatives over neutral buffered formalin (NBF) for lymphoid tissues, and picric acid for gastric biopsies, because of the extra-sharp images they produce. Defining "good" fixation, then, is difficult because of varying personal preferences. However, there are well-documented and accepted minimum staining criteria that specify well-defined nuclear patterns, epithelial cell membranes, and cytoplasmic staining exhibited by well-fixed tissues [19].

Aldehydes are the most commonly used fixatives. They serve to stabilize the fine structural details of cells and tissues prior to examination by light or electron microscopy. **Formaldehyde** is a gas. Its small molecules (HCHO, of which the -CHO is the aldehyde group) dissolve rapidly in water, with which they combine chemically to form methylene hydrate, HO-CH2-OH. This is the form in which formaldehyde exists in aqueous solutions; its chemicalreactivity is the same as that of formaldehyde. Methylene hydrate molecules react with one another, combining to form polymers *(Figure 4)* [9]**.**

The liquid known as formalin contains 37-40% of formaldehyde and 60-63% of water (by weight), with most of the formaldehyde existing as low polymers (*n*=2 to 8 in the formula given in *Figure 4*). Higher polymers (*n* up to 100), which are insoluble, are sold as a white powder, paraformaldehyde [9].

Reaction of formaldehyde with proteins

The aldehyde group can combine with nitrogen and some other atoms of proteins, forming a cross-link -CH_2- called a *methylene bridge.* Studies of the chemistry of *tanning* indicate that the most frequent type of cross-link formed by formaldehyde in collagen is between the nitrogen atom at the end of the side-chain of lysine and the nitrogen atom of a peptide linkage *(Figure 5)*, and the number of such cross-links increases with time (*Gustavson, 1956*). Fixative action of formaldehyde is probably due entirely to its reactions with proteins. Initial binding of formaldehyde to protein is largely completed in 24 hours (*Helander, 1994*) but the formation of methylene bridges proceeds much more slowly. Substances such as carbohydrates, lipids and nucleic acids are trapped in a matrix of insolubilized and cross-linked protein molecules but are not chemically changed by formaldehyde unless fixation is prolonged for several weeks [9].

Gluteraldehyde has fairly small molecules, each with two aldehyde groups, separated by a flexible chain of 3 methylene bridges. It is HCO-$(CH_2)_3$-CHO. The potential for cross-linking is obviously much greater than with formaldehyde because it can occur through both the -CHO groups and over variable distances. In aqueous solutions, glutaraldehyde is present largely as polymers of variable size *(Monsanet al., 1975)*. There is a free aldehyde group sticking out of the side of each unit of the polymer molecule *(Figure 6)*, as well as one at each end. All these -CHO groups will combine with any protein nitrogen with which they come into contact, so there is enormous potential for cross-linking *(Figure 7).*

Reaction of glutaraldehyde with proteins

Fixation of tissue is more efficient with glutaraldehyde than with formaldehyde and relies on its cross-linking properties. For reproducible results, highly purified glutaraldehyde is

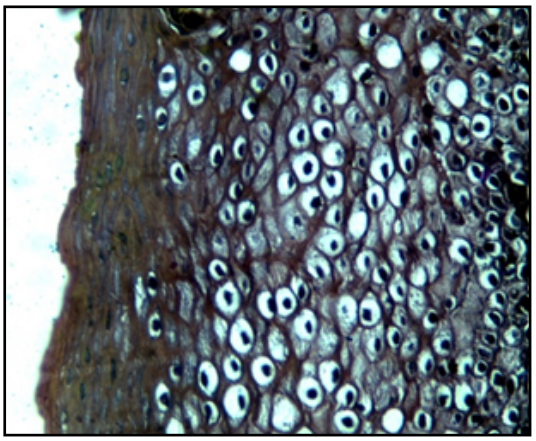

Figure 3. *Osmium tetroxide-fixed H&E stained tissue section in 40X magnification.*

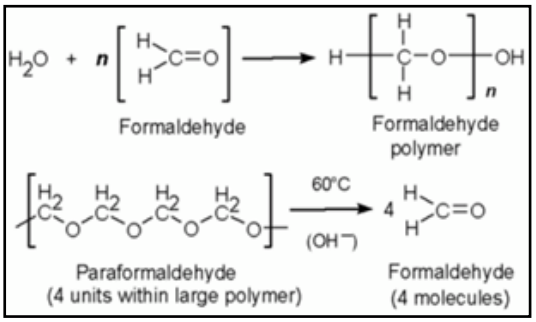

Figure 4. *Formation of formaldehyde polymers(above), and depolymerization of paraformaldehyde (below).*

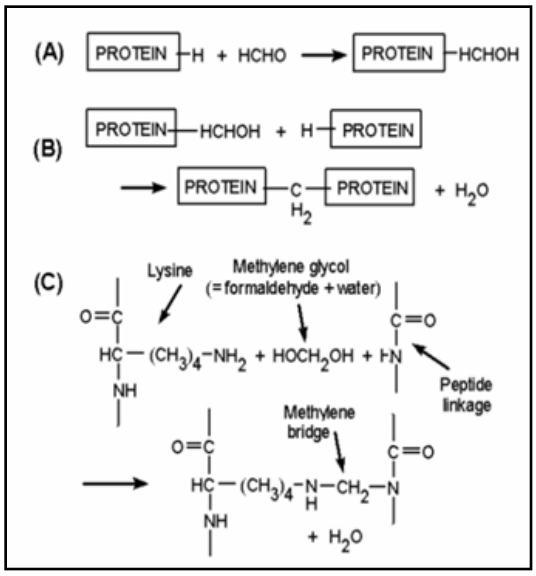

Figure 5. *Reactions involved infixation by formaldehyde, (A) Addition of a formaldehyde molecule to a protein. (B) Reaction of bound formaldehyde with another protein molecule to form a methylene cross-link. (C)A more detailed depiction of the cross-linking of a lysine side-chain to a peptide nitrogen atom.*

needed [20]. The chemical reaction of glutaraldehyde with protein is fast (minutes to hours), but the largermolecules, especially the oligomers, penetrate tissue slowly [9]. Objects fixed for a few hours in glutaraldehyde are no longer osmotically responsive *(Paljarviet al., 1979)*. Both aldehyde groups of a single glutaraldehyde molecule react with proteins to form cross-linkage. Glutaraldehyde can also react with phospholipids containing free amino groups (e.g. phosphatidylserine, phosphatidylethanolamine).

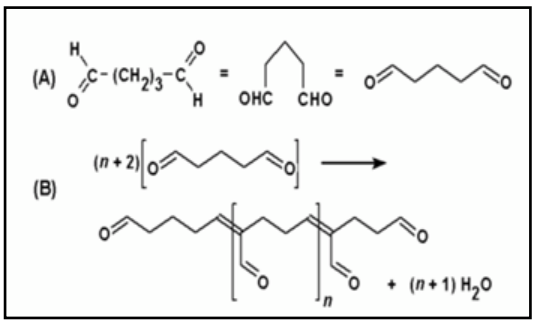

Figure 6. *(A) Three representations of a molecule of monomeric glutaraldehyde. (B) Polymerization reaction of glutaraldehyde, showing an aldehyde side-chain on each unit of the polymer.*

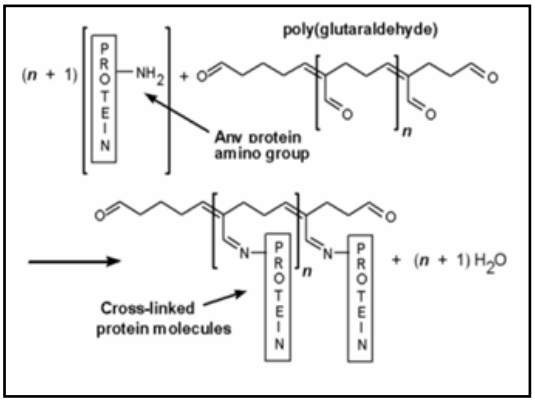

Figure 7. *Reaction of Poly (glutaraldehyde) with amino groups of proteins.*

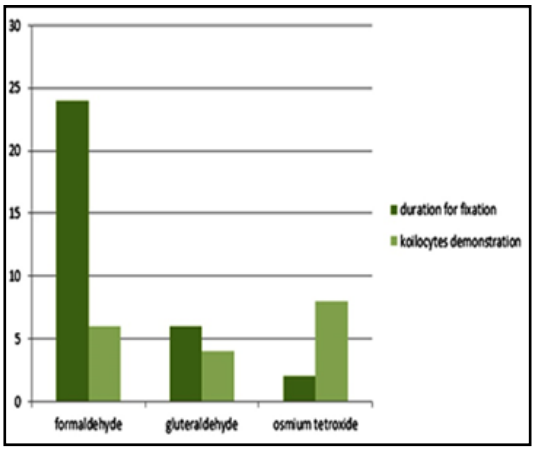

Figure 8. *Graph for time taken for fixation of tissue by respected fixative (in Hrs) and demonstration of koilocytes by them {graded from value 0-10}.*

Glutaraldehyde introduces free aldehyde groups to the fixed tissue [9] *(Figure 7)*. The stabilizing effect of glutaraldehyde is attributed to rapid and persistent intra- und inter-molecular cross-linkages of tissue components. Glutaraldehyde is believed to react by a similar mechanism to formaldehyde. Glutaraldehyde, however, will give a more tightly linked product: its greater length as compared with formaldehyde and its two aldehyde groups allow glutaraldehyde to link more distant pairs of protein molecules as formaldehyde. This type reaction is a factor which makes glutaraldehyde an efficient fixative. The degree of cross-linking is progressive with time, and depends on the accessibility of ε-amino groups by aldehyde groups which leads to the formation of Schiff bases [21].

Osmium tetroxide has come to take a predominant place as a fixative for studies of biological materials by electron microscopy. It is considered as the most suitable substance for preservation of the whole of the cellular constituents [21]. Osmium tetroxide is mostly used in buffered solutions, because it has been suggested that the quality of fixation by osmium tetroxide is largely dependent on the concentration of hydrogen ions. It is believed that when tissues are fixed in an unbuffered solution of osmium tetroxide, a wave of acidity precedes the osmium into the tissue, and that the structure of cells is best stabilized if the fixing solution is buffered slightly on the alkaline side of neutrality *(Palade, 1952)* [21]. The value of osmium tetroxide for the preservation of cell structure has been already reported in 1927 *(Strangeways TSP and Canti RG, 1927)*. With the development of electron microscopy in the early 1950s, it became apparent that osmium tetroxide was by far the best choice for the study of fine structure as compared with the then employed fixatives for light microscopy *(Palade GE, 1952)*. The main disadvantage of osmium tetroxide is its slow penetrability into tissue blocks. Furthermore, the ionic constitution of the buffer influences the cellular fine structure *(Trump BF and Ericsson JLE, 1965)* as well as the rate of osmium penetration. Today, osmium tetroxide has still its important role as secondary fixative in electron microscopy [20].

Reaction of osmium tetroxide with proteins

Previously reported *in vitro* studies [10,22,23] on the free amino acids found in membrane proteins show that they react rapidly with OsO_4 to give black, un-characterizable solids. This reaction, however, is simply due to the presence of the easily reduced α-amino groups (carboxyl groups are relatively inert to OsO_4); for, if they are blocked to give linkages there is no such reaction [10]. By osmium tetroxide the investigation of the fine structure of biological material by means of the electron microscope has depended, to a very large extent, on the deposition of osmium compounds within the tissue during treatment with a buffered osmium tetroxide solution, and the value of such treatment in the fixing of structure and enhancement of contrast is well accepted [24]. The results indicate that nearly all of the osmium reaction occurs with lipid rather than protein. As the percentage protein in the molecule increased, the osmium uptake decreased, and the pure proteins used bound very little osmium. The many observations that OsO_4 does not stain saturated lipids would seem to indicate that the fatty acid double bond is the most likely reaction site. However, in the investigations of *Bahr*, the protein components of tissue were thought to bind osmium as readily as did lipid. It is possible that the physical state of the biological material is of importance here and that tissue lipids and proteins in bulk behave differently from those in macromolecular form [25-28].

Amongst the three fixative on plotting graph for time taken for fixation of tissue by respected fixative *(in hrs)* and demonstration of koilocytes by them. (Scored [0-10] from the mean value obtained from different observers who blindly grades the appearances of koilocytes on the basis of sharpness and contrast of koilocytes in the epithelium of OSCC samples) *(Figure 8)*.

Conclusion

With the aim of 'evaluation of best fixative for koilocytes demonstration', in samples of OSCC we found that

osmium tetroxide consuming minimum time for fixation and demonstrate koilocytes with a clear and sharp image, followed by formaldehyde and then by glutaraldehyde. The possible mechanism behind this result could be that osmium tetroxide readily reacts with lipids which are the chief components of cell membrane of epithelial cells and so does of koilocytes along with the lysed cell organelles [24**].** The results indicate that nearly all of the osmium reaction occurs with lipid rather than protein. As the percentage protein in the molecule increased, the osmium uptake decreased, and the pure proteins used bound very little osmium.

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