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REVIEW ARTICLE

Biopsy and Tissue Processing Artifacts in Oral Mucosal Tissues

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ABSTRACT

Artifact refers to an artificial structure or tissue alteration on a prepared microscopic slide—the result of an extraneous factor. It can result in alteration of normal morphologic and cytologic features that may occur as a result of the way the tissue has been handled, right from the time the biopsy, which is surgically obtained till the entire histopathological procedures of fixation, processing, embedding, sectioning and staining are performed on it. The procedures themselves subject to human and material errors resulting in an artifact may interfere with an adequate diagnosis or render the tissue to be undiagnosable. The present review provides in depth knowledge on the mismanagement of tissue during different biopsy sampling techniques and various processing procedures leading to the appearance of artifacts. Such familiarization in turn will contribute to knowledge of the material and instruments required for correct biopsy performance in dentistry, as well as of the material required for correct sample storage, transport and processing thereby necessitating stringent precision in technique at every step to enable an accurate diagnosis.

Keywords: Artifacts, Biopsy, Processing procedures, Accurate diagnosis.

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INTRODUCTION

Artifact refers to an artificial structure or tissue alteration on a prepared microscopic slide—the result of an extraneous factor.[1] These artifacts result in alteration of normal morphologic and cytologic features or even lead to complete uselessness of the tissue. These have been shown to occur at various stages: During surgical removal, fixation, processing, embedding or staining of tissue sections.[1,2]

Biopsy Artifacts

A biopsy consists of the obtainment of tissue from a living organism with the purpose of examining it under the microscope in order to establish a diagnosis based on the sample.[3] The word biopsy originates from the Greek terms bios (life) and opsis (vision): Vision of life.[4]

According to the procedures applied, oral biopsies can be classified by features of the lesion as direct biopsy and indirect biopsy, by area of surgical removal as incisional and excisional biopsy and depending on the time of biopsy as preoperative, intraoperative and postoperative.[5]

A biopsy technique can be reduced to six steps: Selection of the area to biopsy, preparation of the surgical field, local anesthesia, incision, handling of the specimen and suture of the resulting wound.[5] Numerous types of artifacts can affect the biopsy specimen at any of the above mentioned stages.

Artifacts can be incurred into the tissue from the time area of biopsy is prepared with iodine tincture or other colored solutions [6] or from the time of intralesional injection of anesthetic solution into the tissue leading to vacuolization of cells. It can produce hemorrhage leading to extravasation of RBCs and separation of connective tissue bands with vacuolization[7,8] (Fig. 1).

During handling of tissue, squeeze artifacts can occur which are a form of tissue distortion resulting from even the most minimal compression of tissue that groups crush, hemorrhage, splits (Fig. 2), fragmentation and pseudocysts (Fig. 3), and are usually caused by forceps, by using a stitch for traction or by a dull scalpel blade.[1,9,10,11] Some types of tumor cells, such as small undifferentiated carcinoma are particularly prone to crush artifact which can render a tumor biopsy uninterpretable.[12] Seone et al[13] suggested that crush, splits and hemorrhage are the artifacts most frequently found in incisional oral biopsies.[13]

Often, the difficulty in correct orientation of the tissue is faced during the embedding procedure due to small size of oral biopsies. This is known as curling artifacts (Fig. 4), which is less of a problem in thin lesions having relatively thick keratotic surfaces[14,9] Hence, the ability of the oral

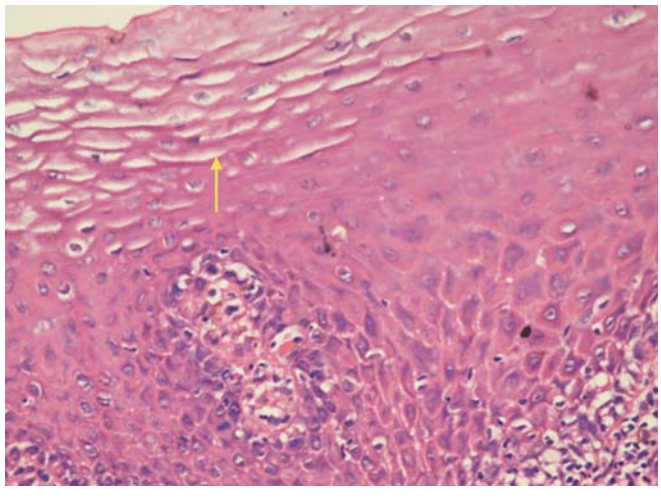


Fig. 1: Intralesional injection of anesthetic solution into a hyperkeratotic oral tissue leading to vacuolization of cells

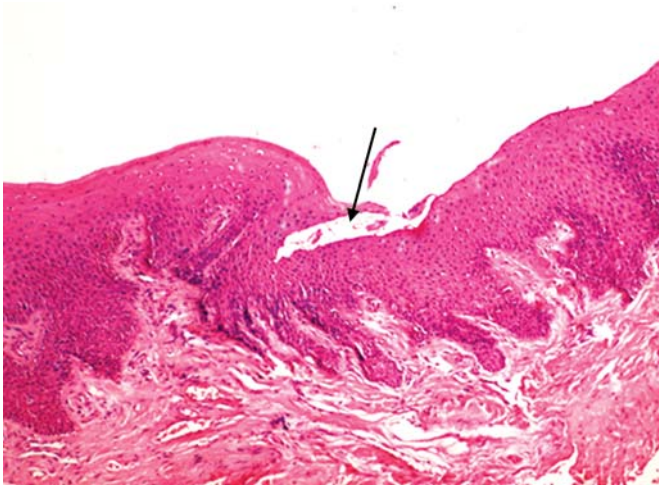


Fig. 2: Squeeze artifacts—tissue distortion like split caused by improper handling of tissue by forceps

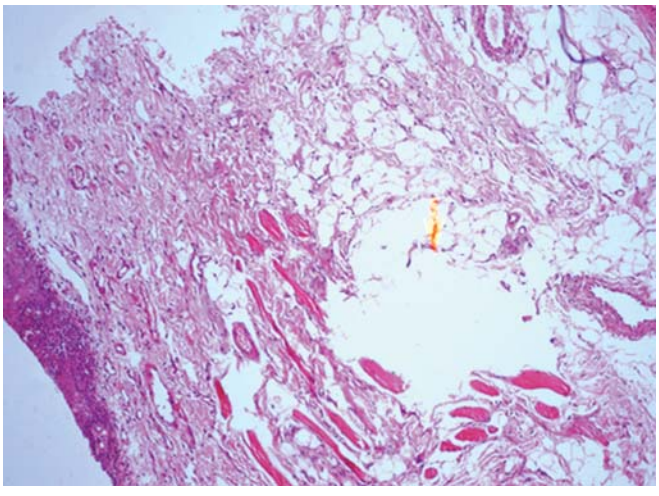


Fig. 3: Squeeze artifacts—tissue distortion like pseudocyst formation caused by improper handling of oral tissue by forceps

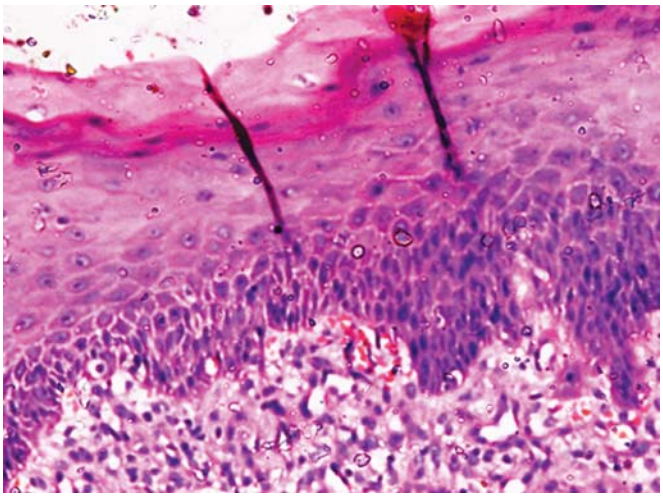
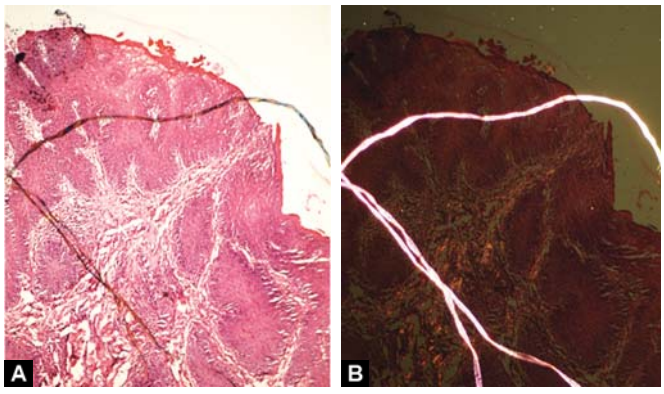


Fig. 4: Curling artifact—due to folding of oral mucosal tissue

pathologist to interpret a biopsy correctly is directly proportional not only to the quality but also to the quantity of the specimen.[6,10]



Figs 5A and B: Cotton in a section in H&E stain (A) and under polarized light (B)

Certain other artifacts such as fulguration artifacts are produced by electrosurgery. Electrosurgery induces profound artifactual alterations by producing heat which may alter both the epithelium and connective tissue. Microscopically it consists of a broad band of basophilic coagulum along the surgical margins giving an amorphous appearance to the epithelium and connective tissue making it useless for diagnosis especially if the specimen is small. The epithelial cells appear detached and the nuclei assume a spindle, palisading configuration. There may also be separation of the epithelium from the basement membrane.[15]

The interpretation of the specimen is made difficult due to the presence of foreign bodies also. Cotton and starch are the most common substances which contribute to this. Cotton in a section may resemble an eosinophilic, amyloid-like or black substances (Fig. 5A) and polarizes under polarized light (Fig. 5B) and contamination of the specimen with starch powder which is used as a lubricant of surgical gloves, may result in starch artifacts. The starch granules may superficially resemble atypical epithelial cells. These are refractile, glassy, polygonal, PAS-positive bodies generally 5 to 20 mm in diameter. These are spore-like structures with dark central area which could be misinterpreted as a pyknotic nucleus or as one undergoing mitosis. They appear light blue with hematoxylin and eosin (H&E), stain blue-black with Lugol's solution and deep lilac-red with PAS. Microscopically under polarized light will reveal the 'maltese cross' birefringence suggestive of starch granules.[16]

Fixation Artifacts

Biopsy is followed by fixation of the tissue. But the morphology of a tissue specimen is altered by the use of different fixatives. Fixation is required to arrest autolysis and putrefaction and to stabilize the protein of the cells.[2] A good fixative will penetrate tissue quickly, will preserve cellular detail at the moment of fixation and will harden the specimen to protect it from various processes of dehydration,

clearing, impregnation, embedding, sectioning, staining and mounting. For optimal fixation, tissue fixed in 10% formaldehyde solution should be left in the fixating solution for 2 to 10 hours, depending on its thickness. The amount of fixative should be approximately 20 times the volume of the specimen.[4,5] Occasionally, solutions such as distilled water or saline are substituted.[17]

Delayed fixation will cause changes in the form of cell shrinkage and cytoplasmic clustering. The nuclear chromatin cannot be distinguished, and the nucleoli are sometimes not visualized. Vascular, nerve and gland structures show a loss of detail, and the impression of scarring or cellularity loss is created.[2]

Freezing during transport before fixation also causes cytoplasmic condensation. This occurs secondary to cell dehydration as a result of freezing leading to the formation of ice crystal artifacts. Interstitial vacuoles form, together with vacuoles within the cell cytoplasm, due to ice crystal formation.[2]

Often formalin binds to heme-forming black precipitations known as pigmentation artifacts when fixed in solutions containing formalin or mercury. Heme from RBC's and formalin bind with each other to form a heme pigment that appears as black precipitates in tissue[18] (Fig. 6).

Change in volume of tissues causes shrinkage artifacts due to an inhibition of cellular respiration and changes in membrane permeability. Tissues are pulled away from each other, leaving empty spaces. False localization also caused by diffusion of unfixed material leads to streaming artifacts. Sometimes small molecules like inorganic ions and biogenic amines can also be lost from the tissue leading to an appearance of diffusion artifact.[15]

Chemical changes can also lead to artifacts. Glutaraldehyde used to fix tissues will add carbonyl groups to tissue in which they were not originally present and these groups will react with Schiff reagent.[15] But heating below optimal temperature (45-55°C) results in poor sectioning quality leading to the formation of microwave fixation artifact whereas overheating produces vacuolation, over stained cytoplasm and pyknotic nuclei.[15] Sometimes ice crystal artifacts are also produced during fixation using freeze drying method. This artifact can cause total distortion of the tissue and pose a diagnostic challenge.[15]

Tissue Processing and Sectioning Artifacts

The processing of an oral biopsy specimen is subject to a procedural protocol that results in a tissue fit for diagnosis and interpretation. The procedures themselves are subject to human and material errors and the result is an artifact that in the least may interfere with adequate diagnosis or at the most render the tissue so distorted as to be undiagnosable. After fixation, tissue needs to be dehydrated slowly starting in 50% alcohol. Tissue immersed in too much concentration of alcohol will usually show a high degree of shrinkage due to rapid removal of water. These are referred to as shrinkage artifacts.[3,4,19] If the tissue is insufficiently dehydrated prior to clearing and embedding, it can still cause alterations in the tissue seen as vacuolization of the specimen.[15]

Poor embedding of tissues most often results in sections that expand and disintegrate when introduced onto the water surface and rarely cause areas in the tissue block to go unrepresented in the tissue sections cut.[18,20,21] Tissue insufficiently dehydrated prior to clearing and infiltration with paraffin wax is hard to section and present with tearing artifacts and holes.[15]

Microtomy, the means by which tissues are sectioned so that microscopic examination is plausible involves some artifacts that can get incorporated if proper technique is not followed. Wrinkling, curling (Fig. 7), nicks in tissue,

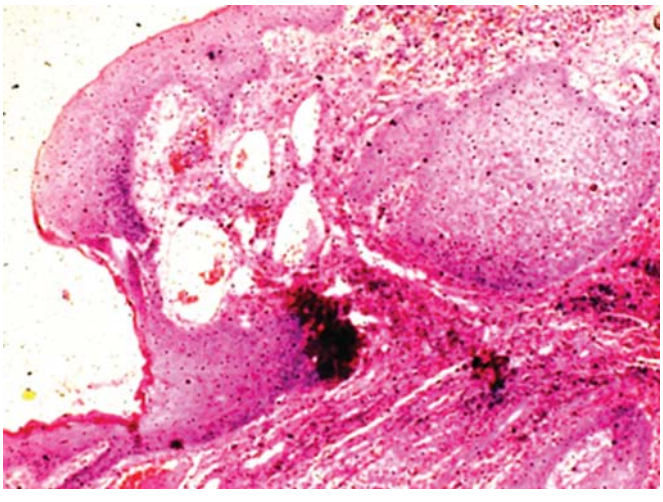


Fig. 6: Pigmentation artifacts in oral leukoplakia lesion—heme-formalin bind to form black precipitations

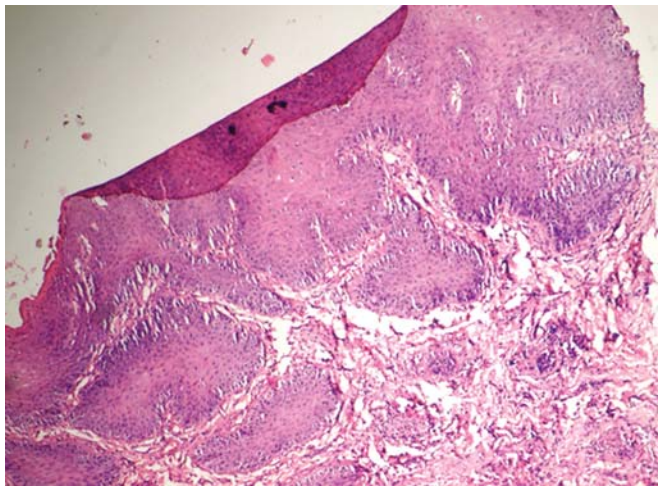


Fig. 7: Curling artifact in oral hyperkeratotic tissue

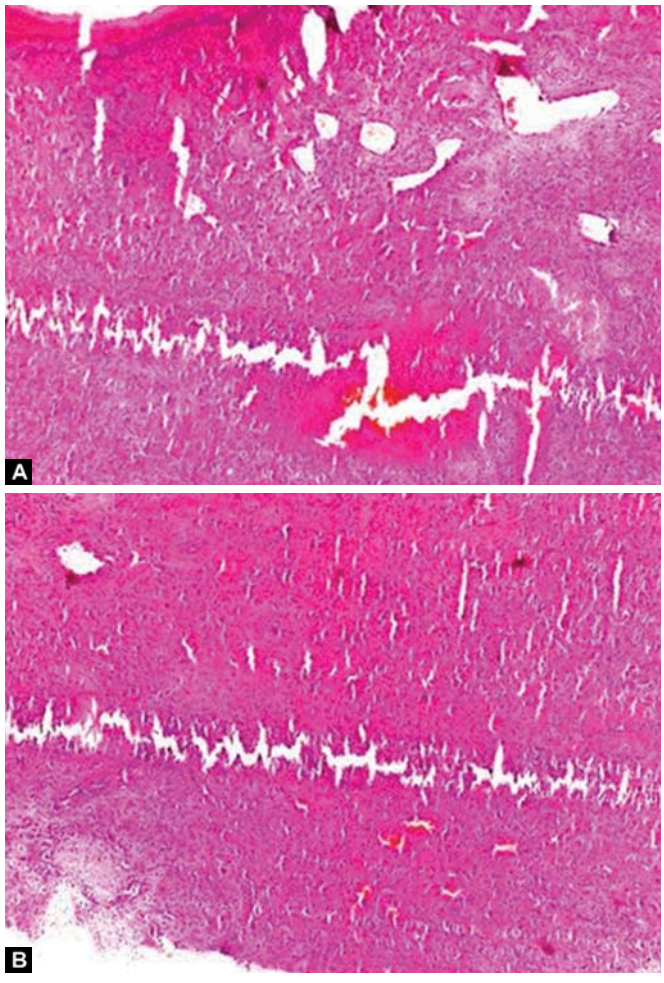
alternate thick and thin sections are some of the artifacts that can be seen at this stage. [18,20,21]

Alternate thick and thin sections are produced either when the embedding wax is too soft or if the microtome knife is loosely attached with an insufficient clearance angle.[15]

Histologic knife artifact, the result of a dull or chipped blade on the microtome in the histology laboratory. Tangential cut artifact—the rete pegs give a false impression of invasive squamous cell carcinoma[12] (Fig. 8).

Chaffer is the visible record of the knife vibrations, appearing as evenly spaced, narrow, parallel bands across the tissue specimen. A blunt knife edge can cause sections to curl or even get compressed as also can any form of knicks on the blade edge result in straight lines appearing across sections[15] (Figs 9A and B).

Wrinkling and folding of tissue sections is seen with alarming regularity is due to very thin sections being unevenly stretched around other structures having different consistencies.[15] Contamination of a dermatome blade by a particle of previously cut tissue causes 'floater artifact' which is then transferred by water bath by a particle of previously cut tissue which is then transferred to the new block.[12] Floaters are pieces of tissue that appear on slides that do not belong there. They can result due to section bits from a previous section cutting that float onto the next which mostly can be ascribed to irregular and improper cleaning of the cutting surfaces and clearing of the water bath.[15]



Figs 9A and B: Sectioning artifact (Chaffer)—knicks formed due to faulty blade

Staining and Mounting Artifacts

The next step of staining the cut sections brings with it the possibility of artifacts in terms of altered intensity and nature of staining mostly due to old, decomposed dyes, impurities present in dyes. Leaching of certain substances from tissues into the dyes (as is seen by weak staining of calcium by Alizarin red S resulting from loss of calcium ions into aqueous fixative). Blotching of sections is caused when sections placed in xylene with the purpose of dissolving wax do not undergo complete removal of wax. At the time of staining this causes patches or blotchiness to appear in slide[15] (Fig. 10).

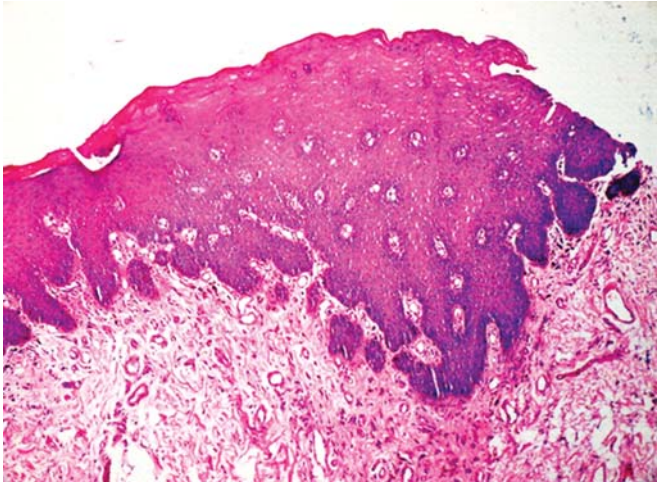


Fig. 8: Tangential cut artifact showing connective tissue cores within a mucosal tissue

The last stage involved in the entire procedure is the mounting of the stained section onto the slide using a mountant. This more often causes bubbles to form under the cover slip especially when the mounting medium is too thin. Preparation of slide in the right way can also be deterred by the presence of a drop of water resting between cover glass and specimen during preparation of slide thereby leading to the formation of artifacts[12] (Fig. 11).

The procedures themselves are subject to human and material errors and the result is an artifact that in the least may interfere with adequate diagnosis or at the most render the tissue so distorted as to be undiagnosable.[1]

The artifacts incurred during the biopsy procedure, fixation and processing procedures along with ways to correct them have been subsequently summarized in Tables 1 and 2.

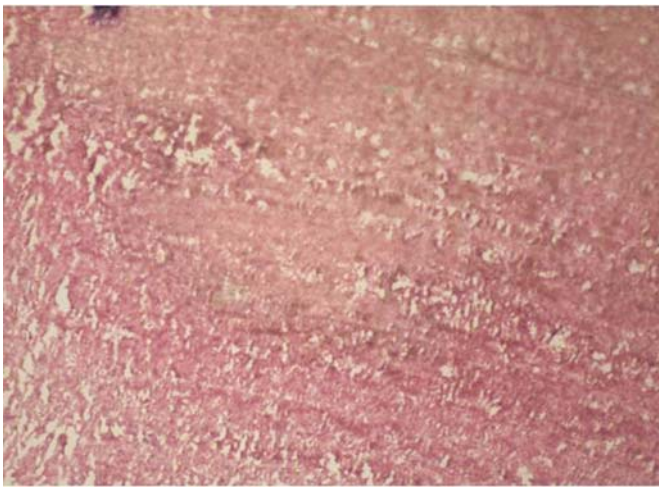


Fig. 10: Blotchiness due to incomplete removal of wax during staining

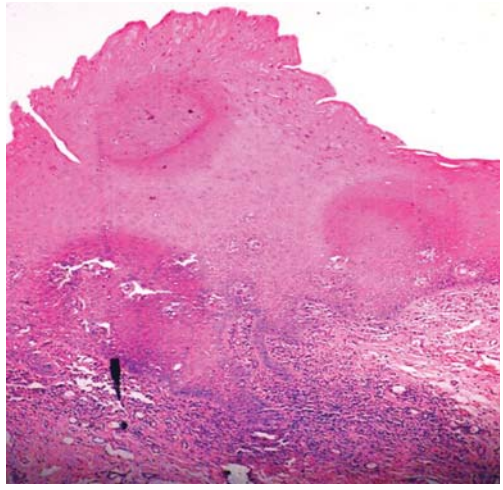


Fig. 11: Artifacts caused by a drop of water resting between cover slip and specimen during mounting

Table 1: Artifacts incurred during oral mucosal biopsy procedures along with ways to correct them

Type of biopsy	*Artifact*	*Corrections*
Incisional biopsy	Squeeze artifacts	• Careful handling of tissue by forceps.
	Curling artifacts	• Thin lesions with relatively thick keratotic surface placed with the mucosal surface upon a piece of the sterile paper that held the suture material. • Injection should be performed with a separation of 3 to 4 mm, and at four cardinal reference points (top, bottom, left and right).
	Artifacts due to injection of local anesthetic solution into the lesion (vacuolations)	• Good clinical judgment is required for selecting the best area for biopsy. • Adequate depth of the specimen. • The specimen should not be allowed to remain unfixed, while the incision is being sutured.
	Starch artifacts	• Prevention of contamination of the specimen with starch powder • This is used as a lubricant of surgical gloves Alternatively, use rubber gloves.
	Foreign bodies causing artifacts	• Correct identification of foreign materials in cytological and biopsy specimens for accurate pathological interpretation.
Excisional biopsy	When the tissue is removed with excessive force, the epithelium and connective component may suffer important damage.	• Tissue should be removed carefully.
Forceps and scalpels	Forceps used to grasp the specimen may perforate the latter, leaving gaps and creating compression zones around the tissue.	• Toothed forceps should not be used. • Use of B forceps which facilitates, simplifies and homogenizes soft tissue biopsies of the oral cavity and of the lesser salivary glands. • Sufficient tissue must then be obtained with care, avoiding sample compression or traction.
Electroscalpel and CO_2 laser scalpels, cauterizes the vessels, causing no bleeding, induction of thermal damage	The heat generated by an electroscalpel gives rise to alterations, such as tissue protein coagulation—resulting in an amorphous epithelial and connective tissue appearance. In such situations, the epithelial cells become fusiform and hyperchromatic.	• Limit it to only relatively large specimen, since the artifact could obscure all detail of a smaller specimen. • Care must be exercized to use the cutting and not the coagulation electrode when obtaining a specimen, so that low milliamperage current will be produced that will allow cutting and liberation of the specimen.

Table 2: Artifacts incurred during various fixative and tissue processing procedures along with various ways to correct them

Procedures	*Artifacts*	*Corrections*
Fixation: Fixation for light microscopic preparations	Shrinkage artifacts	• Immediate and correct fixation of the tissue specimen to interrupt autolysis and putrefaction and stabilize the cell proteins. • Amount of fixing agent should exceed the tissue volume by a factor of 20.
	Pigmentation artifacts—tissues are fixed in solutions containing formalin or mercury	• Treatment with picric alcohol or 1% alcoholic solution of sodium hydroxide. • Formation can be prevented by buffering the formalin saline or it can be removed by treatment with iodine.
	Streaming artifacts diffusion artifact	• Proper fixation for accurate localization and prevention of leaching of small ions from the tissue.
	Artifacts due to chemical changes (changes in the form of cell shrinkage and cytoplasmic clustering).	• Use Bouin-fixation medium for prolonged storage of the specimens.
	Acetone or 70% alcohol sharply dehydrates the tissues, complicating epithelial staining and poorly fixing the connective tissue elements.	• Other simple fixing fluids can also be used, such as picric acid, acetic acid, chromic acid, potassium dichromate, mercury chloride, cadmium chloride, osmium tetraoxide (osmic acid).
Freezing of the tissue	Ice crystal artifacts	• Freezing of the tissue before fixation is not recommended. • Freezing during transport should also be avoided, since cytoplasmic condensation has been described, secondary to cell dehydration as a result of freezing. • Tissue must be plunged into isopentane cooled to –1,600°C to –1,800°C with liquid nitrogen immediately.
	Disruption artifacts	• Low temperature should be used because unless the whole tissue is frozen, large ice crystals are formed.
Fixation for electron microscopic preparations		• Specimen should be immersed in 3% glutaraldehyde in the refrigerator for 24 hours, followed by transfer to a 0.1 M buffer solution until study. The specimen is again dehydrated and sealed with a coverslip.
Tissue processing	Reagents: Contamination, pH, concentration, temperature, timing, shrinkage artifacts, vacuolization of the specimen.	• Tissue needs to be dehydrated slowly starting in 50% alcohol.
Embedding	Tearing artifacts and holes	• Tissue should be sufficiently dehydrated prior to clearing and infiltration with paraffin wax. • Embedding in methacrylate should be used for electron microscopic sections
Tissue sectioning	Wrinkling, curling, nicks, alternate thick and thin sections, chaffer, wrinkling and folding, floaters	• Cool the wax block or rectify the faulty knife. • Gentle teasing of the tissue sections should be done with forceps. • Clean the cutting surfaces and clear the water bath.
Staining	Hydration error, reagents, light staining, dark stain, improper contrast, fading stain, blotching	• Complete removal of wax • Ideal temperature • Ideal timing according to the stain used.

CONCLUSION

Careful handling of the tissue and prompt appropriate fixation and tissue processing will enable a confident histological diagnosis to be reached.[22] Inadequate care at any stage could result in a nondiagnostic biopsy and may necessitate the patient having a repeat procedure with its ensuing physical and psychological morbidity.[22]

Since, Zegarelli[9] in 1978 published an article dealing with common problems in biopsy procedure, very few additional studies appeared on the subject. Thus, through this review we illustrated some of the important artifacts in tissue specimens especially those that prevent accurate diagnosis and to suggest some techniques for alleviating these problems.

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