

Artefacts: A Diagnostic Dilemma – A Review

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ABSTRACT

The significance of proper handling of biopsy specimens is important and obvious for any histopathological diagnosis. Accuracy of tissue diagnosis by the pathologists is heavily dependent upon the competence of histotechnologists. The aim of a good histopathological technique is to produce microscopic preparation of tissues, usually stained, that represents as closely as possible, their structures in life. But this is not always possible and some sort of tissue morphology is bound to be seen. The ability of oral pathologists in interpreting a biopsy correctly is directly proportional not only to the quality, but also to the quantity of the specimen. There are many ways through which the exact interpretation of tissue specimen becomes compromised and the major pitfall is the presence of artefacts. These artefacts may occur during surgical removal, fixation, processing, embedding, microtomy and staining procedures. It is therefore important to identify the common occurring artefacts during interpretation of oral biopsies.

Key words: Artefacts, Sectioning artefacts, Staining artefacts, Squeeze artefacts, Crush artefacts, Histopathology

INTRODUCTION

A biopsy is the removal of part, or all, of a lesion, to enable histopathological examination and definite diagnosis. The word, 'biopsy' has been derived from the Greek words, 'Bios' (life) and 'Opsis' (vision): vision of life. Biopsy is the gold standard diagnostic procedure for obtaining adequate representative tissue for histopathological evaluation, for arriving at a final diagnosis [1]. The application of biopsy in the management of oral lesions includes a sequence of steps: adequate data collection, competent diagnostic skills, proper surgical management, evaluation and representation of the pathologist's report and comprehensive patient follow-up [2]. Numerous types of artefacts can affect the biopsy specimen at any of the above mentioned stages.

Specimens removed from the oral cavity are often small, and the possibility of producing artefacts is thus enhanced [3]. The word, 'artefact' is derived from the Latin words, 'Ars' (art) and 'factum' (made) [4]. According to Bernstein, Artefact refers to "An artificial structure or tissue alteration on a prepared microscopic slide caused by some extraneous factors" [5]. These artefacts result in alteration of normal morphological and cytological features or they may even lead to complete uselessness of the tissue [3], thus creating serious errors and misdiagnosis of correct histopathological impression.

It is important to have thorough knowledge on artefacts, so that appropriate precautionary measures can be taken to avoid or minimize their occurrence [6]. Several papers have reported that artefacts are common in oral mucosal biopsy specimens [1,3,7]. Some papers have also compared artefacts occurred due to different surgical techniques i.e., punch biopsy versus scalpel biopsy [8,9]. Therefore, this article highlights the different types of artefacts that occur right from the time the biopsy is surgically obtained, till all the histopathological procedures are performed.

TEXT

The artefacts can be minor, involving only small portion of the specimen and which therefore, do not interfere with an accurate diagnosis. In some cases, they may be excessive or may involve

the entire specimen, thus rendering it useless for diagnostic purposes [4,10,11].

The potential circumstances that may result in artefacts are during handling of tissues at the time of biopsy, during the fixation process and during the histo-technical procedures used for embedding and staining tissue [3,4,12].

ARTEFACTS DURING ORAL BIOPSY PROCEDURES

Generally, during removal of organs, clamps and ligatures are applied to arrest bleeding. So, the organ is anoxic for some time period, before it is removed from the patient [4]. After obtaining the specimen, if there is delay in its fixation, this also leads to anoxia. Anoxia brings about changes which are noticeable under an electron microscope within 10 minutes, for eg: mitochondrial damage. The longer the agonal interval in fixation, the more mitoses apparently progress to completion. Major effect of delay appears to be poor histology, causes more difficulty in recognizing mitoses [13]. Anoxia also causes release of hydrolytic enzymes from cytoplasmic organelles, "Lysosomes". The hydrolytic enzymes commence to digest the cells, so that details are lacking when they are seen under the microscope [12]. Autolyzed tissues usually show nuclear pyknosis, karyolysis and karyorrhexis, along with cytoplasmic vacuolation and disintegration of tissue structure. These changes are called Agonal changes.

These changes can be prevented by storage of tissues at 40 C, but they can be completely avoided by rapid fixation.

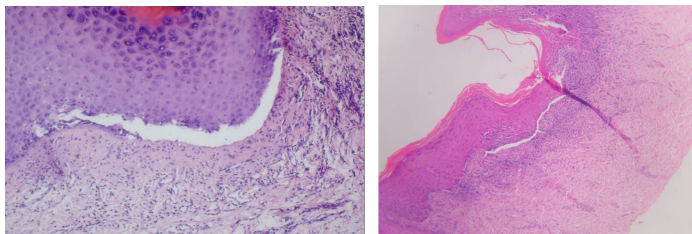
Forceps artefact is seen when the teeth of the instrument penetrates the specimen, resulting in voids or tears [Table/Fig-1 and 2] and compression of the surrounding stroma, making exact interpretation difficult [3]. During improper surgical removal, when the scalpel cuts too shallow a specimen, correct evaluation of the epithelium and its relationship with the underlying connective tissue is impossible.

This type of artefact prevents an accurate evaluation of local invasion in biopsies for epithelial neoplasia [14] and hence, the wrong diagnosis leads to wrong treatment.

This artefact can be prevented by avoiding the use of tooth

forceps. B forceps can be used to obtain biopsies of the oral cavity and salivary glands, as it simplifies and homogenizes soft tissues. Sufficient tissue should then be obtained with care, avoiding compression or traction [22].

Curling artefacts are seen more commonly in Incisional biopsies. Curling is sometimes less of a problem when thin lesions have relatively thick keratotic surfaces [Table/Fig-3],[14]. If the specimen is too small, such as a delicate strip of oral mucosa, the shrinking process caused by formalin fixation causes curling and bending of the tissue, thus making its correct orientation during the embedding procedure difficult [10]. Small biopsies can reduce their size during processing and fixing [15,16].



[Table/Fig-1]: Tissue tear

[Table/Fig-2]: Tissue tear and folds



[Table/Fig-3]: Curling artefact due to folding of tissue

This can be prevented, if after the biopsy, the specimen is placed with its mucosal surface up on a piece of the sterile paper (usually that which held the suture material) and if it is allowed to remain unfixed for some time while the incision is being sutured [14,16]. Since curling is seen in thin biopsy specimens, ensuring adequate depth of the specimens can prevent this artefact [9].

Squeeze artefacts are a form of tissue distortion resulting from even the most minimal compression of tissue that groups together crush, haemorrhage, splits, fragmentation, and are usually caused by forceps, during use of a stitch for traction or by a dull scalpel blade [5, 7, 14, 17].

The incorrect use of forceps produces the formation of pseudomicrocysts, apparently lined with surface epithelium, forced inward by the teeth of the instrument, along with compressed surrounding connective tissue, thus making evaluation impossible [10].

This can be overcome by careful handling of the specimen by forceps. Compression artefacts, a form of tissue distortion, result

from even the minimal compression of tissue. This artefact is more common in incisional than in punch biopsies [9]. This is seen as a distorted tissue with scalloped serrations (produced by beaks of the forceps) and crushed cells, appear as dark chromatin strands and may give a false diagnosis of dysplastic lesions.

This can be prevented by the following factors 1> Handling the specimen carefully, especially at the base with delicacy 2> Using substitute for forceps or using suture as an alternative to forceps.

Intralesional injection of anaesthetic solution should be avoided, as it can produce haemorrhage with extravasation and separation of connective tissue bands with vacuolization [18]. This injection should be given with a separation of 3 – 4 mm, and at 4 cardinal reference points (top, bottom, left and right) [19]. This artefact is called Haemorrhage artefact.

This artefact can be prevented by the following factors: good clinical judgment is required for selecting the best area of biopsy, adequate depth of the specimen and the specimen should not be allowed to remain unfixed, while the incision is being sutured.

During surface preparation (inking), preparation of the area of biopsy with iodine tincture or other coloured solutions should be avoided, as it can interfere with tissue processing and staining procedures [15].

Split artefacts occur on the surface and at the side of the lesion due to scalpel, which causes multiple cuts in the tissue. This artefact may result in a split between epithelium and connective tissue, giving a false impression of vesiculo – bullous lesions. This can be prevented by avoiding excess pressure caused by suture traction.

Foreign bodies' presence often makes the interpretation of the specimen difficult. Cotton and starch are the most common substances which contribute to this. The presence of cotton in a section may resemble eosinophilic, amyloid – like or black substances and it polarizes under polarized light.

These may create problems for sectioning and for the subsequent histopathological evaluation, as it might resemble amyloid like material which is highly characteristic of odontogenic tumours, thus resulting in wrong diagnosis of odontogenic tumours.

Starch artefacts may occur due to the contamination of the specimen with starch powder, which is used as a lubricant for surgical gloves. The starch granules may superficially resemble atypical epithelial cells that may mimic salivary gland disease, autoimmune disorders, granulomatous lesions and benign epithelial lesions. These are refractile, glassy, polyzonal, PAS +ve bodies, generally 5-20 mm in diameter. They appear light blue on H and E staining, blue black with Lugol's solution and deep lilac-red with PAS. Microscopically, starch granules reveal Maltose cross birefringence under polarized light [20].

Starch artefacts can be prevented by the alternate use of rubber gloves and correct recognition of the foreign materials in cytological and biopsy specimens is important for precise pathological interpretations.

Fulgeration or Heat artefacts may render the small biopsy specimens undiagnostic. Tissue distortion may result from excessive heat from surgical electrical – cautery instrument or from laser and it may alter both the epithelium and connective tissue. Such sections are represented by coagulation of protein, giving an amorphous appearance to the epithelium and connective tissue. The epithelium cells appear detached and the nuclei assume a spindle, palisading configuration. There may also be separation of the epithelium from the basement membrane [20].

This can be prevented by using the cutting and not the coagulation electrodes when obtaining the specimen, so that low milliampere current is produced, that will allow cutting and liberation of the specimen.

FIXATION ARTEFACTS

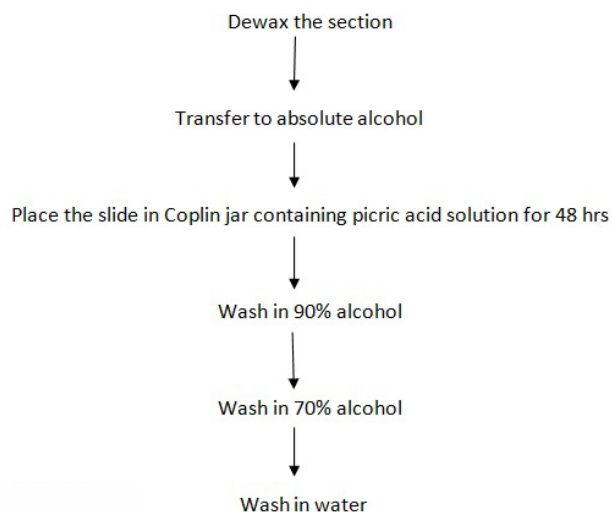
Biopsy is followed by fixation of the tissue. The morphology of a tissue specimen is altered by use of different fixatives. Fixation is required to arrest autolysis, putrefaction and to stabilize the protein of the cell [21]. A good fixative penetrates tissue quickly, preserves cellular details and hardens the specimen as a protective measure [22]. For optimal fixation, the amount of fixative should be about 20 times the volume of the specimen and 10% neutral buffered formalin is considered as the best fixative. Occasionally, solutions such as distilled water or saline can be substituted [10].

Normally, tissues fixed in formalin and embedded in paraffin shrink by 33%.

When tissue specimen is submerged in saline for 24 hours with subsequent fixation in formalin, it results in bizarre appearance of epithelium cells, suggesting malignancy. For example, a dental surgeon had excised the tissue and kept it in saline for more than 24 hours. He had then sent the specimen to the histopathology lab after 2 days for processing. After processing of the specimen, the histopathological features revealed large, round, swollen atypical cells with hyperchromatic nuclei, prominent nucleoli, giving an erroneous diagnosis of lymphoma and thus, completely changing the treatment plan. So, if the tissue specimen is being sent in saline, it is advised to immediately substitute the solution with 10% neutral buffered formalin solution, so as to prevent a misdiagnosis.

Prolonged fixation in formalin may cause secondary shrinkage and hardening and it may result in separation of tissue, giving an appearance of empty spaces.

Shrinkage artefacts are seen as a change in volume of tissues. During fixation, tissue change in volume caused by inhibition of respiration, change in membrane permeability and change in sodium transport activity. Those fixatives that are protein

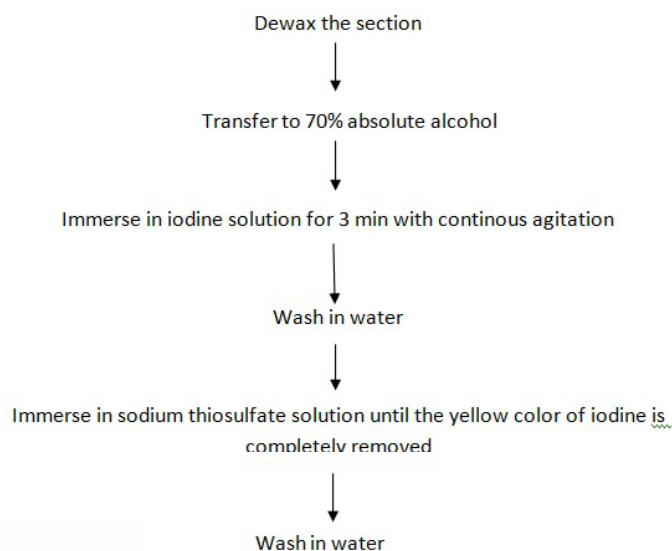


precipitants like ethanol or methanol shrink tissues regardless of their osmotic pressure [4]. As a result, tissues that are attached in life may be pulled away from each other, leaving empty spaces.

This can be prevented firstly, by immediate and correct fixation of the specimen that interrupts autolysis and putrefaction, thus stabilizing the cell proteins and secondly, the amount of the fixing agent should exceed the tissue volume by a factor of [20].

Delayed fixation causes changes in the form of cell shrinkage and cytoplasmic clustering. The nuclear chromatin cannot be distinguished and the nucleoli are sometimes not visualized. Vascular structures, nerves and glands show a loss of detail and an impression of scarring or loss of cellularity is seen [21].

This can be overcome by fixing the specimen immediately in 10% formalin solution as soon as the tissue is removed.



Freezing during transport before fixation also causes cytoplasmic condensation and it occurs secondary to cell dehydration as a result of freezing. This is characterized by the formation of interstitial vacuoles and vacuoles within the cell cytoplasm, which result from ice crystal formation [3,21].

This can be prevented by avoiding freezing before fixation and during transport.

When tissues are being fixed in solutions containing formalin or mercury, care should be taken to avoid formation of complexes in tissues, that give rise to pigmentation artefacts. Heme from RBCs and formalin bind each other to form formalin-heme complex that appears as black precipitate in tissue. This pigment is most often seen in cellular or bloody tissues and autopsy tissues. This pigment has no relationship with the tissue and it can be confirmed by polarized microscopy, because it will polarize a white light.

This can be removed in sections before staining by treatment with picric alcohol or 1% alcoholic solution of sodium hydroxide (Flowchart 1). Formation can be prevented by buffering the formalin saline. Similarly, mercury containing fixative will form a brownish precipitate and can be removed by treatment with iodine (Flowchart 2) [4].

Streaming artefacts are caused by diffusion of unfixed material to give false localizations, as they come to rest in places other than their original locations [4] and are most commonly seen in glycogen.

This can be prevented by using smaller blocks (Reale and Luciano 1970) or stronger fixatives for larger bits. Fixation of tissue for glycogen should be prompt, as there is an initial sharp loss of glycogen postmortem and it should be carried out at 40 C in 80% alcohol or in Rossman's solution.

Another artefact called false localization of extraneous material occurs in autoradiography with H3 labelled amino acids, sugars, thymidine and uridine. Tissues become incorporated into the substances by active metabolism [4]. This false localization of unreacted radioactive sulphate can be removed by washing with cold sodium sulphate. This artefact can also be completely avoided by freeze drying.

Diffusion artefacts refer to materials that may sometimes diffuse out of the tissue. Apart from large molecules, small molecules like inorganic ions and biogenic amines can be lost from tissues [4]. These may result from denaturation of associated protein, chromogranin, in case of adrenaline and nor adrenaline. This can be demonstrated by placing the adrenals in iodate. The catecholamines can be seen leaving the tissue as a red cloud of aminochromes. Biogenic amines which are thus generated can only be retained by precipitation [12].

This can be prevented by proper fixation for accurate localization and also by preventing the leaching of ions from the tissue [22].

Chemical changes can also lead to artefacts. Gluteraldehyde used to fix tissues will add carbonyl groups to tissues in which they were not present and these groups will react with Schiff's reagent [20]. This can be overcome by using Bouin's fixation medium for the storage of specimens.

Optimum temperature for microwave fixation is 45- 55°C. Underheating results in poor sectioning quality, whereas overheating above 65°C produces vacuolization, overstained cytoplasm and pyknotic nuclei [4]. The microwaves generated by commercial ovens penetrate tissues to a thickness of 10-15mm. The mechanism whereby microwaves bring about tissue stabilization involves protein denaturation. The time taken for IHC and in - situ hybridization can be significantly decreased

Crush artefacts are a form of artefact found in surgical specimens and are associated with intense eosinophilia at the centres of tissue in H and E stained sections. These are caused by protein coagulation caused by ethanol of partially fixed protein.

Ice crystal artefacts are produced during fixation using freeze drying method. Here, the tissues must be plunged into isopentane and cooled to -160 to -1800 C with liquid nitrogen immediately. Low temperature is important, because unless the whole tissue is frozen, large ice crystals can be formed, causing disruption artefacts [4]. Such artefacts can cause total distortion of the tissue and pose a diagnostic challenge [20].

This can be prevented by avoiding freezing of the tissue before fixation and during transport also, freezing is not recommended, as it causes cytoplasmic condensation secondary to cell dehydration [22].

ARTEFACTS DURING DECALCIFICATION

Thin slices of calcified tissue can usually be obtained by cutting with a sharp knife. But, when difficulty is encountered, it is better to use a saw; otherwise, the tissue surrounding the calcified area will get damaged.

Generally, decalcification is greatly accelerated by application of heat. Decalcification carried out at a temperature of 55-60°C results in undue swelling of tissues and completion of digestion [12,13].

Fixation for bone marrow is best carried out with Zenker's formol and it is done to prevent tissue damage during decalcification. After decalcification, acids present in the tissues should be neutralized by saturated lithium carbonate or 5-10% aqueous sodium bicarbonate for several hours [4].

ARTEFACTS DURING TISSUE PROCESSING

Dehydration is the first step in processing and it is the process of removal of aqueous fixative fluids from the tissue by using compounds like alcohol, whereas clearing is replacing the dehydrating agent with fluid that is miscible with dehydrating fluid and embedding medium [4,12]. Tissues immersed in too great a concentration of alcohol will usually show a high degree of shrinkage due to rapid removal of water. These are referred to as shrinkage artefacts.

After fixation, tissue needs to be dehydrated slowly. Starting with 50% alcohol can prevent this artefact [13].

If the tissue is placed in acetone – a dehydrating agent, for a prolonged period of time, the tissue becomes very brittle, thereby affecting subsequent procedures like sectioning.

Other simple fixing agents such as picric acid, acetic acid, chromic acid, etc, can be used to prevent tissue changes.

Prolonged immersion in clearing agent also renders the tissues brittle [4,13]. Even a small amount of clearing agent that contaminates the wax leads to crumbling and crystallization of

tissues during cutting.

This can be prevented by taking proper care to use the proper amount of clearing agent and no clearing agent should be left behind to contaminate the wax.

ARTEFACTS DURING EMBEDDING

Incorrect orientation of tissues in a mould results in diagnostically important tissue elements being missed or damaged during microtomy [4]. Exposing the specimen for too long during embedding results in excessive hardening and the tissues becomes friable. They form cracks during sectioning [20]. Loss of soluble substances is seen when neutral fat is dissolved from fat cells, leaving regular ovoid spaces [Table/Fig-4].

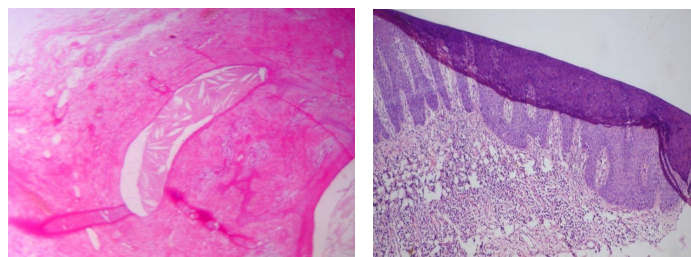
Correct orientation of the specimen in the mould and exposing the specimen to the correct amount of the embedding medium can prevent this artefact. When more than one specimen is being embedded, care should be taken to not layer the specimens and to not embed a larger and a smaller tissue together.

Tissues insufficiently dehydrated prior to clearing and infiltrated with paraffin wax are hard to section and present with tearing artefacts and holes [4,12].

This can be prevented by adequately dehydrating the tissues before clearing and infiltration with paraffin wax.

ARTEFACTS DURING MICROTOMY

The preparation of histological sections with consistently high quality and containing minimal artefacts require properly embedded tissues and a suitable microtome. Microtomy, the means by which tissues are sectioned, so that microscopic examination is possible, involves some artefacts that can get incorporated if proper technique is not followed [20]. Wrinkling, curling [Table/Fig-5], nicks in tissue, alternate thick and thin sections are some of the



[Table/Fig-4]: Loss of soluble substance resulting in empty spaces

[Table/Fig-5]: Curling artefact

artefacts that can be seen at this stage [4,12,23].

Alternate thick and thin sections are produced when the wax is too soft for tissue, block or blade is loose, clearance angle is insufficient or mechanism of microtome is faulty.

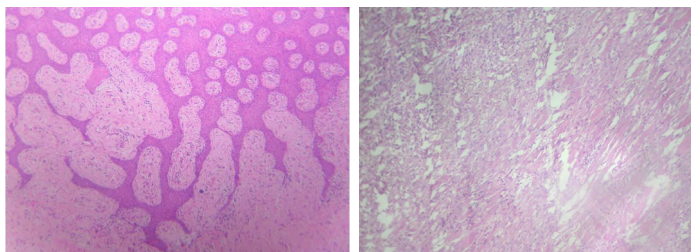
This can be overcome by cooling the block, tightening the block or blade and increasing the clearance angle.

Wrinkles and folding of tissue sections [Table/Fig-2] are seen when very thin paraffin sections are forced to stretch unevenly around other structures which have different consistencies.

If folds have occurred, they may be removed by gentle teasing with forceps [4,13] or folds may be removed by transferring the sections from the slide to another water bath at high temperature [12].

If the tissue is cut tangentially, the connective tissue cores may become entrapped within the epithelium, giving a false impression of invasive squamous cell carcinoma [Table/Fig-6].

Chatters or chaffers are thick or thin zones parallel to knife edge as it cuts the tissue [Table/Fig-7]. This is either caused by loose knives or block holders, excessively steep knife edge or wax which is too hard to be sectioned [4,12]. Chaffer is the visible record of knife vibrations and it appears as narrow parallel bands, usually



[Table/Fig-6]: Tangential cut artefact

[Table/Fig-7]: Chatters: Knicks formed due to faulty blade

evenly spaced across the tissue specimen [20].

The knife vibration can be prevented by altering the thickness of the tissue, changing the orientation and soaking the block face with detergent or water.

Knife nicks are caused by nicks in blade edge, hard particles in the tissues or wax. This will cause straight lines to appear across the sections, as knife cuts through the embedded block. Floaters are pieces of tissue that appear on slides that do not belong there. They may have floated during processing and may result from sloppy procedures on cutting bench using dirty towel, knife, and gloves. These can have tissues that are carried over to the next case [4,13].

Distilled water, rather than tap water, should be used and the bath should be emptied and dried after each cutting section, to rectify the problem.

ARTEFACTS DURING STAINING

In the next step of staining, the cut section brings with it the possibility of artefacts, in terms of altered intensity and nature of staining, due to old, decomposed dyes, impurities present in the dye and leaching of certain substances from tissues into the dye (as is seen by weak staining of calcium by alizarin red S, resulting from loss of calcium ions into aqueous fixative) [20].

These can be prevented by using ideal temperature and time, depending on the stain used and filtration of the staining solution will remove the impurities from the stain.

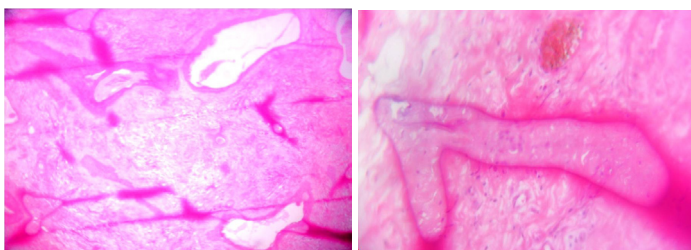
Blotching of sections is caused when the sections are placed in xylol with the purpose of completely dissolving the wax. If all wax has not been removed, during staining patches or blotchiness may appear on slide. Improper clearing of the wax may result in undue staining of the slide, making diagnosis difficult [Table/Fig-8].

Prolonged xylene treatment and re-staining can overcome this problem.

ARTEFACTS DURING MOUNTING

Stained sections are protected from damage by the application of cover-glasses with appropriate mounting media. This procedure may introduce artefacts, which not only interfere with the appearance of the preparation, but also alter the staining results. Bubbles are formed under the cover-slip when the mounting medium is too thin and as it dries, more air gets sucked under the edges [Table/Fig-9] [4, 12].

This can be prevented by using mounting medium of adequate thickness and removal of air bubbles from under the slide. The



[Table/Fig-8]: Improper wax clearing resulting in undue staining

[Table/Fig-9]: Air bubbles seen during mounting

bubbles that are caused by poor floatation technique can be removed by using freshly boiled water in floatation bath [24].

MISCELLANEOUS ARTEFACTS

Bone artefacts can occur during sawing, drilling and decalcification procedures. Bone dust can be seen in two forms – firstly, as splinters of bone or cartilage and secondly, as round masses of debris. In decalcified sections, this leads to difficulty in interpretation and to an erroneous diagnosis of metastatic calcification.

Decalcification defects can be checked by thorough fixation of the specimen before decalcification and radiographic checking of the progress and completion of the process [4].

Undecalcified resin sections contain a fine, grit – like, bone dust or powder in association with the bony trabeculae, either within or in close proximity to it. Bone dust can also occur in sections prepared either using both glass or diamond knives, and no dependable method is available for correcting this artefact [24].

Reprecipitation artefacts are caused by lack of agitation and inadequate volume of decalcifying fluid. They are seen as round granules or crystalline masses which lie mainly in the soft tissues and bone marrow. Brown and Rowles have shown this material to be secondarily calcium phosphate and it stains strongly with alum hematoxylin [12,13].

Overdecalcification may hamper cutting qualities, affects staining properties and histological details are destroyed.

This can be overcome by determining the end point of decalcification or minimized by neutralizing the bone section by 1% aqueous solution of lithium carbonate [4,12]. Surface decalcification of the paraffin block can rectify incomplete decalcification [24].

Artefacts of undecalcified bone consist of cracking of matrix in resin sections. This is virtually impossible to eradicate, but it may be minimized by extending processing times to improve infiltration and also by slow polymerization of epoxy resins, to promote linear polymers and to produce a more flexible resin. The alternate use of diamond knives in place of glass knives can also rectify this artefact [24].

CONCLUSION

The proper preparation of the tissues for microscopic studies or analyses depends on the steps taken by the surgeon, assistant pathologists and histo-technicians for reducing the inclusion of artefacts. These artefacts may go unnoticed clinically, but they can create potential diagnostic problems for the pathologist during histopathological examination. The procedures themselves are subjected to human and material errors and they may result artefacts that may interfere with adequate diagnosis. With the recent advances in laboratory equipments, the need to recognize these artefacts and of attempts to overcome them still remains a challenge.

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