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TOPIC – FIXATION AND TISSUE PROCESSING

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SYNOPSIS

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INTRODUCTION

- **Fixation** is the preservation of biological tissues from decay due to autolysis or putrefaction.
- It terminates any ongoing biochemical reactions and may also increase the treated tissues' mechanical strength or stability.
- Tissue fixation is a critical step in the preparation of histological sections, its broad objective being to preserve cells and tissue components and to do this in such a way as to allow for the preparation of thin, stained sections.
- This allows the investigation of the tissues' structure, which is determined by the shapes and sizes of such macromolecules (in and around cells) as proteins and nucleic acids.

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- In performing their protective role, fixatives denature proteins by coagulation, by forming additive compounds, or by a combination of coagulation and additive processes.
- A compound that adds chemically to macromolecules stabilizes structure most effectively if it is able to combine with parts of two different macromolecules, an effect known as cross-linking.
- One reason is to kill the tissue so that postmortem decay (autolysis and putrefaction) is prevented.
- First, a fixative usually acts to disable intrinsic biomolecules—particularly proteolytic enzymes—which otherwise digest or damage the sample.

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- Second, a fixative typically protects a sample from extrinsic damage.
- Fixatives are toxic to most common microorganisms (bacteria in particular) that might exist in a tissue sample or which might otherwise colonize the fixed tissue.
- In addition, many fixatives chemically alter the fixed material to make it less palatable (either indigestible or toxic) to opportunistic microorganisms.
- Finally, fixatives often alter the cells or tissues on a molecular level to increase their mechanical strength or stability.
- This increased strength and rigidity can help preserve the morphology (shape and structure) of the sample as it is processed for further analysis.

TYPES OF FIXATIVES

- A **fixative** is a stabilizing or preservative agent:
  Dye **fixatives** or mordants, are chemical substances used in processing fabrics to create circumstances in the micro-substrates causing dye molecules to adhere and remain that way.
- Most samples used for staining normal and pathological tissues are embedded in paraffin, and a number of fixatives have been formulated with this in mind.
- There is an abundance of specialty fixatives that will not be covered here but may be found in the references given in the bibliography.

PHYSICAL METHODS OF FIXATION

Heat fixation :

- This is the simplest form of fixation. Boiling or poaching an egg precipitates the proteins and, on cutting, the yolk and egg white can be identified separately.
- Each component is less soluble in water after heat fixation than the same component of a fresh egg.
- Picking up a frozen section on a warm microscope slide, both attaches the section to the slide and partially fixes it by heat and dehydration.
- Even though adequate morphology could be obtained by boiling tissue in normal saline, heat is primarily used to accelerate other forms of fixation as well as the other steps of tissue processing.

MICROWAVE FIXATION

- Microwave heating can reduce times for fixation of some gross specimens and histological sections from more than 12 hours to less than 20 minutes (Kok & Boon, 2003; Leong, 2005).
- Microwaving tissue in formalin results in the production of large amounts of dangerous, potentially explosive vapors.
- In the absence of a hood for extraction or a microwave processing system designed to handle these vapors, this may cause safety problems.
- Commercial glyoxal- based fixatives which do not form vapors when heated at 55°C have been introduced as an efficient method of microwave fixation.

FREEZE-DRYING AND FREEZE SUBSTITUTION

- Freeze-drying is a useful technique for studying soluble materials and small molecules. Tissues are cut into thin blocks, immersed in liquid nitrogen and the water removed in a vacuum chamber at −40°C.
- The tissue can be post-fixed with formaldehyde vapor.
- In freeze substitution, specimens are immersed in fixatives, e.g. acetone or alcohol at −40°C, this slowly removes water through dissolution of ice crystals and the proteins are not denatured.
- Bringing the temperature gradually up to 4°C will complete the fixation process (Pearse, 1980).
- These methods of fixation are used primarily in the research environment and are rarely used in the clinical laboratory setting.

CHEMICAL FIXATION

- Coagulant fixatives
- Both organic and non-organic solutions may coagulate proteins making them insoluble. Cellular architecture in vivo is maintained primarily by lipoproteins and fibrous proteins such as collagen.
- Coagulating these proteins maintains tissue histomorphology at the light microscope level.
- Unfortunately, because coagulant fixatives result in cytoplasmic flocculation and poor preservation of mitochondria and secretory granules, these fixatives are not useful in ultrastructural analysis.

DEHYDRANT COAGULANT FIXATIVES

- The most commonly used in this group are alcohols (e.g. ethanol, methanol) and acetone.
- Methanol is closer to the structure of water than ethanol.
- Ethanol therefore competes more strongly than methanol in the interaction with hydrophobic areas of molecules and coagulant fixation begins at a concentration of 50–60% for ethanol but 80% or more for methanol (Lillie & Fullmer, 1976).
- Disruption of the tertiary structure of proteins (i.e. denaturation) changes their physical properties, potentially causing insolubility and the loss of function.
- Even though most proteins become less soluble in organic environments, up to 13% of protein may be lost, e.g. with acetone fixation (Horobin, 1982).

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- Temperature, pressure, and pH.
- Ionic strength of the solute.
- The salting-in constant, which expresses the contribution of the electrostatic interactions.
- The salting-in and salting-out interactions.
- Alcohol denatures protein differently depending on the choice and concentration of alcohol, the presence of organic and non-organic substances and the pH and temperature of fixation.
- The protein denaturing effect of ethanol is > phenols > water and polyhydric alcohols > monocarboxylic acids > dicarboxylic acids (Bhakuni, 1998).

FORMALDEHYDE FIXATION

- Formaldehyde, as 10% neutral buffered formalin (NBF) is the most common fixative used in diagnostic pathology.
- The reactions of formaldehyde with macromolecules are numerous and complex.

  $H_2C = O + H_2O \rightarrow HOCH_2OH$
- Methylene hydrate reacts with several side chains of proteins to form reactive hydroxymethyl side groups (–CH_2–OH).
- When the current relatively short fixation times are used with 10% neutral buffered formalin (hours to days), the formation of hydroxymethyl side chains is the primary and characteristic reaction and the formation of actual cross-links may be rare.
- Formaldehyde also reacts with nuclear proteins and nucleic acids (Kok & Boon, 2003; Leong, 2005).

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- Formaldehyde reacts with C=C and –SH bonds in unsaturated lipids but does not interact with carbohydrates.
- The side chains of peptides or proteins which are most reactive with methylene hydrate have the highest affinity for formaldehyde; these include lysine, cysteine, histidine, arginine, tyrosine and the reactive hydroxyl groups of serine and threonine.
- Gustavson (1956) reported that one of the most important cross-links in ‘over-fixation’, i.e. in tanning, is between lysine and the amide group of the protein backbone but again with the current shorter fixation times this is unlikely to occur.

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- Over-fixation of tissue may also be partially corrected by soaking the tissue in concentrated ammonia plus 20% chloral hydrate.
- The principal type of cross-link in short term fixation is thought to be between the hydroxymethyl group on a lysine side chain and arginine (through secondary amino groups), asparagine and glutamine (through secondary amide groups) or tyrosine (through hydroxyl groups) (Tome et al., 1990).
- For example, a lysine hydroxymethyl amine group can react with an arginine group to form a lysine–CH2–arginine cross-link.
- Similarly, a tyrosine hydroxymethyl amine group can bind with a cysteine group to form a tyrosine– CH2–cysteine cross-link.

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- When formaldehyde dissolves in an unbuffered aqueous solution, it forms an acid solution (pH 5.0– 5.5) because 5–10% of commercially available formaldehyde is formic acid.
- Acid formalin may react more slowly with proteins than NBF because the amine groups become charged, e.g. –N+H3.
- In solution, this requires a much lower pH than 5.5.
- Acid formalin also preserves immunorecognition better than NBF (Arnold et al., 1996).
- The disadvantage of using acid formalin for fixation is the

formation of a brown/black pigment with degraded hemoglobin.

- Formaldehyde primarily preserves peptide-protein bonds and the general structure of cellular organelles.

GLUTARALDEHYDE FIXATION

- Glutaraldehyde is a bifunctional aldehyde which probably combines with the same reactive groups as formaldehyde.
- In aqueous solutions glutaraldehyde polymerizes forming cyclic and oligomeric compounds (Hopwood, 1985); it is also oxidized to glutaric acid.
- It requires storage at 4°C and a pH of approximately 5 for stability (Hopwood, 1969).
- Alternatively, the aldehyde groups may react with a wide range of other histochemical targets which include antibodies, enzymes or proteins.
- The reaction of glutaraldehyde with an isolated protein such as bovine serum albumin, is fastest at pH 6–7 and results in more cross-linking than formaldehyde.

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- Extensive cross-linking by glutaraldehyde results in better preservation of the ultrastructure, but this method of fixation negatively affects immunohistochemical methods and slows the penetration by the fixative.
- Any tissue fixed in glutaraldehyde must be small (0.5 mm maximum) and, unless the aldehyde groups are blocked, increased background staining will result (Grizzle, 1996a).
- Glutaraldehyde does not react with carbohydrates or lipids unless they contain free amino groups which are found in some phospholipids.
- At room temperature glutaraldehyde does not cross-link nucleic acids in the absence of nucleohistones, but it may react with nucleic acids at or above 45°C

CHOICE OF FIXATIVES

- The choice of fixative depends on the treatments a tissue is going to receive after fixation like

- what is the chemical structure that needs to be stained?
- If fat is to be demonstrated,formalin fixation for the tissue is recommended.
- For the demonstration of glycogen,formalin should never be used instead alcohol be the fixative of choice.

Formaldehyde

- It is a gas but is soluble in water to the extent of 37 to 40 %w/v.
- The solution of formaldehyde in water is called formalin.
- It is one of the commonly used fixative since it is cheap,penetrate rapidly and does not harden the tissues.

PROPERTIES OF FORMALDEHYDE

- It preserves the protein by forming cross linkage with them and the tissue component.
- It denatures the protein.
- Glycogen is partially preserved hence formalin is not a fixative of choice for carbohydrate.
- Some enzymes can be demonstrated in formalin fixed tissues.
- It neither preserves nor destroys fat.
- Complex lipids are fixed but has no effect on neutral fat.
- After the formalin fixation,fat may be demonstrated in frozen section.
- Also pure formalin is not as satisfactory fixatives as it over hardens the tissues.
- A 10% dilution in tap or distilled water is satisfactory.

ALCOHOL (ETHYL ALCOHOL)

- Absolute alcohol alone has very little place in routine fixation for histopathology.

Properties of Alcohol

- It acts as reducing agents,become oxidized to acetaldehyde and the to acetic acid.
- It is slow to penetrate,hardens and shrinks the tissue.
- Alcohol penetrates rapidly in the presence of other fixative hence in combination like Carnoy's fixative is used to increase the speed of tissue processing.
- Ethanol preserves some protein in relatively undenatured state so that it can be used for immunofluorescence or some histochemical methods to detect certain enzymes.
- It is a fat solvent hence it dissolve fats and lipids.
- Methylene alcohol is used for fixing blood and bone marrow smears.

MERCURIC CHLORIDE

Properties of Mercuric chloride

- It brings about precipitation of the proteins which are required to be removed before staining by using potassium iodide in which they are soluble
- The size that is thickness of the tissue to be fixed in mercuric chloride is important since if the tissue is more than 4 mm,then it hardends the tissue at the periphery whereas the centre remains soft and under fixed
- It penetrates rapidly without destroying lipids
- It neither fixes nor destroys carbohydrates,treatment of the tissue with mercuric chloride brings out more brilliant staining with most of the dyes
- Tissues fixed with mercuric chloride containing fixatives contain black precipitates of mercury which are removed by treating with 05% iodine solution in 70% ethanol for 5-10 minutes,sections are rinsed in water,decolourized for 5 minutes in 5 % sodium thiosulphate and washed in running water

DEHYDRATION

- Paraffin wax is hydrophobic (immiscible with water), most of the water in a specimen must be removed before it can be infiltrated with wax.
- This process is commonly carried out by immersing specimens in a series of ethanol (alcohol) solutions of increasing concentration until pure, water-free alcohol is reached.
- Ethanol is miscible with water in all proportions so that the water in the specimen is progressively replaced by the alcohol.
- A series of increasing concentrations is used to avoid excessive distortion of the tissue.

CONT.....

- A typical dehydration sequence for specimens not more than 4mm thick would be:
- 70% ethanol 15 min
- 90% ethanol 15 min
- 100% ethanol 15 min
- 100% ethanol 15 min
- 100% ethanol 30 min
- 100% ethanol 45 min

CLEARING

- The tissue is now essentially water-free, we still cannot infiltrate it with wax because wax and ethanol are largely immiscible.
- We therefore have to use an intermediate solvent that is fully miscible with both ethanol and paraffin wax.
- This solvent will displace the ethanol in the tissue, then this in turn will be displaced by molten paraffin wax.
- This stage in the process is called “clearing” and the reagent used is called a “clearing agent”.
- The term “clearing” was chosen because many (but not all) clearing agents impart an optical clarity or transparency to the tissue due to their relatively high refractive index.
- Another important role of the clearing agent is to remove a substantial amount of fat from the tissue which otherwise presents a barrier to wax infiltration.

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- A popular clearing agent is xylene and multiple changes are required to completely displace ethanol.
- A typical clearing sequence for specimens not more than 4mm thick would be:
- xylene 20 min
- xylene 20 min
- xylene 45 min

EMBEDDING

- The specimen is thoroughly infiltrated with wax it must be formed into a “block” which can be clamped into a microtome for section cutting.
- This step is carried out using an “embedding centre”where a mould is filled with molten wax and the specimen placed into it.
- The specimen is very carefully orientated in the mould because its placement will determine the “plane of section”, an important consideration in both diagnostic and research histology.
- A cassette is placed on top of the mould, topped up with more wax and the whole thing is placed on a cold plate to solidify.
- When this is completed the block with its attached cassette can be removed from the mould and is ready for microtomy.

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- If tissue processing is properly carried out, the wax blocks containing the tissue specimens are very stable and represent an important source of archival material.

CONCLUSION

- **Fixation** is the preservation of biological tissues from decay due to autolysis or putrefaction.
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