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An Introduction to Pathology Techniques

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Learning Objectives

- Understand the role of study personnel in necropsies.
- Understand the various steps involved in producing glass slides from harvested tissues.
- Understand the ancillary techniques used in toxicological pathology.
- Discover what carcinogenicity, inhalation and crossreactivity studies entail.

This book is aimed at all study personnel – including study monitors, study directors and toxicologists – who are exposed to pathology reports, necropsies, peer review, haematology and biochemistry results and adversity on a regular basis. The secret to an informative, relevant and useful pathology report is an open and collegial relationship between the study director and the study pathologist (Keane, 2014). This chapter aims to describe the various stages of the pathological process (e.g. necropsy, fixation of tissues, cutting of slides) in order to demonstrate where crucial errors which can cause problems at a later stage, may arise. In addition, it includes a brief overview of ancillary techniques that pathologists sometimes use (e.g. electron microscopy). Finally, it discusses carcinogenicity studies, digital pathology, biological drugs and crossreactivity studies, and their impact on study personnel. Throughout the chapter, the client is referred to as the 'sponsor' of a particular pharmaceutical study.

Pathology is the study of disease, particularly the structural and functional changes in tissues and organs. Toxicological pathology is concerned predominantly with cell and tissue injury in animals treated with introduced chemical compounds or biological drugs. Studies are regulated by international bodies such as the Organisation for Economic Co-operation and Development (OECD), the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Animal testing to determine the safety of pharmaceuticals, medical devices and food/colour additives is required by the FDA before it will give approval to begin clinical trials in humans. Pathology data may be quantitative (haematology, chemistry data, organ weights) or qualitative (microscopic diagnoses), and the toxicological pathology report is divided into macroscopic and

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microscopic findings. Study personnel are ultimately responsible for the study report, including the pathology report and data. Thus, study personnel need to understand what the pathology report means and how it has been generated. This chapter aims to help the study director understand the processes involved in a study, from harvesting tissues from the animal to generating glass slides to producing a pathology report.

1.1 Animal Considerations

The main species of animal used in the pharmaceutical industry are rats, mice, dogs, non-human primates, minipigs and rabbits. Occasionally, farm animals, hamsters, cats and gerbils are also used. There are no absolute reasons for selecting a particular animal species for systemic toxicity, but for acute oral, intravenous, dermal and inhalation studies and studies of medical devices, the mouse or rat is preferred, with the option of the rabbit in the case of dermal and implantation studies. Non-rodent species may also need to be considered for testing, although a number of factors might dictate the number and choice of species. Carcinogenicity studies generally use rats and mice.

All animal studies must be conducted according to the animal welfare laws of the country in which they are based, and in general studies may use protected animals only if there are no other reasonable, practicable choices for achieving a satisfactory result. Laboratory animals may only be used in minimal numbers, where they have the lowest possible degree of neurophysiological sensitivity and where the study causes minimal pain, suffering, distress and lasting harm. Animal suffering must be balanced against the likely benefits for humanity, other animals and the environment. In general, in the planning of all preclinical studies, due consideration must be given to reduction, refinement and replacement (the '3 Rs') (Tannenbaum and Bennett, 2015).

Generally, only healthy purpose-bred young adult animals of known origin and with defined microbiological health status should be used in pathology studies (i.e. health monitoring of sentinel animals must be performed before the study starts). Health monitoring involves testing for the various bacteria, viruses, parasites and protozoa that may infect experimental animals and compromise study results (McInnes et al., 2011). The weight variation within a sex should not exceed 20% of the mean weight, and when female animals are used, they should be non-pregnant and should never have borne young.

Laboratory animals should be given a short acclimatisation period at the start of the study. Control of environmental conditions and diet and proper animal care techniques are necessary throughout the study in order to produce meaningful results. The number of animals needed per dose group depends on the purpose of the study. Group sizes should increase with the duration of treatment, such that at the end of the study sufficient animals are available in every group for a thorough biological evaluation and statistical analysis.

1.2 Necropsy

Necropsies or post mortem examinations (Figure 1.1) on experimental animals are a fundamental part of toxicological pathology (Fiette and Slaoui, 2011). They generally take place at the end of a study, but are also conducted if an animal dies early. The necropsy and

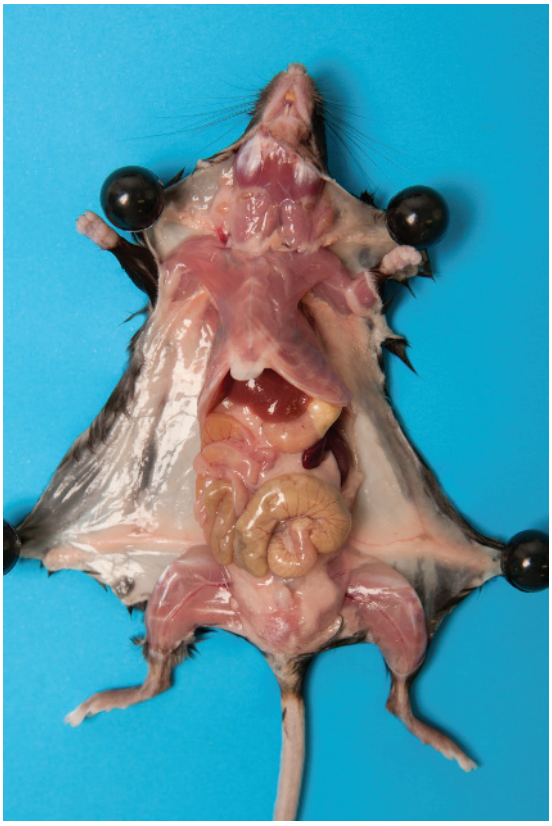

Figure 1.1 Necropsies or post mortem examinations on experimental animals such as this mouse are a fundamental part of toxicological pathology.

pathology data are the single most important aspect of the pathology process, and study personnel get only one chance to retrieve them: once the tissues have been discarded, potentially valuable information is lost forever. At the necropsy, all macroscopic findings and abnormalities visible to the naked eye (e.g. enlarged liver, ulcerated skin, the presence of diarrhoea) are noted and recorded. In addition, tissues are collected for examination under the microscope. The pathologist, necropsy supervisor, prosector, phlebotomist and weighing assistant are all responsible for the recording the macroscopic data (Keane, 2014). Some studies collect all tissues (a full tissue list is indicated in the study plan or protocol), while others harvest only a limited list. A copy of the study plan should be available in the necropsy room to ensure that study personnel collect the correct tissues. Sometimes, all the tissues are collected into formalin, but slides are only cut if the sponsor decides there is a need later on. Harderian glands and draining lymph nodes are examples of tissues that are not always collected: study personnel should check the study plan before beginning the necropsy.

Study directors and toxicologists are often required to attend the necropsies of the animals on their studies. Although not directly involved in the necropsy process, it is nevertheless important that study personnel understand the process and are able to provide advice and management, particularly if unusual tissues are to be collected, severe test article-related findings are observed in high-dose animals, or deviations

from standard operating procedures (SOP) occur. The study pathologist may be consulted if there is an unusually high rate of unscheduled deaths in the study or it is difficult to characterise macroscopic findings (such as very white teeth noted when treatment causes defects in enamel formation) (Keane, 2014).

Carbon dioxide asphyxiation provides a rapid form of euthanasia for mice (Seymour et al., 2004) and rats, but it can cause severe lung haemorrhage, which may make microscopic examination of the lungs difficult. Barbiturate overdose is an effective form of euthanasia, but it requires the use of pentobarbital sodium (Seymour et al., 2004). Larger animals (e.g. rabbits, non-human primates and dogs) are euthanised by an overdose of sodium pentobarbitone, which may cause congestion of some organs and is highly irritant if injected into the tissues around the vein.

Control and treated animals should always be necropsied by the same team of technicians, and the animal numbers and order of examination should be randomised. The identity of an animal is given in a tattoo, ear notch or microchip and should be recorded on all necropsy storage containers in indelible ink.

Organs should be weighed at necropsy; increases and decreases in organ weight can often be correlated with the microscopic findings observed by the pathologist. To ensure meaningful organ weights are recorded, the organs should be taken from an exsanguinated animal (if possible), and excess moisture and adipose tissue should be removed. Guidelines on the weighing of organs are outlined in various papers (Michael et al., 2007; Sellers et al., 2007).

The macroscopic lesions observed at necropsy may be the only pathological data generated from certain studies and must be presented in the form of an incidence table. Consequently, lesions should be described consistently throughout the necropsy process, and standardised terms should be used. The use of an agreed, standardised macroscopic glossary will help to reduce the incidence of different personnel using different terms to describe the same lesion (Scudamore, 2014). In studies in which histopathology will be performed, the macroscopic lesions observed at necropsy are very important to the pathologist, as they often correlate with the lesions observed under the light microscope (e.g. an enlarged yellow liver at necropsy will often have lipid vacuolation in haepatocytes revealed under light microscopy).

Macroscopic lesions at necropsy should simply be described: no attempt at interpretation or diagnosis should be made at this stage (e.g. necropsy staff should not describe an enlarged, mottled liver as 'hepatitis' or a yellow tissue colour should not be described as jaundice). This is because once signed, the anatomic pathology report cannot be easily reinterpreted. All macroscopic lesions observed at necropsy should be described in terms of size and distribution (focal, multifocal and diffuse), colour and consistency (soft, friable, firm, hard, fluid filled, gritty, etc.). The location, size and number of a mass or lesion should be recorded. A standard diagram of the dorsal and ventral aspects of the animal is useful for recording the exact locations of lesions and masses. All measurements should be made in millimetres, and terms such as 'enlarged', 'pale' and 'small' should be avoided or should be accompanied by an actual measurement or colour. In particular studies, it may be useful to photograph certain lesions in order to illustrate their exact nature and severity to future study personnel. However, although photographs are a good record of macroscopic lesions observed at necropsy, there may be Good Laboratory Practice (GLP) and legal issues to contend with (Suvarna and Ansary, 2001).

Autolysis occurs within 10 minutes of the death of an animal (Pearson and Logan, 1978), so necropsy should be performed as quickly and efficiently as possible, with

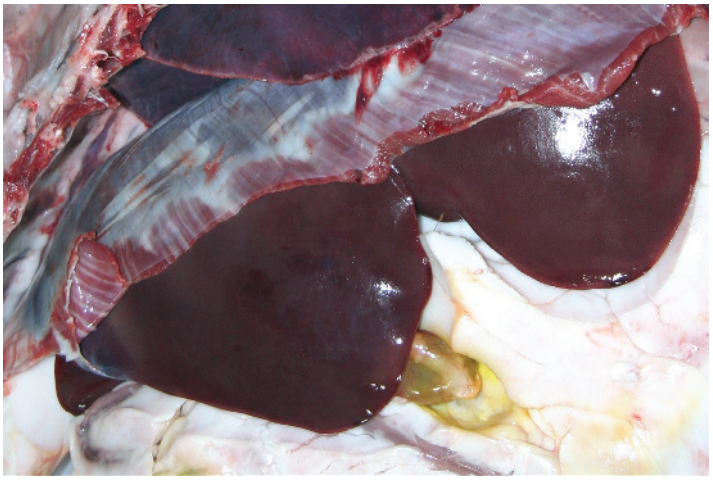

Figure 1.2 Post mortem imbibition of bile pigment in the mesenteric fat tissue adjacent to the gall bladder. Photograph taken from a bovine necropsy.

limited tissue handling, squeezing and tissue damage. Post mortem change occurs as a result of autolysis (action of enzymes from the ruptured cells on the dead animal's cells) and putrefaction (degradation of tissue by the invasion of certain microorganisms); changes include *rigor mortis* (stiffening of limbs and carcase), clotting of the blood, hypostatic congestion (pooling of blood into the dependent side of the carcase, termed '*livor mortis*'), imbibition of blood (or bile pigment; Figure 1.2) and gaseous distension of the alimentary tract. In addition, pseudomelanosis (the greenish or blackish discolouration of tissues due to ferrous sulphide) tends to occur in organs that lie adjacent to the intestines, such as the liver. Most of these changes will be visible if an animal dies during the night or on the weekend, and every effort should be made to store the carcase in a fridge and to perform a necropsy as soon as possible thereafter.

1.3 Lung Inflation with Fixative

Tracheal instillation of the lungs with fixative at necropsy is required to improve the histology of the pulmonary architecture in mice and rats, and is recommended for all rodent studies. Tracheal instillation of fixative may be performed either after removing the lungs from the thoracic cavity or with the lungs in situ (Braber et al., 2010). It may sometimes be easier to inflate only one lung lobe, using a needle and syringe to inject formalin (Knoblaugh et al., 2011).

1.4 Fixation

In general, fixation of tissues maintains cellular integrity and slows the breakdown of tissues by autolysis. The most common fixative is 10% neutral buffered formalin, which ensures rapid tissue penetration, is easy to use and is inexpensive. However, formalin is

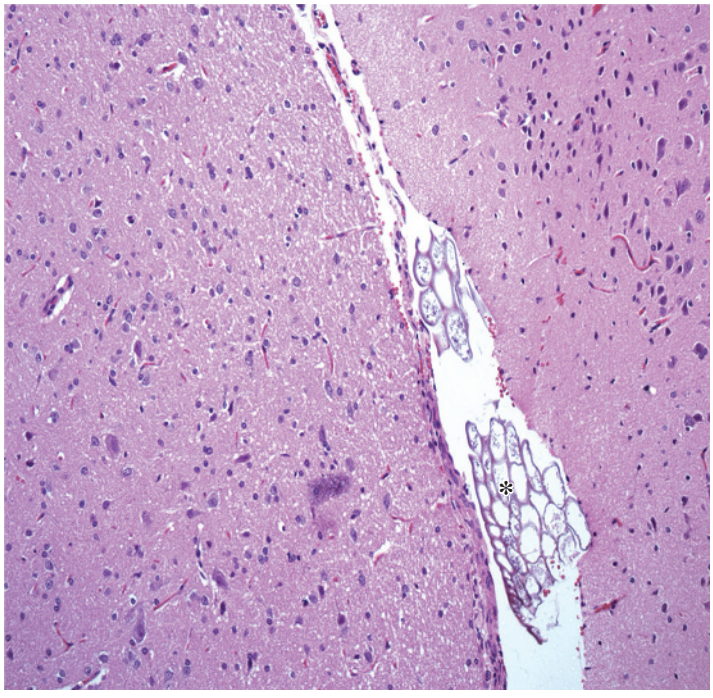

Figure 1.3 Artefacts induced at necropsy include inclusions of foreign material into the issue, such as plant material (*) during brain removal.

highly toxic and carcinogenic and may have effects on the immune system (Costa et al., 2013). Tissues should be fixed at a 1 : 10 or 20 ratio of fixative to tissue for at least 48 hours. Modified Davidson's is the recommended fixative for eyes and testes, as it prevents retinal detachment in the eye and separation of cells lining the seminiferous tubules in the testes. Glutaraldehyde or osmium tetraoxide is used for the fixation of tissues intended for electron microscopy. Artefacts which occur at necropsy include inclusions of foreign material into the tissue (e.g. plant material during brain removal (Figure 1.3) and the incorporation of sharp shafts of hair into soft tissues) and pressure and pinch effects (from forceps) (McInnes, 2011). These can be confused with lesions by an inexperienced pathologist.

1.5 Making Glass Slides

The production of glass slides suitable for histopathological analysis involves a number of steps performed in the histology laboratory (Figure 1.4).

1.5.1 Trimming

In the first step, formalin-fixed tissues collected during the necropsy are further cut up in order to fit into the embedding cassettes (Knoblaugh et al., 2011). Two steps in the pathology phase of the study cannot be repeated: the necropsy and the macroscopic tissue trimming. This is because if tissues are discarded after the necropsy or at trimming,

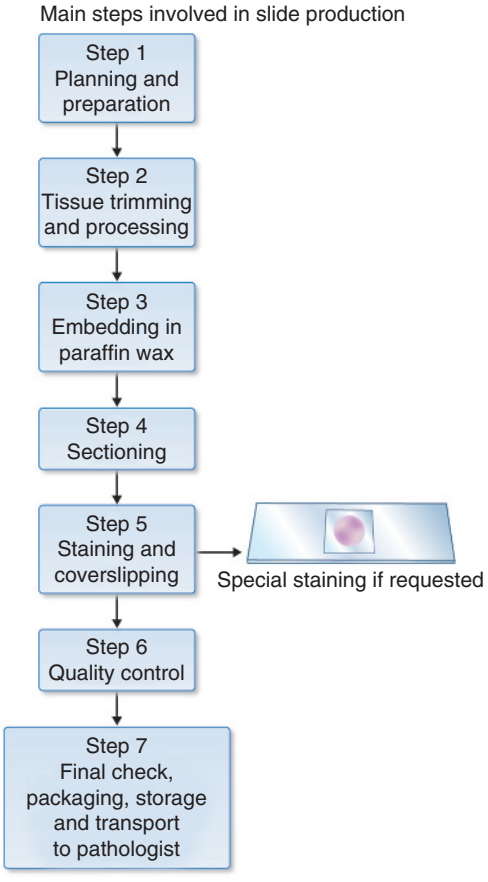

Figure 1.4 Production of glass slides suitable for histopathological analysis.

it is impossible to return to them. Great care should thus be exercised at the trimming stage. All tissues should be trimmed in the same way, from the same area in the organ, and all described gross lesions must be identified and included in the cassette (Figure 1.5). The cassette lid will cause impression marks on the tissue surface if the tissue is too big for the cassette (Knoblaugh et al., 2011). Excellent trimming and blocking patterns indicating how to section each tissue and which tissues should be placed together in a cassette are contained in Ruehl-Fehlert et al. (2003), Kittel et al. (2004) and Morawietz et al. (2004). The staff involved in trimming should be aware that variations in the incidence of certain lesions (such as thyroid C-cell findings and thyroid tumours) can be associated with the type of section taken (i.e. transverse compared to longitudinal).

It is essential that the cassette be marked correctly with the animal's identification number, sex and group, and with either the tissue name(s) or a number indicating which tissues are always trimmed into that particular cassette (Figure 1.6). The blocking sheet (Figure 1.6) indicates which tissue has been processed in which cassette. Multiple tissues may be grouped in one cassette (e.g. different tissues from the gastrointestinal tract), but certain tissues (e.g. adrenal and bone) should not be grouped together, since differences in consistency will cause problems during the microtoming of the wax

Figure 1.5 Tissue trimming and placement in a cassette.

Figure 1.6 The blocking sheet.

blocks. The collection and histological examination of heart valves was overlooked in the past, but with the introduction of reporting of valvular lesions associated with the use of antiobesity drugs (fenfuramine-fentermine) (Connolly et al., 1997), these tissues must now be examined. Thus, histology technicians now endeavour to cut the heart into sections, which allows for the visualisation of the major aortic and pulmonic heart valves. Artefacts introduced at this stage include small pieces of tissue from the previous cassette superimposed on another tissue (due to failure to clean the knife between tissues and between animals) (Figure 1.7) (McInnes, 2011).

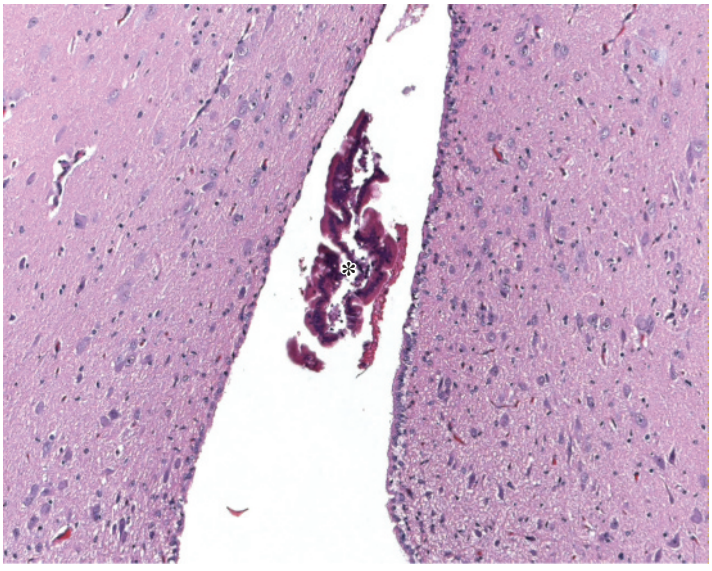

Figure 1.7 Small pieces of tissue (*) from a previous cassette superimposed on another tissue.

1.5.2 Tissue Processing

After trimming, the cassettes are placed in a machine that allows the tissues to undergo a series of steps which include tissue dehydration, clearing and impregnation with paraffin wax (Figure 1.8). Paraffin wax serves to keep tissue firm and intact, and in the correct orientation for sectioning.

1.5.3 Embedding

During embedding, a trained technician places the paraffin wax-infiltrated tissues and additional wax into a mould, which is chilled to produce tissue blocks.

1.5.4 Microtoming

During microtoming, thin sections (~4–6 μm) are cut from the wax block using a rotary microtome (Figure 1.9). The operation of this device is based upon the rotary action of a hand wheel, which advances the specimen (wax block) towards a rigidly held blade. The thin wax sections are then floated in a water bath, and appropriately identified glass slides are used to scoop them out of the water. The glass slides are placed in an oven to melt off the wax, leaving only the unstained tissue. Histology technicians require fine motor skills in order to ensure that all of the anatomic features of small tissues such as the adrenals and pituitary (e.g. cortex and medulla of adrenal) are displayed on a single slide. The formalin fixed wet tissues may be discarded after the study is finalised, however the wax tissue blocks and the glass slides are archived according to the protocol (Keane, 2014).

1.5.5 Staining

Before staining, all tissues are transparent, and it is hard to make out any cellular detail under the microscope. For this reason, stains are bound to different parts of the tissue,

Figure 1.8 Machine for tissue dehydration, clearing and impregnation with paraffin wax.

allowing these components to be visualised. Histochemical stains are made up of chemical dyes that bind to tissues by the same mechanism as a chemical interaction. The most commonly used stain is haematoxylin and eosin (H&E), which stains acidic tissue components pink (the eosin binds to the cytoplasm, making it pink) and basic tissues blue (the haematoxylin binds to the nuclei of the cells, making them blue) (Figure 1.10).

After staining, the section is mounted with a coverslip to prevent the tissue from drying out, prevent surface damage to the tissue and improve tissue transparency. If the pathologist discovers that a tissue is missing, all attempts must be made to find the missing tissue (by going back to the wet tissues, or by cutting deeper into the original block if no wet tissue remains); if a tissue is inadequate, it must be improved (by re-embedding). Occasional missing or inadequate tissues are acceptable in a study, but large numbers will compromise its completeness, and the study may have to be repeated – at great cost.

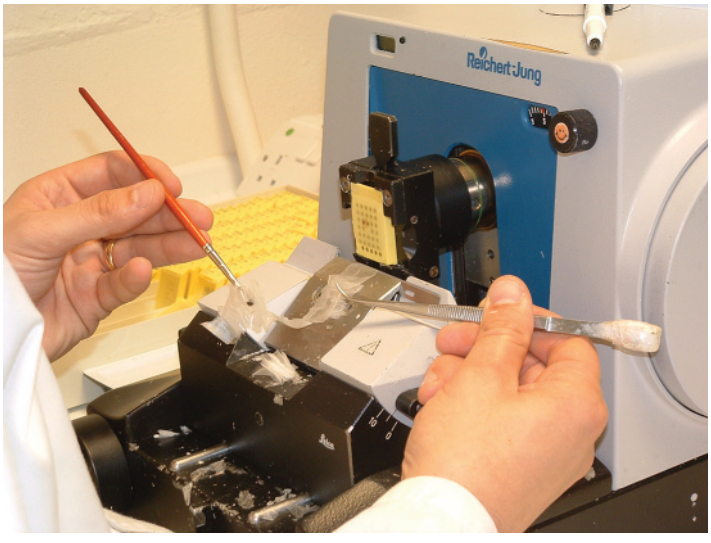

Figure 1.9 Rotary microtome.

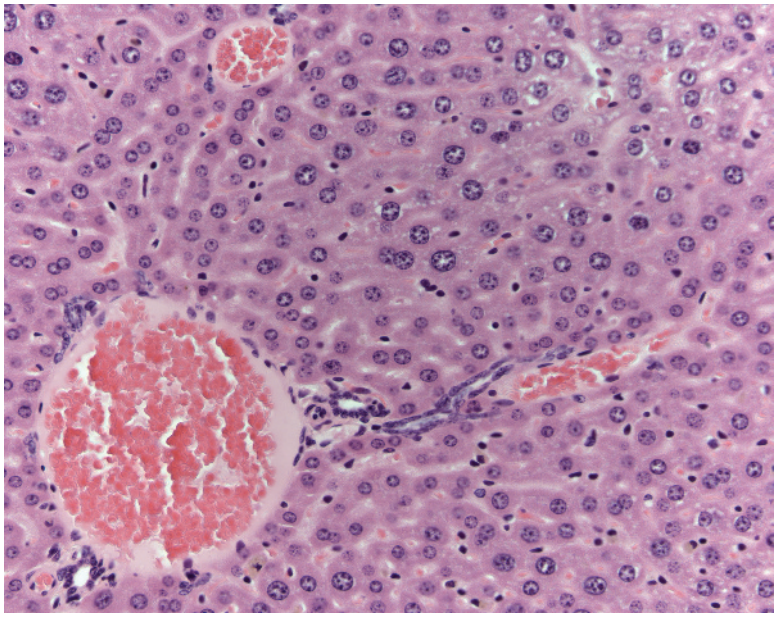

Figure 1.10 H&E staining.

1.5.6 Quality Control

The final step in slide processing is to carefully check the glass slide for artefacts, to make sure that the information on the block is the same as that on the slide and to ensure that the slides have been arranged in the correct order according to the blocking sheet (which pertains to one animal) (Figure 1.11).

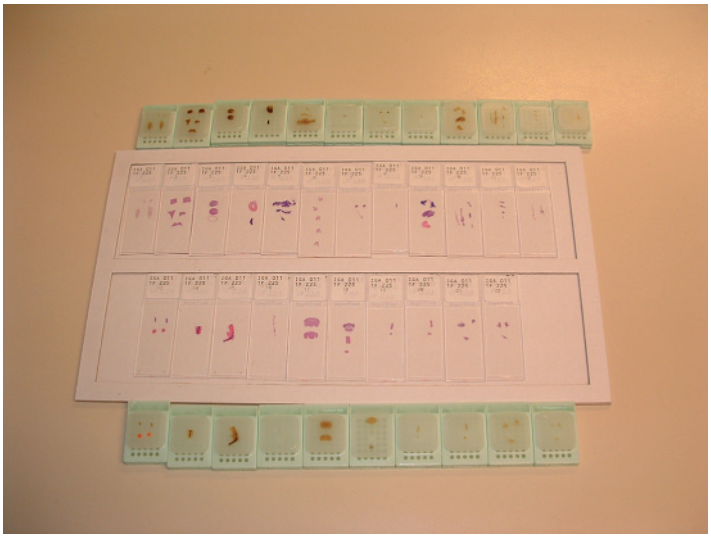

Figure 1.11 Quality control.

1.6 Special Histochemical Stains

Stains with specific affinities for different tissue components (e.g. calcium, fat) can be used to confirm the identity of these tissues or substances (see Table 1.1). Examples include phosphotungstic acid haematoxylin (PTAH) (Figure 1.12), which demonstrates muscle striations; periodic acid–Schiff (PAS), which stains glycogen and carbohydrate; Congo red, which stains amyloid; and Oil Red O, which stains lipid (can only be used on fresh-frozen tissue that has not been fixed in formalin). Pathologists may ask for special

Table 1.1 Special histochemical stains.

Substance	Tissue seen in	Special stain used
Bile	Liver	Fouchet's
Lipofuscin	Various (generally older animals)	PAS, Sudan black B, Schmorl's, Long Ziehl-Neelsen technique
Glycogen	Liver, muscle	PAS with diastase
Haemosiderin (golden brown)	Spleen, others	Perl's Prussian blue
Formalin pigment (artefact)	Blood-rich tissues, large areas of haemorrhage	- (need to extract it from sections using picric acid)
Melanin (more dark brown–black)	Lung, muscle, others	By exclusion
Fat	Liver	Oil Red O on frozen non-formalin fixed tissue
Collagen	Scar tissue, tumour	Masson's trichome

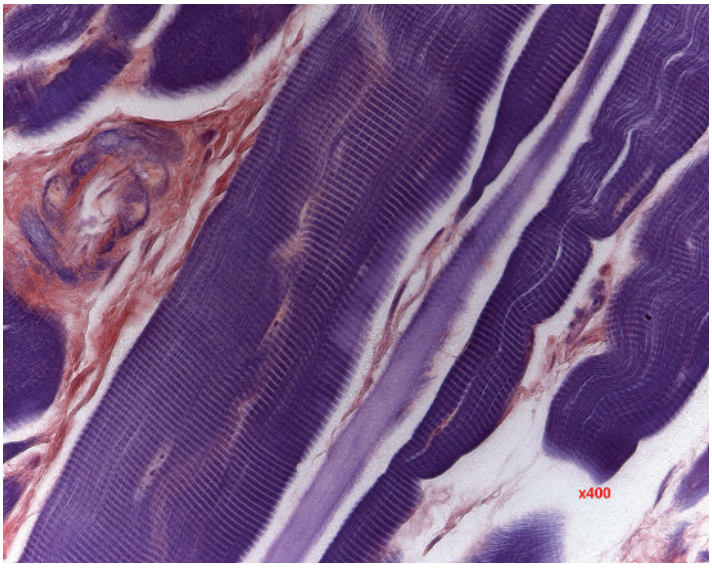

Figure 1.12 Phosphotungstic acid haematoxylin (PTAH).

stains after examining a slide (e.g. to confirm the presence of collagen and thus the diagnosis of a fibrosarcoma) and this will necessitate a protocol amendment (Keane, 2014). Alternatively, special stains may be incorporated into the study plan from its inception (e.g. when a sponsor requests special staining of all fat tissue in order to assess the effect of compounds such as peroxisome proliferator-activated receptor (PPARs) agonists on white and brown fat; Long et al., 2009).

1.7 Decalcification

Tissues which contain high levels of calcium slats such as bone and teeth are difficult to cut so they require decalcification with solutions such as formic acid to remove the calcium salts, soften the tissue and make it less brittle and easier to cut. Artefacts associated with excessive periods of tissue decalcification have been described (McInnes, 2011).

1.8 Immunohistochemistry

Immunohistochemistry (IHC) is used when H&E staining does not give sufficient information about the cell type of interest. Immunohistochemistry is requested by the pathologist when he or she wants definite confirmation or detection of a specific cell type. For instance, H&E staining cannot distinguish between T- and B-cells but IHC using antibodies against CD3 and CD19 can; thus, IHC is a method of detecting a molecule or epitope on a cell, in situ, in a tissue section, using an antibody to that molecule and a visible label.

Immunohistochemistry is useful in the identification of unknown tumours and in the detection of infectious agents. Toxicological pathologists may need to know the

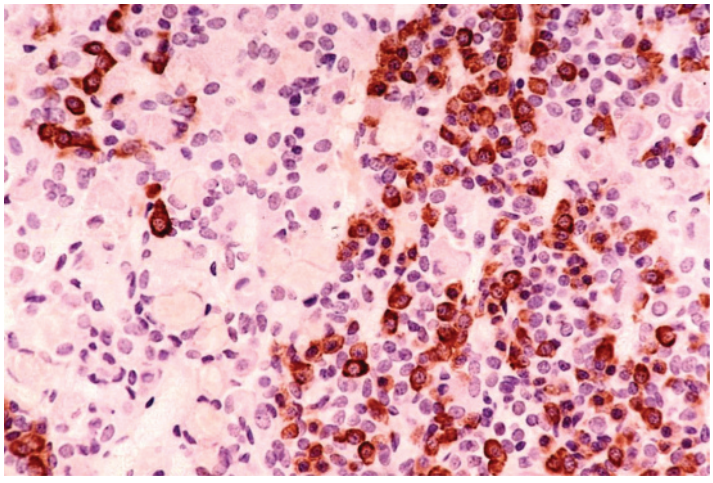

Figure 1.13 Growth-hormone IHC stains positive cells in pituitary.

exact identity of a cell that displays test article-related changes; markers such as glial fibrillary acid protein (GFAP) (which stains reactive astrocytes), synaptophysin and chromagranin (which stain neuroendocrine cells), growth hormone in pituitary cells (Figure 1.13) and LAMP-2(+) for hepatic phopholipidosis are thus all useful. Other common antibodies include proliferating cell nuclear antigen (PCNA), which stains cells in active proliferation (G1, G2, S and M phases), and the TUNEL and caspase 3 antibodies, which stain cells in apoptosis.

Fresh tissue samples frozen in OCT compound (Tissue Tek, UK) are optimal for IHC or in situ hybridisation, but formalin fixed tissue may also be used provided various techniques are applied to break down the formalin bonds (such as microwaving the tissue in citrate buffer, which is called 'antigen retrieval'). Cell morphology is generally poorer in fresh-frozen sections than in paraffin wax-embedded sections, but more antibodies tend to work on fresh-frozen tissue.

Mast cells are usually fairly easy to detect, due to their prominent granulation, but they can be highlighted, if required, using the histochemical stain toluidine blue or by using IHC with the antibody against CD117. Mononuclear immune cells such as macrophages and lymphocytes in rodents may be identified using a basic panel of IHC markers for macrophages (F4/80, Mac2), T-cells (CD3, CD4, CD8) and B-cells (CD19, CD23) (Ward et al., 2006).

Challenges in IHC include the fact that most antibodies are developed against human antigens, and thus toxicological pathologists are never sure whether a given antibody will work in rodent tissues (good positive and negative controls are essential); the difficulty of cutting suitable cryostat sections from frozen tissues; the problems involved in unmasking antigens (antigen retrieval) in formalin-fixed tissue; and high levels of background staining. Polyclonal antibodies bind to several different epitopes and are thus more sensitive but less specific than monoclonal antibodies, which bind to a single epitope.

1.9 Tissue Crossreactivity Studies

Tissue crossreactivity (TCR) studies are conducted in pharmaceutical environments and contract research organisations in order to check for off-target antigen binding and on-target binding in unusual tissues with new monoclonal antibodies intended for therapeutic use. The value of TCR studies lies in the prediction of toxicity that would not have been detected using in vivo pharmacology and toxicology studies in pharmacologically relevant animal models (Geoly, 2014). Generally, a complete set of frozen human and animal tissues is available for immunolabelling with the new antibody. The TCR assay by itself has variable correlation with toxicity and efficacy (Leach et al., 2010), and crossreactivity studies often involve a great deal of background staining in various animal species and in human tissues, which is difficult to interpret and may not always be the best method of determining the safety of a new antibody.

1.10 Electron Microscopy

Electron microscopy is used to confirm findings observed in H&E-stained tissue sections, such as hepatic hypertrophy (increase in peroxisomes and in endoplasmic reticulum), and in conditions such as phospholipidosis (lamellar bodies) (Figure 1.14). Transmission electron microscopy (TEM) produces two-dimensional (2D) photograph

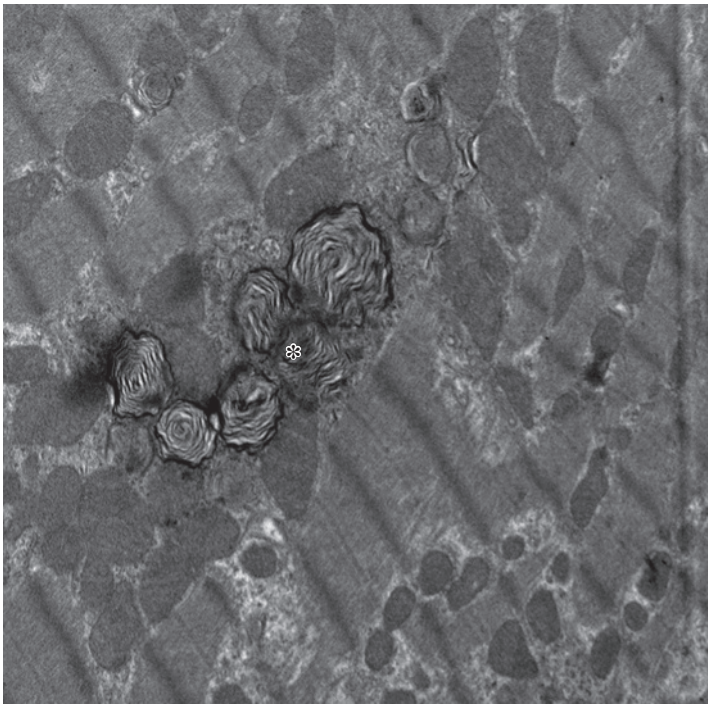

Figure 1.14 Phospholipidosis is characterised by lamellar bodies (*), which are visible in this electron micrograph of a rat heart.

of a thin section of tissue, whilst scanning electron microscopy (SEM) produces a three-dimensional (3D) photograph of the surface of a tissue. Generally, TEM is used because it provides greater resolution than does a light microscope and because it produces pictures of actual cells and their organelles and nuclei. In general TEM provides photographs which demonstrate cellular organelles, mitochondria, rough and smooth reticulum and lysosomes, phopholipidosis, inclusions and peroxisomes. In contrast SEM is useful in assessing the surface of the intestine in order to see bacterial attachment or the destruction of the villi. The disadvantages of electron microscopy include the cost, the use of particular fixatives, resin-embedding and the requirement for trained technicians to produce useful photographs. This has led to a recent reduction in its use.

1.11 In Situ Hybridisation

In situ hybridisation (ISH) can be used to detect RNA or DNA of interest in tissue sections. Polymerase chain reaction (PCR) and other gel/blot methods do not identify in which cell(s) the RNA has been increased (upregulated). Thus ISH is used to allow the pathologist to see which cells are producing a particular DNA or RNA product, because it provides the advantage that the architecture of the tissue is maintained (which is not the case in PCR).

In common with IHC, ISH requires different types of probe. Probes can be lengths of DNA or RNA. The RNA probes are often called 'riboprobes'. The most commonly used DNA probe in ISH is the oligonucleotide probe. Probes must have a sequence of bases (A,T,C,G) that is complementary to the mRNA of interest (i.e. if the base on the mRNA is C, the probe needs to be G). A number of companies will synthesise probes and label them. Binding of probe to target is termed 'hybridisation'. A label is used to visualise the probe in a particular cell or tissue.

Tissue microarrays are increasingly used to screen large numbers of tissues using either IHC or ISH in pharmaceutical environments. Tissue microarrays consist of multiple tissue samples embedded in a single wax block, used to produce a single glass slide containing many small samples of different tissues.

1.12 Laser Capture Microscopy

Areas of interest within a tissue section can be cut out of the section using a laser. The tissue can then be used in a number of subsequent techniques, such as amplification of mRNA in the area of interest by reverse transcriptase polymerase chain reaction (RT-PCR) or IHC.

1.13 Confocal Microscopy

Confocal microscopy provides high-resolution photographs of 2D or 3D fluorescent images. The confocal microscope has a pinhole and is efficient at rejecting out-of-focus fluorescent light. The practical effect of this is that an image comes from a thin section

of the tissue. By scanning multiple thin sections in a sample, one can build up a very clean 3D image of the sample (Prasad et al., 2007).

1.14 Image Analysis

Image analysis provides quantitative assessments of cell number, cell size, lesion extent and the staining of a particular antibody in an organ, amongst other things. A computer program is used to generate the data, to which statistics can be applied. Further information about image analysis can be found in Scudamore (2014) and in Chapter 8 of this volume.

1.15 Digital Imaging

Digital imaging allows glass slides to be scanned and examined remotely online. This enables the slides to be viewed by multiple parties without the need for transport to different countries. Digital imaging is useful for quantitative analysis of cell and nuclear sizes and for examination of difficult lesions by multiple pathologists in different locations. It also facilitates the peer-review process.

1.16 Spermatocyte Analysis

Recognising the different cellular associations that make up the spermatogenic cycle within seminiferous tubules of the testis is essential to identifying when cell populations are missing. A further challenge in the male animal is how to recognise immaturity and distinguish it from degenerative conditions (Creasy, 2011). This is a problem in the dog and non-human primate. The use of the correct fixative to preserve the testes (Lanning et al., 2002; Latendresse et al., 2002) is essential for spermatogenesis testing. Examination of the different stages of the spermatogenic cycle in the testis of the rat and mouse using PAS-stained testis is a valuable technique that allows pathologists to evaluate spermatocyte development and identifies test article-related effects at various stages, if they exist (Creasy, 1997). Generally, most rodent studies will require the examination of a PAS-stained slide of the testis of the male rats and mice to ensure that no treatment-related findings have occurred in the male reproductive organs.

1.17 Good Laboratory Practice

Most studies in contract research organisations are conducted according to GLP (HHS, 1992). Medical devices, drugs and biological products intended for veterinary or human use are regulated by the EMA or FDA and must undergo preclinical testing for safety (generally in laboratory animals) before clinical trials in humans can begin. Adherence to GLP is intended to ensure that the quality and integrity of the data sent to the regulatory body can be relied upon and that no alteration of data have occurred! All

regulations concerning GLP studies are comprehensive; they include standards for all personnel, facilities, equipment, test articles and records. Briefly, GLP requires the identification of individuals who fulfil responsibilities of management (as defined by GLP), as well as GLP-compliant facilities, equipment and materials. Training is essential for all personnel who are required to be GLP-compliant, and a quality-assurance (QA) department is required to inspect facilities and examine protocols and reports. The SOPs underpin GLP and should be in place for the histology, haematology, biochemistry and other laboratories. The SOPs should be available for the collection and identification of specimens, conduction of necropsies and the reception, identification, storage, and testing of control and test materials (e.g. serum, formalin-fixed tissues).

1.18 Inhalation Studies

Inhalation pathology concerns test article-related findings in the tissues of the upper respiratory system, which includes the larynx, nasal turbinates, trachea, tracheal bifurcation and associated lymph nodes. Rats, mice, dogs and non-human primates are commonly used in inhalation studies. Large structural differences have been described amongst these animal species in the anatomy of the nasal cavity, which cause variations in the intranasal airflow and deposition of particles, as well as total air volume. In addition, there are species differences in the turbinate sizes and consequent epithelial surface areas. Inhalation studies are more specialised than routine safety studies, and study personnel need to know the significance of animal inhalation lesions and background lesions in order to assess the risk of a particular inhaled compound for humans.

Four epithelial types are found in the nasal cavity/nasal turbinates (Monticello et al., 1990): squamous epithelium, transitional epithelium, respiratory epithelium and olfactory epithelium. Standardisation of nasal sections for histopathological evaluation is essential, so the four sections of the rat and mouse nasal turbinates are always cut in the same locations of the upper palate (Young, 1981). In rodents, the base of the epiglottis is an important predilection site for xenobiotic damage, where sensitive epithelium overlies the submucosal glands. Ultimately, cynomologus monkeys may be a better laboratory animal for testing human inhaled compounds, as their nasal structures more closely resemble those of humans than do those of rodents. Good laboratory practice ensures that raw data are uniform, consistent, reliable and reproducible (Keane, 2014).

1.19 Continuous-Infusion Studies

Continuous-infusion studies involve the use of medical pumps attached to the animal (generally in cloth pouches), which allow for the continuous infusion of a compound into a particular vein (via a catheter). They have a number of particular problems, including how to choose the species best suited to an indwelling catheter, the infusion technique, the surgical method used to attach the pump and insert the catheter, the chemical composition of the catheter material and the injected compound and the choice of blood vessel and its diameter, as well as how to keep the catheter open during the in-life period (Weber et al., 2011). Polyurethane catheters are preferred over polyethylene catheters, which can harden, become brittle and release small amounts of

catheter substance into the circulation (Weber et al, 2011). A continuous-infusion study in the dog using the saphenous vein will not be sustained for more than 3 days, because the animal scratches the wounds and pulls the catheter out of the blood vessels (Weber et al., 2011).

The length of the jugular is often overestimated in rabbits and dogs, and the catheter extends into the ventricles of the heart, where it causes inflammation of the endocardium (Weber et al., 2011). Lesions such as chronic inflammation, fibrosis, foreign-body reactions to sutures (granuloma formation), abscess and thrombosis and haemorrhage are typical at the site of insertion of the catheter into the blood vessel (Weber et al., 2011) and should be distinguished from test article-related lesions. High incidences of abscesses and necrosis in the study animals suggest poor technique and make the study invalid.

1.20 Carcinogenicity

Carcinogenicity studies are generally conducted in mice and rats, which are exposed to test compounds for a period of approximately two years in order to establish whether they cause an increased incidence of tumours in the treated animals compared to non-treated controls. Carcinogenicity studies require large numbers of animals (generally 50 per sex group) in order to ensure that sufficient numbers will still be alive at the end of 104 weeks. Expected benefits of carcinogenicity studies include an increase in the safety of people exposed to chemicals that have passed toxicity tests, increased efficiency during the development of human pharmaceuticals and decreased wastage of animals, personnel and financial resources. Carcinogenicity studies are expensive and require excellent management and organisation skills amongst study personnel. In addition, statistical design issues (e.g. proper randomisation of animals, sample size considerations, dose selection and animal allocation issues, as well as the control of potentially confounding factors such as littermate and caging effects) will need to be addressed (Haseman, 1984). Since carcinogenicity studies take a long time and consume many resources, they should only be performed when human exposure warrants the collection of data from life-time studies in animals.

Recently, the poor predictability and correlation between treated rodents and humans in conjunction with their substantial animal-welfare and economic costs, have brought into question their purpose and future (Knight, 2007).

1.21 Biologicals

Biological medical products, or 'biologicals', are compounds manufactured in or derived from biological sources. This means that they are different to pharmaceutical products which are chemically produced, such as anti-inflammatory drugs. Examples of biologicals include living cells (such as stem cells), vaccines, gene therapy, recombinant proteins and blood and blood products. Biologicals can consist of sugars, proteins or nucleic acids, or they may be cells and tissues. Biologicals may be regulated through different methods than conventional chemical compounds.

1.22 The Pathology Report

Pathologists record their findings in a secure and validated computer data-capture program (such as Provantis), which allows for table generation and statistical analysis (see Chapter 2). Peer review is required for GLP-compliant studies and is conducted to produce consistent and reliable data and report (see Chapters 2 and 8). Once the study pathologist and the peer-reviewing pathologist have agreed on the findings, the study pathologist makes the necessary changes to the data, locks the data and generates a final set of tables and a final report. This report is then sent to the study director for incorporation into the final study report. Quality pathology reports are generated when motivated study personnel and the study pathologist communicate and work together (Keane, 2014).

The pathology report must be concise and accurate, and it should state the significance of its findings (i.e. what is and what is not test article-related). The macroscopic and histopathological findings should be interpreted in conjunction with the haematology and biochemistry results, as well as the organ weights and macroscopic findings should be correlated with histopathology findings if possible. A statement about the no-observed-adverse-effect level (NOAEL) should also be included (see Chapter 7). The pathology report should also identify any treatment related, unscheduled deaths which occurred before the study was terminated (Keane, 2014).

1.23 Conclusion

It is impossible to list all of the potential pathological techniques used by all pharmaceutical companies and contract research organisations. However, this chapter has attempted to cover some of the more common techniques. It should also provide useful information about how the glass slides used in toxicological pathology studies are made.

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