

Morphological studies in modern teratological investigations

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Despite the variety of modern molecular techniques available, examination of foetal anatomy is still a fundamental part of teratological studies in evaluating the developmental toxicity of xenobiotics or other non-chemical factors. The article presents contemporary methods of embryotoxicity and foetotoxicity assessment. A single alizarin red S and double alcian blue followed by alizarin red S staining, as well as various methods of soft tissue examination are discussed.

Key words: bone, cartilage, developmental toxicity, foetus, methods, risk assessment, soft tissue examination, skeleton, staining, visceral examination

INTRODUCTION

Teratology is an interdisciplinary science, which investigates causes, mechanisms and manifestations of abnormal development. Until the 20th century the science was mostly theoretical and described only observed congenital malformations. However, a pioneer experimental study by Hale revealed the adverse developmental effect (anophthalmia) of vitamin A deficiency in pigs. The detrimental prenatal effect of maternal dietary deficiency was also the subject of the first conventional teratological experiment on laboratory animals by Warkany and Nelson in 1940. Their observations were confirmed in humans only a few months later among victims of starvation in Holland and in Leningrad in the Soviet Union during World War II [4, 37]. However, until the 1960s, when the public was shocked by the thalidomide tragedy, prenatal toxicity studies were not regulated. The first

principles were drawn up by the experts of World Health Organization in 1967 [35]. These were later improved after observations of humans. In 1952 behavioural and functional complications such as ataxia, mental retardation and disturbances of gait, vision and speech, later named Minamata disease, were noted in children prenatally exposed to methylmercury. In addition, in the 1970s, an increased incidence of cancer was found in offspring after *in utero* exposure to diethylstilbestrol [4, 12].

As a result of the many modifications and differences between one country and another concerning factors such as the duration of the administration period, the species used and the number of animals, the International Conference for Harmonization (ICH) prepared new Guidelines for the Detection of Toxicity to Reproduction for Medicinal Products [14, 16, 21]. This was approved by the US Food and Drug Admin-

istration (FDA) and similar governmental authorities in the European Union and Japan. Separate regulations for testing biotechnological products, vaccines, food ingredients and specific chemicals were also drawn up [12, 20, 36].

At present, 6 types of study must be carried out before drawing a final conclusion on reproductive and developmental toxicity. All have to be conducted exclusively on laboratory animals and with good laboratory practice [18, 19, 28, 34]. According to the administration period of the tested compound and evaluated outcome, the following ICH stages (experiments) are performed [12, 15, 16]:

- stage A (from the pre-mating period to conception) evaluates reproductive functions in adult animals, including spermatogenesis, oogenesis and fertilisation;
- stage B (from conception to implantation) evaluates adult female reproductive functions, as well as the pre-implantation and implantation phases of the *conceptus*;
- stage C (from implantation to the closure of the hard palate) evaluates adult female reproductive functions and embryo development on the basis of the formation of the major organs;
- stage D (from the closure of the hard palate to the end of pregnancy) evaluates adult female reproductive functions, organ development and foetal growth;
- stage E (from birth to weaning) evaluates adult female reproductive functions and the adaptation of the neonate to extra-uterine life;
- stage F (from weaning to sexual maturity) evaluates post-weaning development and growth, adaptation to independent life and attainment of full sexual function.

The first 5 types of experiment (stage A–E) may be performed as single or multi-generational studies. In addition, the experts agreed to longer periods of continuous drug administration and breeding [14].

EVALUATION OF MATERNAL, EMBRYONIC AND FOETAL TOXICITY

According to the ICH regulations, reproductive toxicological investigations should be conducted by a single developmental study on two different species, including rodent and non-rodent laboratory animals. Rats and rabbits are usually used. The period of xenobiotic administration for these animals at ICH stage C, which is the most commonly performed mode for the study, is from the 6th/7th to the 17th gestational day for rats (day 0 = insemination) and from the 6th/7th to the 19th

day for rabbits [12, 23, 24]. The dams are terminated on days 20/21 and 29 respectively, and caesarean section is performed. Gross pathological examination of the thoracic and abdominal viscera of each dam is routinely performed. On the basis of the general toxicity of the compound tested, the target organs should be histologically examined and the blood collected for biochemical or other specific analyses. Finally, the uterus and ovaries are removed, dried in absorbent paper, and weighed. The implantation sites are counted through the transparent uterine wall. To reveal early implantation sites which are not clearly visible, staining with 10% ammonium sulphide solution should be applied. Both ovaries are removed and carefully examined to determine the number of *corpora lutea*. The uterine horns are cut along the anti-mesometrial (greater) curvature and macroscopically examined to evaluate the position of early or late resorptions and live or dead foetuses, which are numbered subsequently from the ovarian end of each horn. The number of *corpora lutea*, implantations, resorptions and foetuses are necessary to calculate pre-implantation and post-implantation loss using the following formulae [12]:

$$\begin{aligned} \text{Pre-implantation loss (\%)} &= [(\text{no. } \textit{corpora lutea} - \\ &\quad - \text{no. implantations}) / \text{no. } \textit{corpora lutea}] \times 100 \\ \text{Post-implantation loss (\%)} &= [(\text{no. implantations} - \\ &\quad - \text{no. live foetuses}) / \text{no. implantations}] \times 100 \end{aligned}$$

Finally each foetus is separated from its amniotic sac and placenta, which is individually weighed and grossly inspected. During this procedure the amniotic fluid may be collected for various biochemical and immunological tests.

In our laboratory the live foetus investigation includes observation of breeding and examination of some reflexes only. Other studies are performed after hypothermia euthanasia done in liquid nitrogen mist [3, 6, 7]. Carbon dioxide, oral barbiturane administration or intraperitoneal injection of pento-barbital is also used [24]. After euthanasia each foetus is carefully inspected externally (including palate examination) to evaluate potential external developmental abnormalities, and then measured. Foetal weight and crown-rump length are usually checked but the tail length is also routinely measured. In the event of expected cranial anomaly other head measurements may be carried out [37].

After completion of the external examination the foetuses are divided into two study groups. Half the animals from each litter are used for soft tissue examination and the remainder for skeletal examination [12].

SKELETAL EXAMINATION

Two methods of skeletal examination of laboratory animals are currently used, namely single and double staining.

Double staining is labour-intensive and time-consuming, since it requires eviscerated and skinned foetuses [8, 12]. However, it is the only method for easy examination of the cartilage skeletal elements and is presently preferred [25]. According to some authors the costal cartilages should be cut on one side during the procedure [12]. However, on the basis of our experience the skin can be removed using a single longitudinal cut along the posterior midline and a transverse cut on the umbilical level and along the long axis of all 4 extremities. Using a pair of small forceps, the eyes, heart and most of the abdominal organs can be removed without damage [3, 6, 7]. Eviscerated and skinned foetuses are washed several times in tap water, then kept in distilled water for about 12 hours and finally re washed 5 times in running deionised water. The foetuses are then dried on a moist paper towel and placed for 24–36 hours in a solution of 9 mg alcian blue, 60 ml absolute ethanol and 40 ml glacial acetic acid. The staining should be checked several times a day and stopped before the alcian is visible in the remaining soft tissues such as the muscles. The procedure is stopped when the costal cartilage, the epiphyses of the long bones and the apices of the spinous processes are blue and clearly visible. The specimens are then drained, blotted and transferred to absolute ethanol. Dehydration usually takes up to 5 days and the alcohol is changed every day or every second day. The final step of the whole procedure is maceration and bone staining. For clearing, 1% potassium hydroxide solution (KOH) is used for 1–2 days. Four drops of 0.1% alizarin red S solution per 100 ml of KOH must be added for bone staining [25, 26]. In our laboratory 2% solution is used with good results [5–8]. However, the rate of the clearing process should be monitored several times a day. The staining can be performed after clearing but the KOH concentration should be reduced to 0.25–0.5%.

There are also other double staining methods. In these techniques the eviscerated and skinned foetuses are dehydrated in 95% ethanol for 5 days, placed in acetone for 2 days to remove fat, and then transferred for 3 days to a solution of 0.3% alcian blue in 70% ethanol, 0.1% alizarin red S in 95% ethanol, acetic acid, and 70% ethanol (1:1:1:17 v/v) [40]. The method for soft tissue clearing is the same as that described above. In this modification the final effect is similar to the

classic method. It should also be noted that, in keeping with our observations, Yong et al. [40], who used this new technique, found that the longer foetuses were allowed to fix in the alcohol before staining, the longer it took to clear the specimens.

A single alizarin red S staining is more common because the procedure is easy and inexpensive. After evisceration the foetuses are washed in tap water several times and then placed in 95% ethanol or isopropyl alcohol [3, 25]. The interscapular fat conglomeration should be removed by a small dorsal cut to gain a better view of the cervical and upper thoracic vertebrae [12]. When dehydration is complete the KOH maceration and alizarin staining should be performed as described above.

Before examination or storage the single or double stained specimens must be placed in graded concentrations, 25%, 50% and 75%, of glycerine for 24 hours and, finally, stored in 99.5% glycerine. The graded series of glycerol solution is necessary to prevent skull compression. It is also important to add a few crystals of thymol to each jar to preserve the specimens [12, 40].

It is worth mentioning that routinely processed single stained foetal specimens may later be examined for cartilage formation. According to Boardman et al. [2], an unskinned eviscerated rat foetus, rinsed in distilled water and kept for 4 to 13 days in 3% acetic acid solution, showed the purple colour transferred from the bone to the adjacent cartilage. The rate of the process depends on acetic acid clearing time and specimen size. In another method, effectively used in our laboratory, single alizarin-stained specimens are washed in 70% ethanol at pH 4 for 1–2 hours and then stained with 0.005% bromophenol blue in 40% ethanol adjusted to pH 4 for 2 hours [39]. It is important to protect the specimens from water or a higher pH environment, which causes the blue staining to fade.

The final examination is performed on glycerine-stained specimens under a 10× magnifying glass. Each bone is checked for size, shape and degree of ossification. It is possible to dissect the skeleton and separate the bones for more precise study (Fig. 1). In our laboratory the absence of alizarin staining in single stained specimens is considered non-ossification, in contrast to the absence of any staining in double stained skeletons, which is defined as a missing bone [3, 6, 7]. All the examinations must be performed by experienced staff who are familiar with the differences between foetal and adult skeletons as well as between human and laboratory animal skeletons. The rat, for example, has 13 thoracic and

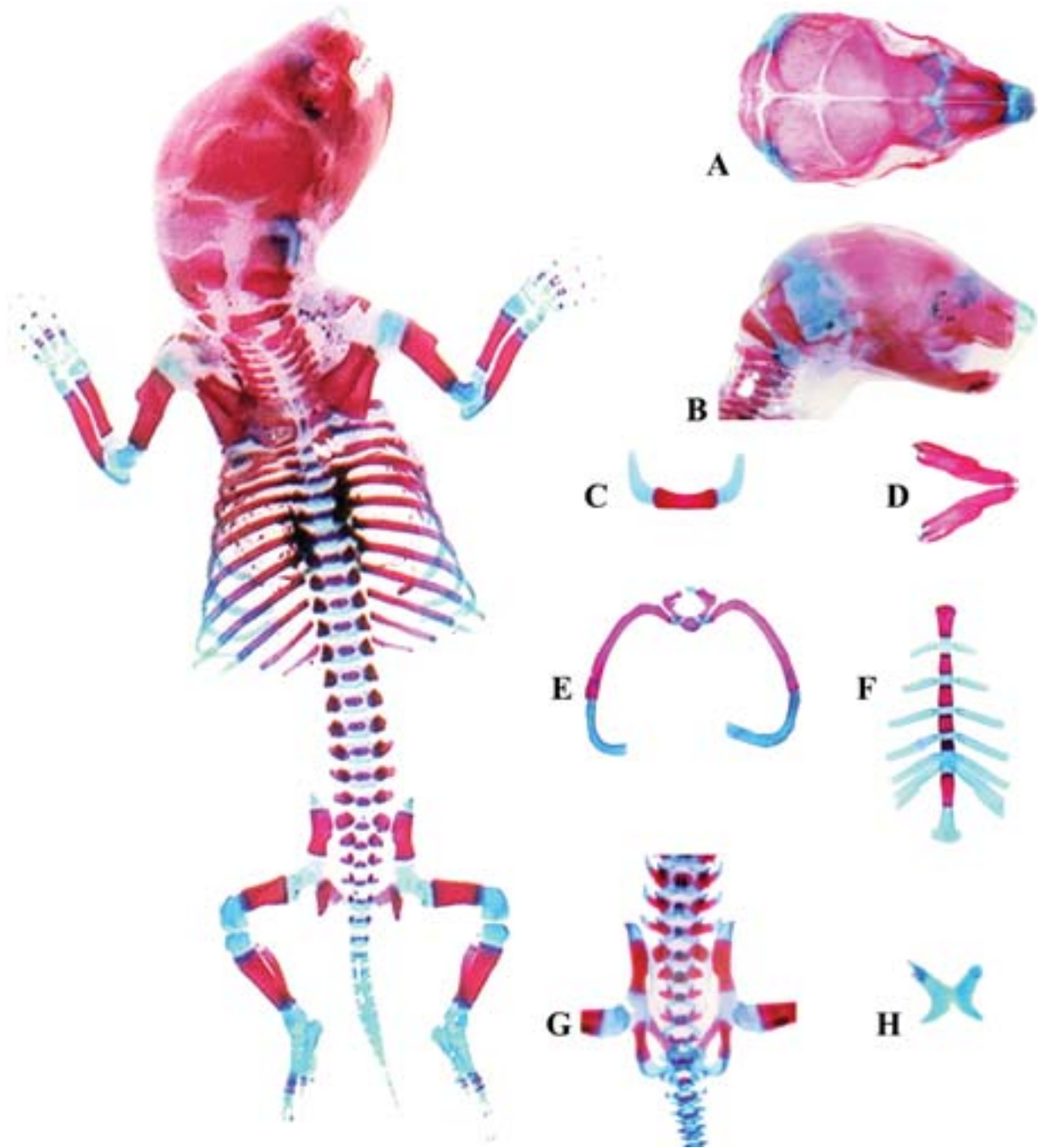


Figure 1. Well formed skeleton of a 21-day-old rat foetus (the posterior view). **A.** Skull (upper view); **B.** Skull (lateral view); **C.** Hyoid bone (upper view); **D.** Mandible (inferior view); **E.** 6th thoracic vertebrae with concomitant ribs (upper view); **F.** sternum (anterior view); **G.** Pelvis with lower lumbar and upper coccygeal vertebrae (posterior view); **H.** Pubic symphysis (anterior view).

6 lumbar vertebrae and cranial bones which are not found in man, such as the interparietal, supraoccipital, exoccipital, paramaxilla, basisphenoidal and basioccipital [11, 17, 22, 26, 33, 37]. The detailed foetal anatomy of various laboratory animals can be found in other publications [12, 17, 22, 23].

Foetal bone ossification can currently be evaluated using various radiological procedures. However,

even when digital radiography, micro CT or MRI equipment is used, the cartilage structure should be evaluated using a double staining method [8, 29].

SOFT TISSUE EXAMINATION

Soft tissue examination can be performed as a gross dissection of unfixed foetuses or after Bouin's fixation using the Wilson free-hand razor cross-

-sectioning technique, both performed under magnification [12, 23, 37].

In our laboratory a modified gross dissection is performed on most of the foetuses selected for skeletal staining during evisceration or on at least half the foetuses primarily selected for visceral examination. The undamaged thoracic viscera are studied for the presence of the great vessels, including branches of the aortic arch. The presence, position and shape of the *ductus arteriosus* are also checked. Finally, the right ventricle is opened by an incision made from the apex of the heart to the pulmonary trunk. The apex is then removed to open the left ventricle and to examine the interventricular septum and the atrioventricular valves [6, 7]. Other internal organs are dissected as for adult animals [12]. Their position, shape, size and colour should be checked.

The most common technique for visceral examination was described by Wilson in 1965 and it is still the standard teratological method [37]. Before examination the foetuses are fixed in Bouin's solution of saturated solution of picric acid, 37% formaldehyde solution and glacial acetic acid (15:5:1 v/v). The foetuses should be immersed for 14 days and then washed twice in tap water and stored in 70% ethanol or isopropyl alcohol until examination. Since the solution is used to harden the soft tissues and decalcify the bones, the histological sections obtained are not useful for ossification assessment e.g. von Koss staining. However, most other histological and immunohistochemical staining can be performed on such fixed tissue [5]. According to Wilson, the initial horizontal section is made through the naris [12, 37]. The remaining part of the head is dissected by a number of frontal cuts but the body is dissected by a series of transverse cuts. In our modification the foetal head is dissected with transverse cuts similar to human coronal computer tomography scans (Fig. 2). This modification enables a more precise view to be obtained of the brain morphology. As in adult macro-radiography [27], whole body sections along the sagittal and frontal planes are also occasionally performed in our laboratory (Fig. 3).

Because of the lack of contrast between the tissues examined after Bouin's fixations, other methods are described for foetal staining. The easiest is freezing in dry ice or liquid nitrogen (freeze fixation) [1, 27]. Some laboratories are starting to use Davidson's techniques with a solution made from ethanol (14 ml), glacial acetic acid (6.25 ml), 37% formaldehyde (37.5 ml) and distilled water (42.25 ml) [13]. This chemical fixation, which unlike Bouin's keeps the original

tissue colour, provides enhanced resolution and increased definition of the visceral structures and preserves the moisture of the specimen, enabling the evaluation to be made over an extended period of time. Furthermore, the fixation and decalcification times are reduced to 7–10 days [13].

Currently, all morphological abnormalities should be classified as a developmental variations or malformations [9, 30, 31, 38]. In contrast to congenital malformations, variations occur within the normal population and do not affect survival or health. These include growth retardation or morphogenesis that has otherwise followed a normal pattern of development [9, 10, 30, 31]. At present there are two similar classifications of congenital abnormalities published by the International Federation of Teratology Societies [22] and the European Teratology Society [17]. Sometimes the borderline is very narrow and, according to the international nomenclature committee, the final decision must be taken by the investigator on the basis of experience and own historical control data [30, 31].

It should be stressed that regardless of the development of new *in vitro* techniques, their results will still simply be an addition to *in vivo* observation conducted using different morphological methods [32]. Improving these is the only guarantee of reducing the risk of a tragedy similar to that of thalidomide.

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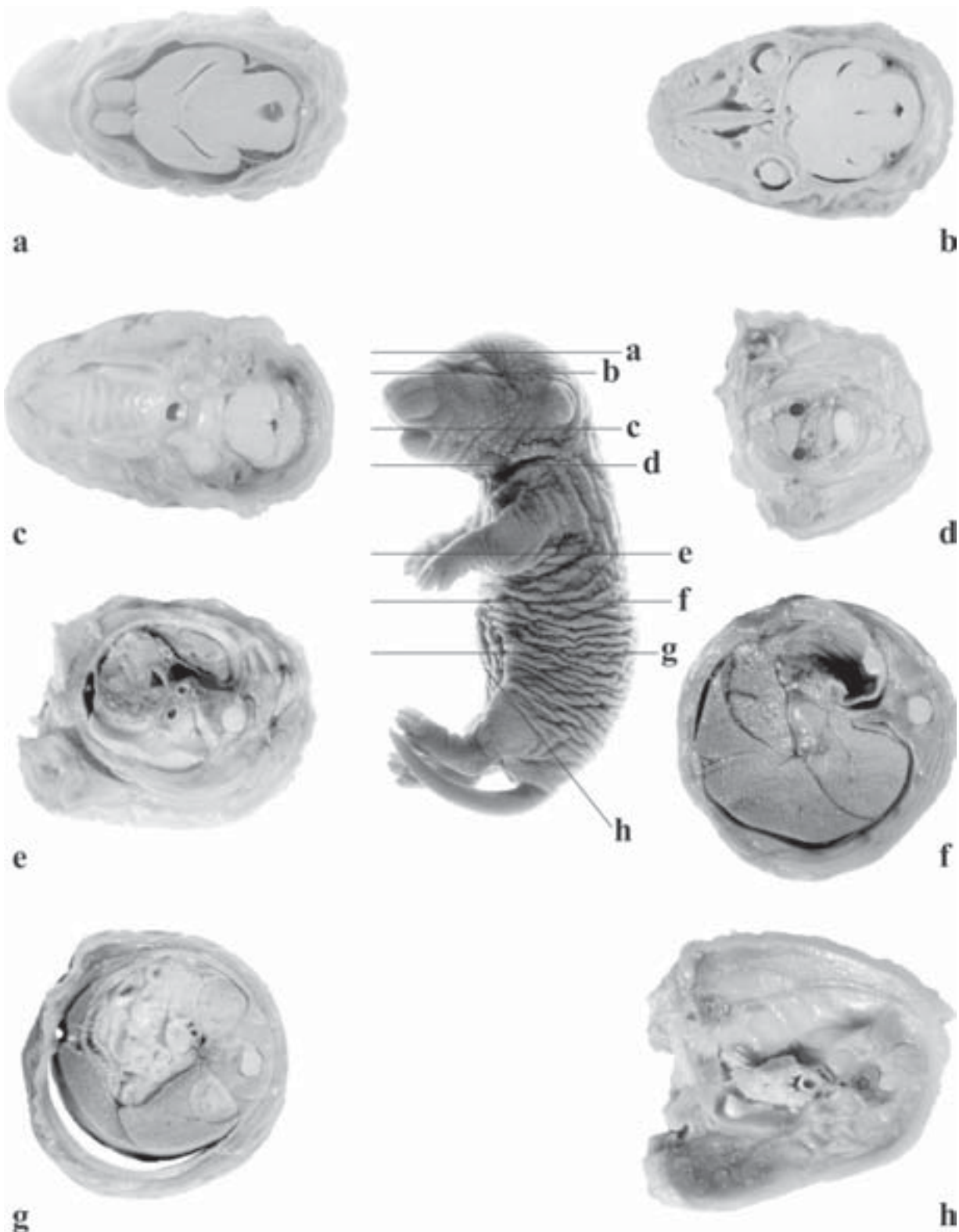


Figure 2. Transversal sections (a–h) of a 21-day-old male rat foetus (Bouin's fixation, Wilson's free-hand method, with own modification for the head examination).

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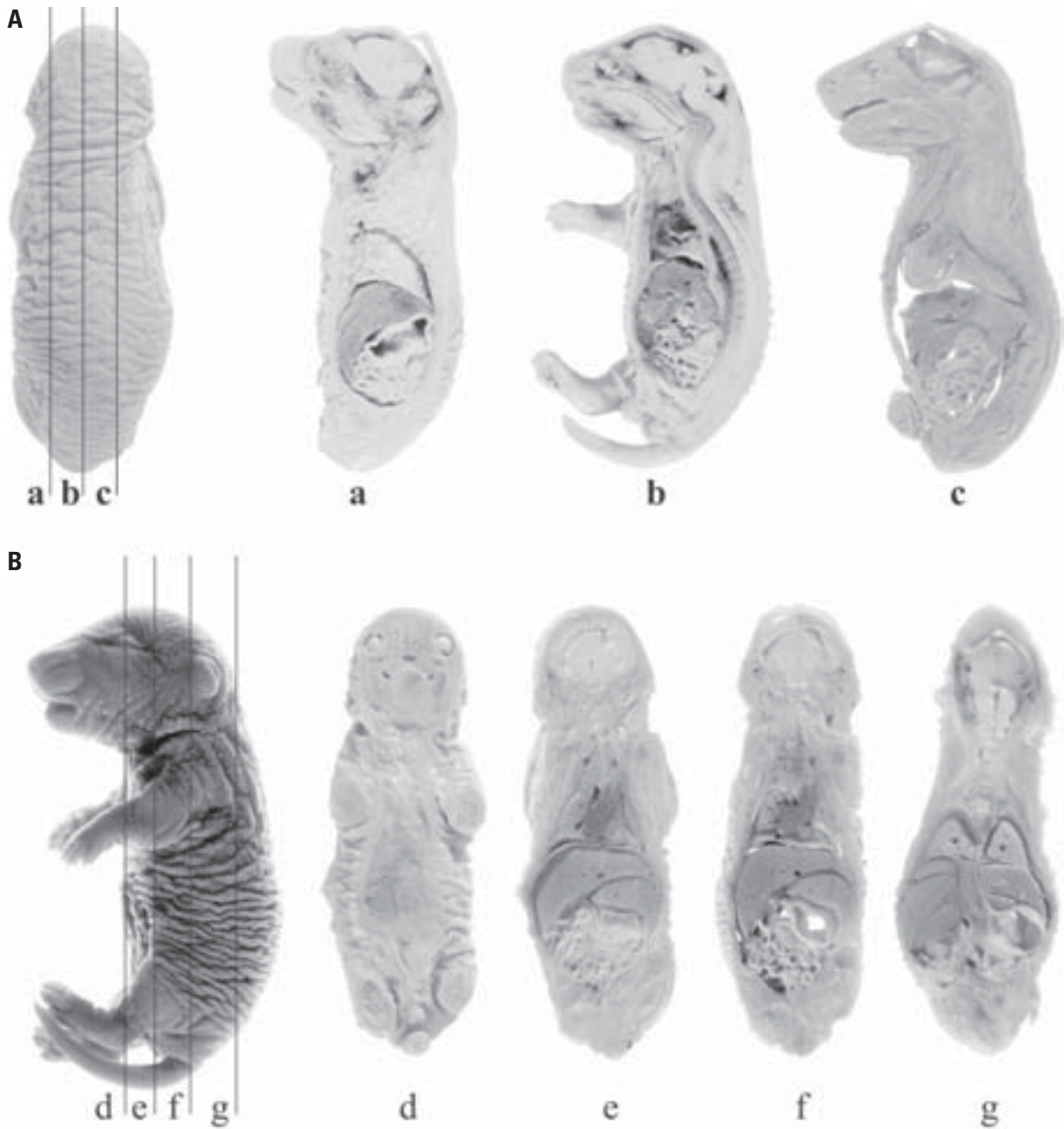


Figure 3. Sagittal (A) and frontal (B) sections of a rat foetus (Bouin's fixation, free-hand method).

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