Interdiscip Toxicol. 2021; **Vol. 14**(4): 97–101.
doi: 10.2478/intox-2021-0014

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SHORT COMMUNICATION

A methodological framework for assessing foetal skeletal and visceral development in teratological investigations

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ITX140421A02 • Received: 18 July 2021 • Accepted: 10 December 2021

ABSTRACT
This paper outlines the methodical approaches essential for the accurate and reproducible evaluation of foetal skeletal and visceral abnormalities in teratology studies. Teratological investigations are critical for assessing the potential of drugs, chemicals, or environmental agents to induce developmental toxicity. The established methodology involves a systematic two-part process. Firstly, visceral examinations use techniques like microdissection and serial sectioning to identify gross and microscopic changes in internal organs. Secondly, skeletal evaluations utilize staining procedures, such as Alizarin red S and Alcian blue, to visualize and analyze the ossification and structural integrity of the foetal skeleton. These standardized procedures are vital for the reliable detection and characterization of malformations and variations. A rigorous, methodical approach ensures the scientific validity of the data, which is fundamental for effective risk assessment and regulatory decision-making concerning human and environmental health.

KEY WORDS: developmental toxicity; foetal abnormalities; skeletal malformations; visceral changes; methodological evaluation

Despite various modern methods in molecular biology, foetal anatomy examination still remains a solid basis for teratological studies in the evaluation of developmental toxicity of drugs and other xenobiotics (Burdan *et al.*, 2005; Tyl and Marr, 2006). In this process, specific staining of foetal tissues and organs plays a key role. Without staining, it would not be possible to distinguish changes in structure that have been caused by a teratogenic noxa. Thanks to many years of research in this area, we currently have methodological approaches that allow accurate detection of congenital malformations (anomalies, variations, malformations) (Chahoud *et al.*, 1999; Solecki *et al.*, 2001, 2003).

During endochondral ossification, condensation of the mesenchyme leads to the formation of cartilage, which can then be replaced by bone tissue. Two basic staining techniques can reliably distinguish between developing bone and cartilage tissue. Free (ionic) calcium forms clots with Alizarin. Therefore, calcium-containing bone tissue changes its colour to dark red. On the other hand, unossified cartilage parts of the skeleton do not change colour by using Alizarin. In order to thoroughly distinguish cartilage from bone, parallel staining with Alcian blue is used (Puchtler *et al.*, 1969; Peters, 1977; Ovchinnikov, 2009). Examination of the soft visceral foetal tissues requires its fixation. For this purpose, a special fixing solution, Bouin's solution, is used. It is an aqueous yellow solution composed of picric acid, acetic acid and formaldehyde. This solution has the unique ability to soften (decalcify) hard skeletal structures and at the same time fix and strengthen soft internal tissues (Carson and Hladík, 2009).

Foetal processing and evaluation

After surgical withdrawal of the foetuses from uterus (mice on the 19th day, rats on the 20th day and rabbits on the 29th/30th day of pregnancy), those which are alive, are randomly divided into two equal groups and prepared to have structural changes in skeleton (Figure 1) and internal organs evaluated. Foetuses have their colour

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of skin, weight, sex (determined by the distance between genital and anal papilla) examined. We also cannot forget to define sex ratio and the vitality of the foetuses. In the foetuses alive, we evaluate obvious external malformations by stereo magnifying glass (Lorke, 1977; Till and Marr, 2006; Estevan *et al.*, 2017, Reynaud and Monrozier 2013).

Figure 1. Total growth retardation in mouse foetuses and occurrence of radioulnar synostosis (Z) in the upper limb (Ujházy *et al.*, 1979) .

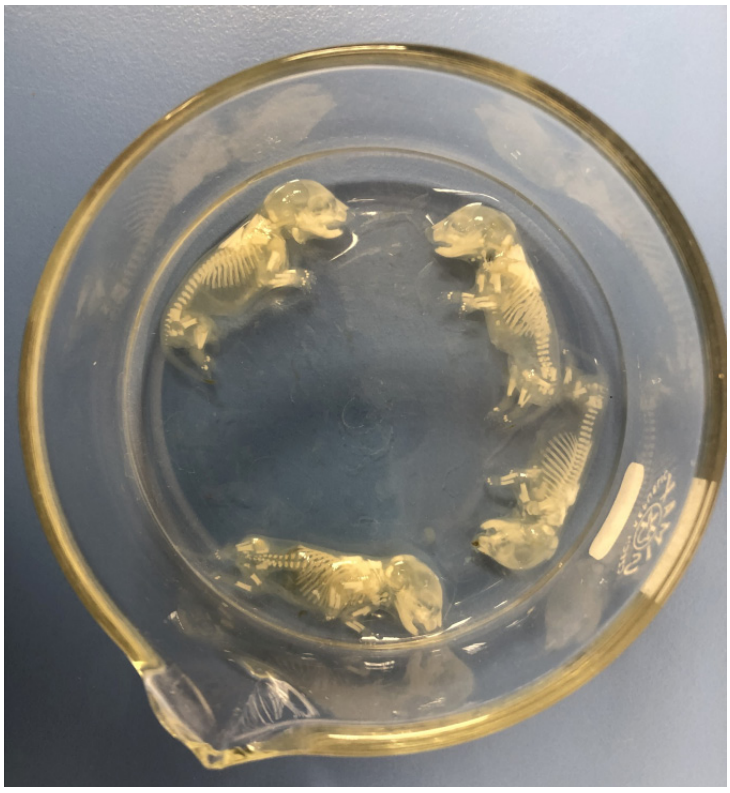

Figure 2. Transparent skeletons of rat foetuses in 1% potassium hydroxide solution.

Methodical procedure for evaluation of skeletal changes

The Peter´s method is used for parallel staining of cartilage and ossified parts of the foetal skeletons (Peters, 1977).

After the sacrifice of a pregnant female, her foetuses are extracted from the uterus. In some parts of the foetuses, which are intended to use for the examination of skeletal changes, the carotids and jugular veins are cut. The foetuses are then placed in distilled water for 2 hours. All internal organs are afterwards dissected from the thoracic and abdominal cavity. For optimal test results, skin withdrawal is recommended for rabbit foetuses. In the next stage, foetuses are fixed for at least one week in 96% denatured alcohol. This process is followed by the first foetal staining in Alcian blue, which takes 2 to 3 days. The foetuses are repeatedly dehydrated in 96% alcohol for 5 days and then stored in 1% KOH for 3 to 4 days until the skeleton appears transparent (Figure 2).

The process continues with second staining of the foetal skeletons in 0.001% solution of Alizarin red S in 1% KOH solution for 2 days (Figure 3).

Last step of the staining process is clearing the skeletons in the ascending glycerol series (25–85%). Finally, coloured foetuses are stored in 85% glycerine. The result of the whole procedure is ossified parts coloured red and the cartilage parts coloured blue (Figure 4).

Methodical procedure for evaluation of visceral changes

The Staples method is used to examine visceral changes in rat foetuses (Staples, 1974).

After removing foetuses from the uterus and examining for possible external malformations, these foetuses are placed in Bouin's solution (10 g of picric acid in 700 ml of warm distilled water, 500 ml of 40% neutral formaldehyde and 100 ml of concentrated acetic acid) for 1 week, what is an optimal time period for the intended degree of bone decalcification and soft tissue fixation (Figure 5).

After the fixation, examined foetus is placed on a paraffin plate and the head is separated from the torso with a razor blade. By using scissors, limbs and a tail were removed from the torso beforehand. Both head and torso are then cut separately into approximately 1 mm thick sections. Subsequently, the head and the torso of the foetus are examined within individual sections. Vertical cuts were made for the head and body to observe malformations. Selected

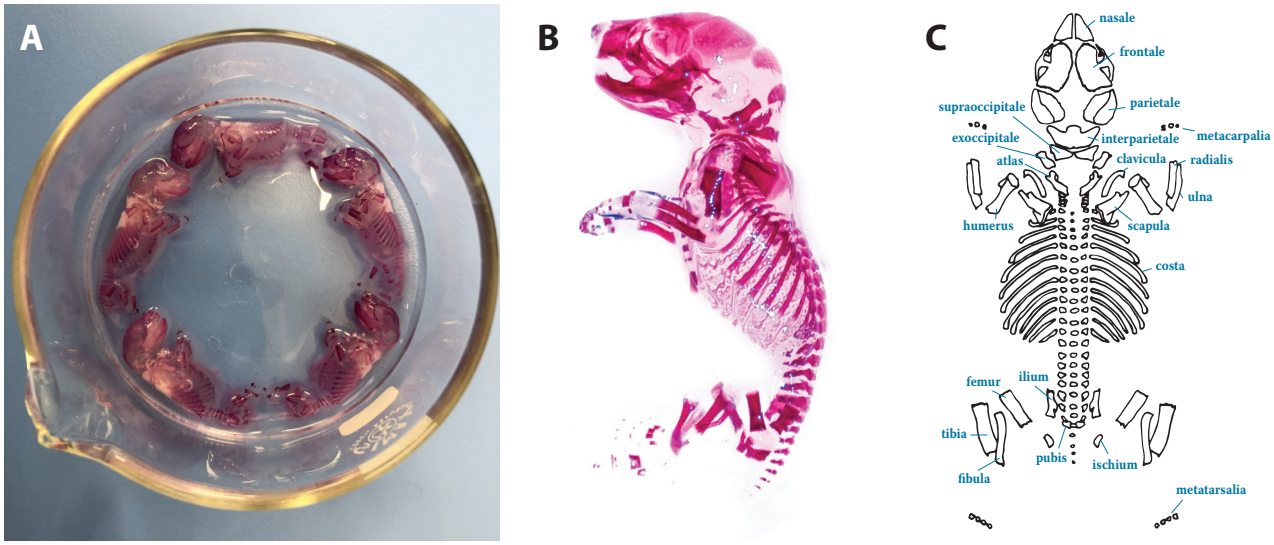

Figure 3. A, B: Skeleton of rat foetus stained with Alizarin red S. **C:** Maquette of rat foetal skeleton.

Figure 4. Parallel staining of the mouse foetal skeleton with Alcian blue (cartilage) and Alizarin red S (bone).

Figure 5. Foetus after fixation in Bouin's solution.

organs were removed carefully and examined for hydrocephalus, cardiomegaly, hydronephrosis, etc. (Critchell, 2013).

Examination of the head part:

1st section is made perpendicular to the lower jaw and microcirres. The composition of the mandible, front section of palate, nasal cavity, sublingual muscles and tooth bases are monitored.

2nd section is led through the center of the eyes while the olfactory mussels and the composition of the eye are observed.

3rd section captures the brain and its chambers, pineal gland, pituitary gland and hypothalamus.

4th section is made just behind the previous one. The composition of the inner ear, parts of the brain and the elongated spinal cord is monitored.

Examination of the torso:

1st section is led through larynx, esophagus, trachea, cerebrospinal canal and large vessels.

2nd section goes through organs placed in the thoracic region, right behind the forelegs (heart, lungs, esophagus and spinal cord).

3rd section is led through liver and diaphragm.

4th section proceeds behind umbilical cord. The target of examination is stomach, intestines, pancreas, kidneys, ureters, urine bladder, rectum and genitals.

Sections from individual organs are stored in Bouin's solution (Figure 6).

Conclusion

Based on a qualitative evaluation of the monitored indicators and significance of the differences, teratogenic effect of the test substance can be either confirmed or ruled out (Wilson, 1973). Based on the large number of teratological studies performed these days, all morphological abnormalities should be classified as developmental variations or malformations (Wise *et al.*, 1997; Chahoud *et al.*, 1999; Solecki *et al.*, 2001, 2003). These morphological developmental abnormalities of mouse, rat and rabbit foetal skeletons and organs are convincingly documented by various staining techniques (Lorke, 1977; Burdan *et al.*, 2005; Till and Marr, 2006). Nowadays, there are two similar classifications of congenital abnormalities – published by the International Federation of Teratological Societies and European Teratological Society. Sometimes the limit of their occurrence is very narrow and according to the International Nomenclature Committee, the final decision must be made by the evaluator of the experiment

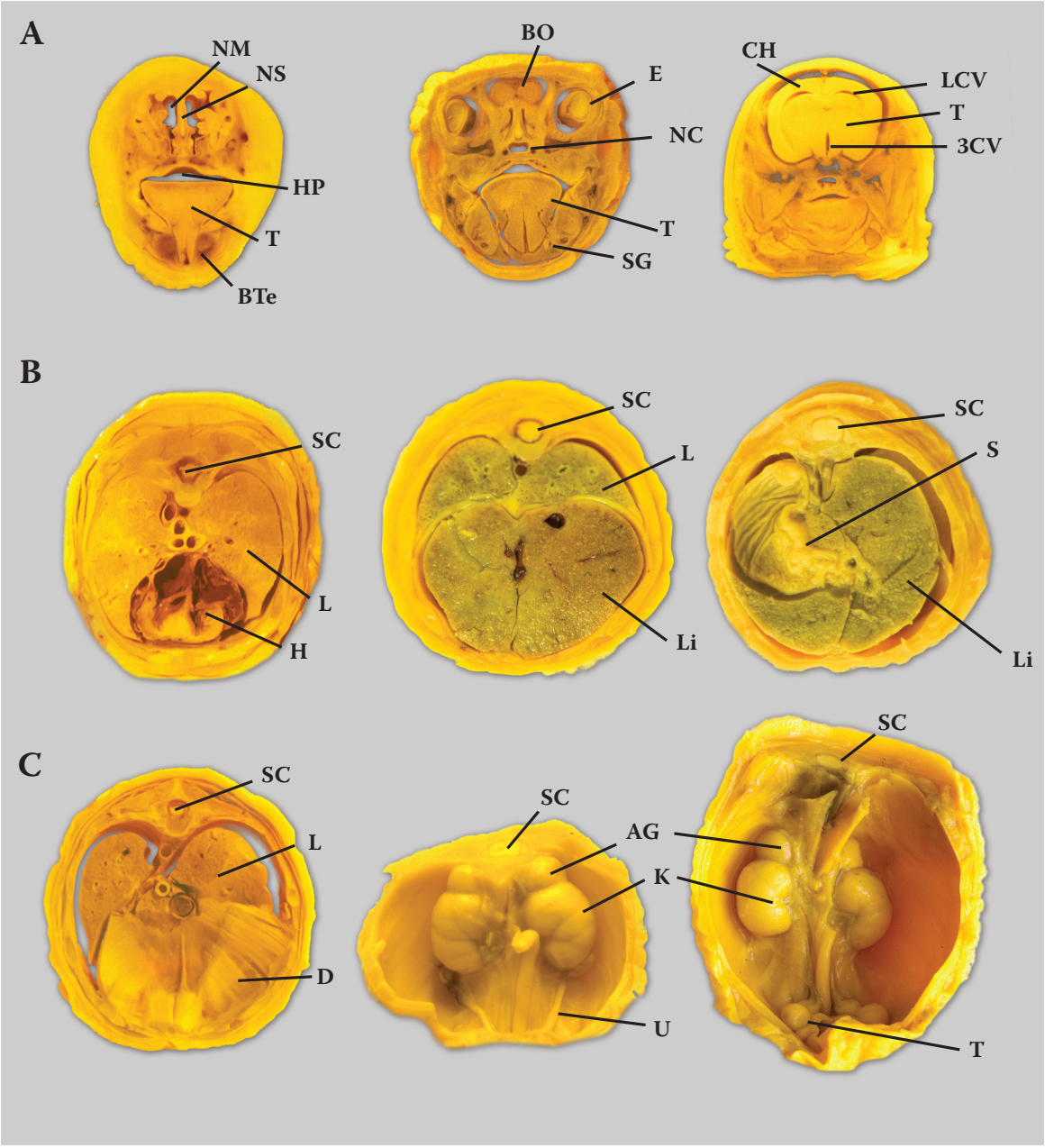

Figure 6. Examples of Wilson sections of rat foetuses. **A. Head area:** NM – nasal mussels, NS – nasal septum, HP – hard palate, T – tongue, BTe – bases of teeth, BO – *bulbus olfactorius*, E – eye, NC – nasopharyngeal canal, SG – sublingual gland, CH – cerebral hemisphere, LCV – lateral cerebral ventricle, T – thalamus, 3CV – third cerebral ventricle. **B. Thoracic area:** SC – spinal cord, L – lungs, H – heart, Li – liver, S – stomach. **C. Abdominal area:** D – diaphragm, AG – adrenal glands, K – kidneys, U – uterus, T – testes.

ISSN: 1337-6853 *(print version)* | 1337-9569 *(electronic version)*

based on his experience and his own historical control data of the biomodel used (Solecki *et al.*, 2001, 2003). It should be emphasized, that regardless of the development of new *in vitro* techniques, the results of the currently used staining morphological methods will always be a useful adjunct to *in vivo* observations. Their improvement is the only guaranteed way to avoid tragedies, such as the thalidomide tragedy in the 1960s.

Peculiarities and pitfalls of teratological research and usage of staining

- By using the methodics of cartilage and bone parallel staining, it is possible to accurately distinguish skeletal maturation stages during pregnancy.
- Specific type of staining and fixation of the foetal tissues and organs makes it possible to distinguish the altered structures that have become the target of the teratogenic noxa.
- Teratogenic effects of drugs and other chemicals identified in reproductive teratological studies using appropriate animal models are considered to be important indicators of potential developmental toxicity in humans.
- Appropriately chosen methodical approaches in teratological studies contribute to a more accurate assessment of new drugs' and other substances' relative safety in terms of reproduction and development of a foetus and a newborn.

Acknowledgement

Special thanks belongs to Mrs. Danka Zborovanová for technical assistance.

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