

Toluidine blue-alizarin red staining of the forelimb bones of 24 days old rabbit embryos (*Oryctolagus Cuniculus*)

Abstract

A skeletal examination of foetuses is required in regulatory embryo–fetal development studies. This paper describes ossification of forelimb bones of the rabbit embryos at a certain age. Six embryos were collected on post-mortem of female rabbit. The age was ascertained, and the embryos were subjected to Toluidine Blue-Alizarin Red staining. Most of the forelimb bones had started the process of ossification, with some exceptions like suprahemate process of the scapula and olecranon process of ulna, which were still cartilaginous in nature at this age.

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Introduction

Skeleton is a supporting system for vertebrate body. It provides attachments for muscles, houses the brain and the spinal cord, and serves for locomotion.¹ Staining of animal skeleton is important in anatomical studies. Toluidine Blue-Alizarin Red staining is one of the most successful staining methods for foetal and young skeletons. Some authors have described the skeletal examination of animals by Alizarin Staining² but there is scarcity with regard to staining of appendicular system in rabbits.

Materials and methods

Animals used in this study were 24 day old rabbit embryos collected on post-mortem from the Division of Veterinary Pathology, FVSc and AH Shuhama Alusteng. Equipments used in this study are the surgical scissors, scalpels, and a glass container (jar). Materials used in this study were Alizarin red, toluidine blue, ethanol, distilled water, glacial acetic acid, glycerine and KOH (Potassium hydroxide). The method described in this paper is Toluidine Blue-Alizarin Red staining of cartilage and bone.³ Using this method cartilage stained blue, bone stained red, whereas muscles and other tissues were transparent. The age of the embryos were determined by the formula, $W = 0.02(T - 12.5)^4$ and it was found to be 24 days. The embryos were first preserved in the solution of formalin, acetic acid and alcohol in the ratio of 1:1:8 for 45 minutes. The embryos were then put into the 0.06 per cent Toluidine blue made in 70 per cent alcohol for 48 hours. Then embryos were destained in 35 per cent alcohol for 20 hours, 50 per cent for 28 hours and 70 per cent for 8 hours respectively. The embryos were counterstained in a freshly prepared aqueous solution of KOH to which 2-3 drops of 0.1 per cent alizarin red S per 100ml of solution was added. This process of transferring the embryos into aqueous solution of KOH to which 2-3 drops of 0.1 per cent alizarin red per 100ml of solution was repeated for 3 days or until the bones had reached the desired intensity of red. The embryos were rinsed in water and mounted on glass slide by DPX (Digital Picture Exchange) mountant.

Results and discussion

Alizarin Red is an anthraquinone derivative used to identify calcium in tissue sections. Calcium forms an Alizarin Red calcium complex in a chelation process, and the end product is birefringent. In the present study, ossification of the bones of rabbit embryos was determined. Most of the forelimb bones were already in the process of ossification at 24-day-old age (Figure 1). It is reported that a primary ossification centre was visible at day 15 of pregnancy in mouse.⁵ The diaphysis of the long bones like of humerus, radius and ulna showed the ossification but their epiphyses were still cartilaginous in nature. It is recorded that the primary ossification centre formation in diaphysis of radius and ulna in 43-day-old embryos.⁶



Figure 1 Ossification of rabbit forelimb bones; Toluidine blue- Alizarin red stain.

1. Scapula
2. Humerus
3. Radius-ulna
4. Phalanges

The glenoid cavity, suprahemate process of the scapula, olecranon process of ulna was also cartilaginous in nature. In case of sheep embryos of 45 days old primary ossification centers were observed in the shaft of scapula and diaphysis of humerus.⁷ Carpals, metacarpals were cartilaginous but the phalanges showed ossification centres (Figure 2). The sheep embryos at 47th day showed process of

ossification in diaphysis of 3rd and 4th metacarpal.⁶ The total length of humerus was 1.3cm with 0.9cm as ossified part. Similarly, total length of radius and ulna was 0.7 and 1.1cm but the ossified part was 0.6 and 0.9cm respectively.

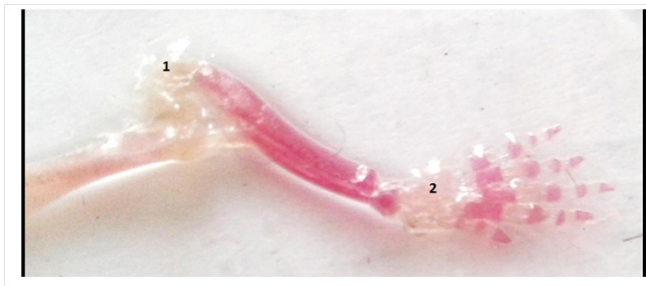


Figure 2 Non-ossified parts of rabbit forelimb bones; Toluidine blue- Alizarin red stain.

1. Olecranon process of ulna
2. Carpo-metacarpal area

Conclusion

As the gestation period of the rabbit is very less being around one month the process of ossification of the appendicular system is almost complete before birth. At the age of 24 days the rabbit embryos in the present study showed a high degree of ossification in the long bones of the forelimb with some unossified parts.

Acknowledgments

None.

Conflicts of interest

The authors declare there is no conflicts of interest.

References

1. Kardong KV. *Vertebrates Comparative Anatomy, Function, and Evolution*. 3rd ed. New York. USA: McGraw-Hill Higher Education; 2002:233–236.
2. Reynaud L, Jocteur-Monrozier A. Skeletal examination by alizarin staining. In: Barrow P, editor. *Teratogenicity Testing. Methods in Molecular Biology (Methods and Protocols)*. Totowa, New Jersey: Humana Press; 2013:947.
3. Burdi AR. Toluidine blue-alizarin red S staining of cartilage and bone in whole-mount skeletons *in vitro*. *Stain Technol*. 1965;40:45–48.
4. Huggett, Widdas. The relationship between mammalian foetal weight and conception age. *J Physiol*. 1951;114:306–317.
5. Patton JT, Kaufman MH. The timing of ossification of the limb bones and growth rates of various long bones of the fore and hind limbs of the early postnatal laboratory mouse. *J Anat*. 1995;186(Pt 1):175–185.
6. Ahmed NS. Development of fore limbs in indigenous sheep foetuses. *Iraqi Journal of Veterinary Science*. 2008;22(2):87–94.
7. Shazia N, Sabiha HB, Geetha R, et al. Prenatal study on ossification and growth of long bones in sheep foetus. *Indian Journal of Veterinary Anatomy*. 2017;29(1):52–55.