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MUSEUM PRESERVATION OF SKELETON OF FETUS & SMALL VERTEBRATES

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Abstract

Transparency technique to demonstrate cartilage and skeleton has been used from years. It is superior to the method of obtaining fetal skeleton by boiling and burying, consisting basically of muscle digestion and staining of cartilage and skeleton. The process involves maceration of soft tissues in 1% KOH and staining of specimen using Alcian Blue 8GX and Alizarin Red S allowing detection of morphology of whole vertebral column and single vertebra, long bones and primary ossification centers.

Keywords: Maceration, Alcian Blue, Alizarin Red S, KOH, Primary ossification centre, Fetal skeleton, Small vertebrate skeleton

Introduction

Earlier methods of skeleton preparation involved burying of foetus for 2-3 months and slowly recover the bones to construct the skeleton or to display loose foetal bones. Foetal remains contain many very tiny bones which have not yet fused with others and are extremely easily lost into the soil. Another method involved process of maceration in which the specimen is boiled in a solvent (water) to remove the last of flesh, grease, and cartilage (3). Sometimes bleaching agents are added to the water. The method described in this article is simple and effective to evaluate bone in small embryos, fetus, small vertebrates.

The specimen is digested in KOH, stained with Alcian Blue 8 GX and Alizarin Red S, cleared in increasing concentration of glycerin, when completed the cartilage are blue in color and bones are red in the transparent muscle. The specimen can be stored in pure glycerin. The principle used here is the affinity of Alizarin Red S to bind with Calcium of bones; this dye stains only the ossified areas of bone. It gives red colour to the bone. The dye was originally made from plant madder (Ruba tinctorum), it was first used in 18th century experiments on bone growth by John Hunter and others (4). Before 8th week of foetal life the bones of lower limb are composed not of bone, but hyaline cartilage. They are often called cartilage models. Cartilage is an essential growth tissue of bones and it can grow very quickly. The story of bone development in the lower limb is story of gradual replacement of cartilage by bone tissue, first in the shaft then later at the ends of the cartilage models, as rate of growth declines.

Objectives

Study the state of cartilage & bones in different stages of development using Alcian blue & Alizarin Red S. To demonstrate museum specimens skeleton preserved in fluid or as dry mount. Preparation of small vertebrate skeleton

Materials and Method

We collected fetuses between the age group of 2 months to 7 months, from Yenepoya Medical college hospital & small adult vertebrates for our study.

Bone cartilage double stain technique includes following steps.

Fixation I : Fix specimens immediately in 10% Formalin (12), for a day to a week depending on the size of the specimen.

Evisceration: Remove the skin, eyes, thoracic & abdominal viscera, adipose tissue (this step can be omitted in case of early embryos). (11).

Washing: wash the specimens thoroughly in running tap water for 2 days

Fixation II: specimen is fixed in 95% alcohol for 2 to 4 days(This step helps skeletons withstand maceration).

Defattening: specimen is put in acetone for 2 to 3 days(till acetone doesn't show yellow discolouration.)

Maceration: Keep the specimen in 1% KOH till the bones are visible through the surrounding tissue.(3)

Cartilage staining: place specimen in 20mg Alcian Blue in 70% alcohol and 30ml glacial acetic acid for 12 to 24 hours. The whole specimen turns blue along with the cartilage.

Destaining: Place the specimen in 70% alcohol. Keep changing the alcohol till the solution shows no blue colouration. (This dehydration will fix the Alcian Blue in

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cartilage and help destain surrounding soft tissues.) (11).

Bone staining: Place specimen in 0.1% Alizarin Red S in 1% KOH till the bones are stained red.(3).

Clearing: The specimen is placed in a solution containing KOH-1g, Glycerin-20ml, Water-79ml, till the excess red colour is removed.(3).

The specimen is placed in 10% glycerin, followed by 50%, 70%, and finally mounted in 100% glycerin. A few crystals of phenol or thymol can be added. All the chemicals used can be obtained from commercial biological supply.

Discussion

The degree of progression of ossification from primary centre, which is endochondral in appendicular and axial skeleton, except for clavicle and intra membranous centers, the majority of cranial bones are visible (4). The carpus and talus are wholly cartilaginous except for primary centre of calcaneous as are the epiphyses of long bones. The central and neural arches of vertebrae are separate. The sternum is still unossified. The membranous anterolateral and postero lateral fontanelles are obvious (fig 4 &7). According to Dawson's technique (3), the specimens are fixed in 95% alcohol directly without first fixing in 10% formalin, in the first case maceration of tissues take lesser time when compared to specimens first fixed in 10% formalin but are liable to greater degree of damage if not handled carefully as specimens are fragile and not hardened as in formalin fixation. Staining of cartilages has been done in the past using methylene blue or toluidene blue (2) but the colour fades with time. Different procedures have been given for preparing alcian blue stain for cartilage staining.3% alcian blue 8GXin glacial acetic acid, alcian blue 8 GN -10mg, 80ml 95% ethyl alcohol& 20% glacial acetic acid (12), 1 vol 0.3% alcian blue 8 GS in 70% ethylalcohol(5). As suggested by Simons & Van Horn(1971) staining of cartilage is now done with Alcian blue 8 GX stain. In our study we used 20mg alcian blue in 70% Absolute Alcohol and 30ml Glacial acetic acid (11), some have used toluidine blue instead of alcian blue, the former gives problem of decolourisation of cartilage when placed in alkaline solution (5).Monitoring of specimen kept in alcian blue staining is important to prevent overstaining as staining cannot be removed from cartilage by any of the chemicals used in the clearing and staining process (15).Phenol removes alcian blue staining (17), the chemical is highly volatile and produce severe burns if it comes in contact with skin, destaining should be done in a well ventilated area [phenol can cause headaches] , in glass jar, using gloves while handling the specimen but when we tried to destain our specimen using phenol we didn't get the desired result, According to Goldstein and Horobin cartilage stained dye is removable by Magnesium chloride which we have not tried. The specimen should not be kept in alcian blue for long, destaining should be done immediately in 95% alcohol, with repeated change of solution. Cartilage staining is not reversible so careful observation of specimen is a must. We can skip this step of cartilage staining and go directly for alizarin staining, here only the bone will take up red colour and cartilage will appear transparent (fig 5 and 6). In case of pigmented specimens bleaching is done. Specimen should not be bleached for long as gas bubbles will form within the skeleton, change to 1% KOH solution immediately if bubbles are formed and repeat changes until no air bubbles are formed, then the specimen is subjected to clearing (15) but we found that we get good results if bleaching is done after digestion. Most of the earlier published work has suggested clearing of soft tissues[digestion] in trypsin (12), which is expensive, we have used 1% KOH for clearing with good result.We macerated the specimen in 1% KOH before cartilage staining in foetus above 6 months instead of staining the specimen first and then subjecting it to maceration (11). Our method took less time to destain. Here we would like to add that destaining after cartilage staining is very important as the specimen when put in Alizarin Red S the cartilage turns to green colour .When specimens are being cleared they soften and if left too long, macerate and are destroyed. Fixation in 10% formalin hardens the tissues and helps to control the maceration, though it prolongs the clearing process (2). 95% ethyl alcohol is used for fixation or 10% formalin followed by 95% alcohol, this makes fragile skeleton hard. It has been suggested that concentration of KOH can be increased for larger specimens (5), but careful monitoring is very important lest the specimen will be damaged. In (fig8) the finer bones (carpal bones, tarsal bones, phalanges, cartilaginous ends etc) have been disarticulated. Evisceration of specimens hastens the maceration process; small embryos which are already transparent need not be eviscerated. Skin of frog should be removed first before fixation and the tail of the lizard should be slit length wise so that subsequent swelling will not break the tail off(2). For bone staining we placed the specimen in 0.1% Alizarin Red S in 1% KOH (3) and kept the specimen in clearing solution (1g KOH, 20ml Glycerin and water 79ml) for a longer time till the solution didn't show red colour The specimen should be kept on white surface in sunlight to speed up the process (6). For the next clearing step we used 10%, 50%, 70% & finally 100% of Glycerin instead of 10% to 100% glycerin(3, 11). The skeletons which were over macerated were dry mounted (fig 8). The bone cartilage double staining technique reveals even slight amounts of cartilage and mineralized tissues, making it valuable in studies of skeletal development in

embryos, fetuses specimens, and small adult vertebrates. We have prepared these to be displayed in the museum for students to observe the ossification centers & to study comparative anatomy of skeleton displayed.

Fig-1

Fig-2

Fig-3

Fig-4

Fig-5

Fig-6

Fig-7

Fig-8

Fig-9

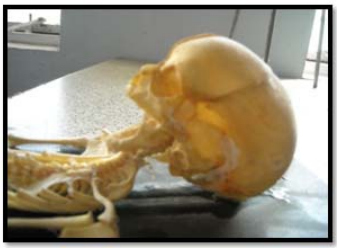

Fig-10

Fig-11

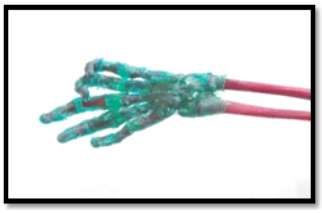

Fig-12

Fig-13

There are some permanent cartilage that do not ossify such as cartilage of trachea and articular cartilage of joints, intramembranous bone formation occur directly without a cartilage template.(4) When preparing double stained specimens, the overlying skin of the specimen must be removed to allow Alcian blue to penetrate and stain the cartilage (13), the skin is difficult to remove from the extremities of fixed specimens so if cartilage formation in digits is of interest, it is important that the specimen is not placed in fixation until the skin is removed.

In the early stained skull there are several obvious fontanels, the single frontal fontanel on the top, between frontal and parietal bones,(fig 4) bilateral sphenoid fontanel above eye and jaws, bilateral mastoid fontanel behind ears, single occipital fontanel at the back of the skull (fig 7).These sutures can be easily identified from rest of the bones because they are more triangular instead of narrow elongated spaces. The roof and sides of the skull form as membrane bones without formation of cartilage first, where as the base and palate form cartilage and then bone. The fontanels are in membrane bones. There are similarity between all the vertebrae with a vertebral canal and neural arch which surrounds it. Only small dots of bones are present in the stained specimen (fig 7). As per our study by using alizarin Red S and alcian blue it is easy to demonstrate the nature of appearance of ossification centers in fetus and small animals, and cartilaginous skeleton can be prepared without distortion from shrinkage.

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