VISUALISATION OF THE FETAL SKELETAL SYSTEM BY DOUBLE STAINING WITH ALIZARIN RED AND ALCIAN BLUE

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SUMMARY : *The full - term balb/c mice fetuses were skinned and fixed in 95 % ethanol for 10 days period. They were kept in acetone for 24 hours, and double stained in a solution of 0.015 % Alcian blue and 0.05 % Alizarin red - S for 4 days. Fetuses were washed in running water, made transparent in the 2 % Potassium hydroxide solution. Then, put into 100 % glycerine solution for conservation after being kept in glycerine solutions of 20 %, 50 %, 80 % grades respectively. All these process resulted in showing the cartilages blue and bones red.*

Key Words : *Double Staining, Alizarin red - S, Alcian Blue.*

INTRODUCTION

Science of Teratology has close relations primarily with Histology-Embryology and Anatomy, and also with many disciplines such as Biology, Genetics and Biochemistry.

Initial teratological studies dates back to the beginning of 1960's. Between the years of 1960-1962, serious skeletal defects were diagnosed on new born babies as a result of extensive use of Thalidomide during pregnancy. Following the diagnosis of these defects, the use and sale of this drug both in USA and Europe have been completely banned.

Studies on the visualisation of skeletal system in fetuses go back to the beginning of this century. Alden B. Dawson carried out the first successful skeletal staining in 1926 (3).

Double staining method developed by Inouye in 1976, was applied in our studies with some changes. Compared with the methods applied by Dawson, Wassersug, Love and Vickers, Jensh and Brent, Yamada; the Inouye method, modified by us, has been much more successful (3, 5, 7, 8, 10, 11).

MATERIALS AND METHODS

BALB/C type full-term laboratory mouse fetuses weighing 0,8 to 1,5 gr were used in this study. The fetuses were throughly skinned. Such skin removal was essential to permit satisfactory penetration by the stain. The unskinned fetuses were found to be non-uniformly stained. In many studies, the animals had been eviscerated prior to fixation but in our study, since we obtained complete clearing, we did not have to remove the viscera considering the risk of losing portions of the skeleton.

The Method Consisted of 3 Major Steps

A. Fixation Process

The skinned mouse fetuses were fixed in 95 %

ethanol for 10 days. After hardening of the specimens, they were placed into pure acetone for 24 hours to remove fat. The ethanol and acetone solution volumes were prepared so as to be 10 times bigger than the volume of the fetuses.

B. Staining Process

Staining was performed at 40°C for 4 days. The following staining solution was prepared:

a) 300 mg Alcian blue dissolved in 100 ml 70 % ethanol : 1 volume (100 ml).

b) 100 mg Alizarin red - S dissolved in 95 % ethanol : 1 volume (100 ml).

c) Glacial acetic acid : 1 volume (100 ml).

d) Ethanol 70 % : 17 volume (1700 ml).

Solution "a" and "b" were mixed and then "c" and "d" were added. At least 100 ml of the resulting staining solution was used per full-term mouse fetus and after the staining, specimens were washed for 2 hours in tap water.

C. Clearing Process

Fetuses were placed in 2 % Aqueous KOH solution for 3 days and then in aqueous solution of 20 % Glycerin containing 1 % KOH until skeletons were clearly visible through the surrounding tissue. Cleared specimens were placed successively into 50 %, 80 % and 100 % glycerin solutions, for 7 days each step. The specimens were stained in 100 % glycerin to which a few thymol crystals have been added to prevent mold formation.

RESULTS

A. Fixation Process

In this study, fixation period for whole skeletal staining was found out to be 10 days or longer. In Inouye's method this period was defined as 4 days or more. Depending on our findings; we think that when the fixation period is held short; the clearing period is increased with a fixation period of 10 days; the staining and clearing procedures were more successful.

B. Staining Process

While investigating the simultaneous staining of bone and cartilage with Alizarin red-S and Alcian blue; we also placed the fetuses into the individual stains consecutively:

a) When Alizarin red - S was used as a prelude to Alcian - blue; while bones were stained with Alizarin red - S, cartilage staining was not satisfactory (Fig. 6).

b) When Alcian blue was applied before Alizarin red - S, the surface contours and cartilage of the fetus were stained but bones were not evenly penetrated by Alizarin red - S.

Due to these shortcomings of consecutive staining; simultaneous staining with a mixture of Alizarin red - S and Alcian blue solutions was preferred. We have concluded that when the staining was performed at 40°C for 4 days; the results obtained were better as compared to the

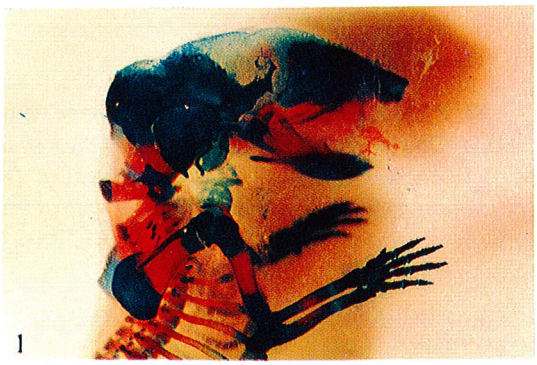

Fig - 1 : Lateral view of the skull and upper extremity of balb/c mice fetus stained with Alizarin red - S and Alcian blue. (Bones in red and cartilages in blue). X 6.7.

Fig - 2 : Lateral view of the ribs of balb/c mice fetus stained with Alizarin red - S and Alcian blue. X 10.

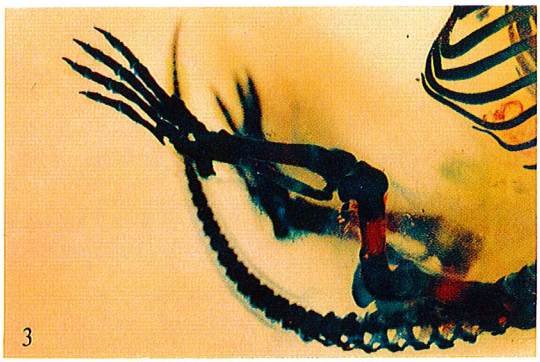

Fig - 3 : Lateral view of the lower extremity and tail of balb/c mice fetus stained with Alizarin red - S and Alcian blue. X 8.

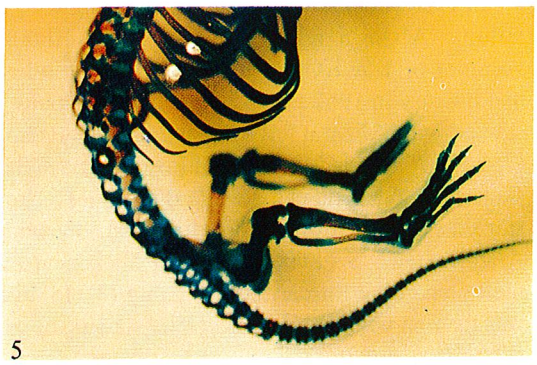

Fig - 5 : Lateral view of the ribs, hindlimb and tail of balb/c mice fetus prepared by Inouye method and stained with Alizarin red - S and Alcian blue. X 6.7.

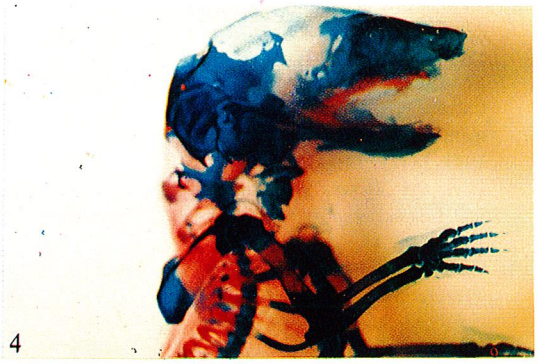

Fig - 4 : Lateral view of the skull and forelimb of balb/c mice fetus prepared by Inouye method and stained with Alizarin red - S and Alcian blue. X 6.7.

Fig - 6 : When Alizarin red - S is used prior to Alcian blue; while bones are stained with Alizarin red - S, cartilage staining is not satisfactory. X 6.7.

staining made at 37°C for 2-3 days, as defined by Inouye. When the fetuses were observed after these 4 days; the osseous and cartilageous staining at the costae, vertebrae and skull was apparent even without the clearing process. When the staining was performed according to Inouye's method; humerus - ulna - tibia and fibula displayed unsatisfactory bone staining with Alizarin red - S and the osseocartilageous border was not visible clearly on the costae. On the other hand; by our modified Inouye's method; osseous parts of humerus - ulna and fibula were optimally stained and the osseocartilageous borders of the costae were clearly demonstrated.

C. Clearing Process

In this study, 2 % KOH solution was used for the clearing process. Clearing began in the 30th minute from the distal parts of the extremities and penetrated into the central parts. At the end of the first day; clearing process was complete except the abdominal and back parts of the fetuses. Inouye had used 1 % KOH solution in his study. We observed a quicker and better clearing with 2 % KOH solution. An additional finding was the quickening effect of

higher temperatures on the clearing procedure. Despite these findings; 3 days were allowed to pass in order to have the best clearing possible.

DISCUSSION

In 1926 Alden B. Dawson made staining with 1 volume Alizarin red - S dissolved in 10.000 volumes of 1 % KOH solution (3). On the other hand, Crary in 1962 tried to clear the specimens - stained with Alizarin red - S using benzyl alcohol (2). In 1970, Ojeda, Barbosa and Bosque stained the skeleton using Alcian blue while Burdi and Flecker stained cartilage with toluidine blue and bone with Alizarin red - S (1, 9). Love and Vickers used methylene blue for cartilage staining (8). Inouye was the first who reported successful staining of the skeletal system; by consecutive staining of osseous and cartilageous tissue using Alizarin red - S and Alcian blue (5).

In this study; some modifications were made in Inouye's double staining method. Staining at 40°C in an incubator for 4 days yielded satisfactory results. With this method; the cartilageous portions of the skeleton were stained in blue and osseous parts in purple to red colour. Due to the period in glycerin, the colour of the bones gradually became red.

About the bone staining by Alizarin red - S; the affinity of the stain for the Ca^{2+} ions is extremely high. Alcian blue is specific for the mucopolisaccarides. Washing can not fade these colours due to the strong bonds (4, 6).

For clearing, in 1926 Dawson used a combination of 79 volumes of water, 20 volumes of glycerin and 1 volume of KOH solution. Crary tried this clearing procedure using benzyl alcohol on his Alizarin red - S stained specimens. In 1977 Dingerkus and Uhler; and in 1983 Kelly and Bryden performed clearing with trypsin (4, 7).

In this study; the most important aspect in the success of the staining was the adequacy of the fixation step. If the fixation period is short; then the clearing process time is lengthened.

In conclusion; using this modified method, satisfactory entire skeletal views were obtained that facilitated many precious teratologic investigations.

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