Two Separated Protocols with the Most Important Comments for Skeletal Staining in Embryonic and Adulthood Period in Laboratory Animals

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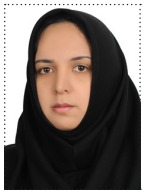

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Article info:
Received: 06 Sep. 2013
Accepted: 25 Jan. 2014

Key Words:
Alizarin red, Alcian blue, Double skeletal staining

ABSTRACT

Introduction: Skeletal staining is an important part of anatomical studies which can evaluate macroscopic disorders in bones and cartilages development. The aim of the present article is to illustrate two distinct protocols by all details in order to stain skeletal system of the lab animals in all ages of their lives so it becomes easy for future studies in this field.

Methods: In this paper, staining method was based on sample's ages. So, all the steps of double skeletal staining described in 2 protocols, one for embryos and newborns and another for adults. Fixing by ethanol, alcian blue staining, dehydration by ethanol, alizarin red staining, washing by potassium hydroxide and glycerol, storage in pure glycerol were the stages of embryos and newborns skeletal staining. Fixing by neutral formalin buffer, placing the samples in ddH2O, fixing again by ethanol, skinning and eviscerating, alcian blue staining, trypsin digestion, alizarin red staining, potassium hydroxide clearing, KOH and glycerol clearing and finally storing the samples in pure glycerol were the steps of adults staining.

Results: Outcomes of a good and reliable procedure for skeletal staining with alizarin red and alcian blue are the samples whose cartilages and bones are stained blue and red respectively. They are completely transparent so that the skeletal parts can be seen through them.

Conclusion: It is shown that skeletal staining procedures differ in embryos and adults although in the most surveys were tried to use a same protocol in different ages of laboratory animals.

1. Introduction

Skeletal staining in embryonic and also in postnatal period is known as an important step in most of teratological investigations. Since many years ago, alizarin red which has a high affinity for binding with calcium ions has been used for staining boney parts of the skeletal system. Few years later differential staining which attracted many investigators was known.

The 1st available paper about skeletal staining is a protocol in which alizarin red is used to stain bones and potassium hydroxide for clearing the samples in 1897 [1]. The next staining protocol was expressed in 1906

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[2] and after that modifications of these two methods were published as single- staining protocols.

The main concerns of the presented protocols were decolorization of the samples taking high amount of color. In 1930, the embryos were bleached in hydrogen peroxide and cleared in benzol and synthetic oil of winter-green [3]. Hollister recommended the use of ultra-violet light in the clearing process [4].

Staining bony part of skeletal system had continued until Toluidine blue as a color for staining cartilaginous part of the skeletal system was introduced in 1941 and then in 1965 it was again employed as cartilage indicator [5, 6]. The mentioned staining processes were not done properly and on the other hand, variable results were gained if the protocols had been repeated in other laboratories. The important defect of these methods of staining is that the stained samples lost their color over the period of time.

Alcian blue as another color for staining cartilages part of skeletal system was proposed in 1970 and the author stained the cartilages part of a chick embryo [7].

Dingerkus and Uhler suggested another protocol that applied enzyme digestion after alcian blue staining. Although it had many advantages compared to the previous protocols, but the samples which are cleared with enzyme digestion lost their rigidity and changed to jelly – like samples so that their handling became difficult [8].

In a suggested rapid way for differential staining of bones and cartilages, the samples were cleared after the staining stage, so optimal stain absorption was not obtained. Potassium hydroxide which was used did not have enough strength to clear the samples in adulthood period and excessive use of it caused fragility [9].

Another protocol claimed to stain all specimens in different ages of their lives. The important point is that clearing stage in embryonic period can be done easily by potassium hydroxide instead of enzyme digestion, however, in this protocol the clearing process in all the period of ages was done by enzyme digestion [10].

Since 1897, different protocols have been proponed. Each of them had some defects, so the next paper tried to do some modifications in order to make a better one.

Nowadays, many investigators use alizarin red and alcian blue staining in their studies in order to evaluate skeletal development in study of the effects of many chemical or herbal substances, but absence of a paper in which the skeletal staining protocol is described completely by all the details was felt.

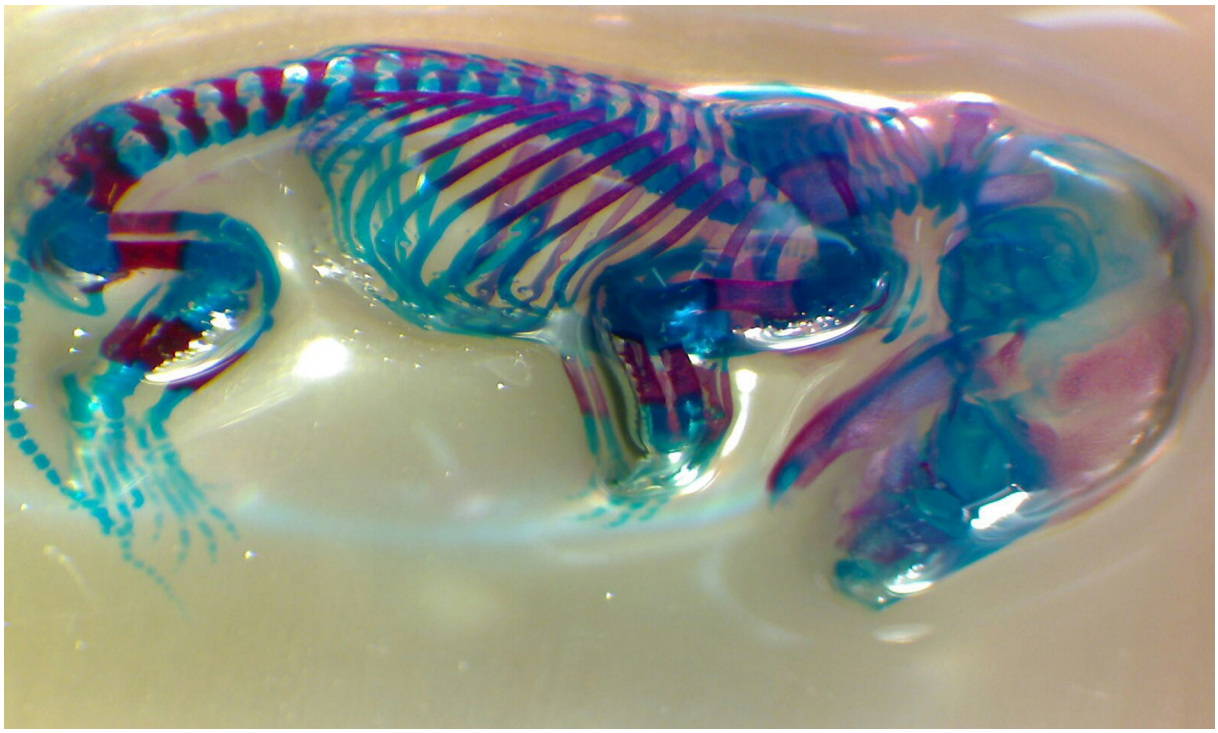

Figure 1. Stained 17th day embryo by mentioned double skeletal staining technique.

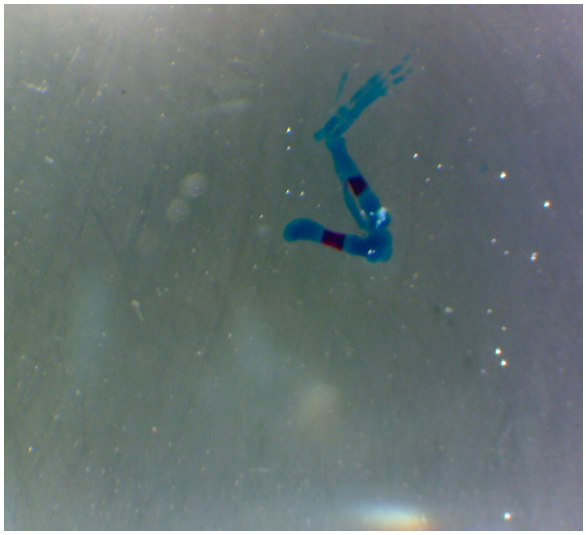

Figure 2. Showing the start of ossification process in hind limb of 15th day embryo.
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In 2003, an experiment was done about the anomalies in fetal alcohol syndrome "a study on developing mice embryos". In this study the author claimed to stain bony part of skeletal system by the technique described by Dawson in 1926 and no details like the time of each step, concentration and etc. were given [11].

In two papers in 2007 and 2013 a same protocol was used in order to stain mice in different ages without regarding the time of each step. The most interesting comment about the proposed protocol is that one protocol was used for embryos as well as adults [12, 13].

Another study was done in 2012 in order to evaluate fetal skeletal malformations by using alizarin red and alcian blue staining. In this article the used colors concentrations were not considered [14].

Therefore, the present study was aimed to present two different and independent protocols which are comprehensive, precise with the same result in any lab situations without regarding the specific condition such as temperature, PH, humidity and etc. One for staining samples in prenatal and early postnatal period and another for adults. At the end of this paper at discussion part the most important comments for this technique is given in order to upgrade the level of skeletal staining investigations.

2. Materials & Methods

In this investigation as a basic study we used three ages of mice;embryos,newborns and adults.

96 mice were used in each group in order to be insure about all the steps of the illustrated protocols.

2.1. Staining embryos and newborns

Alcian blue and Alizarin Red stain, acid acetic glacial, distilled water, ethanol, glycerol, potassium hydroxide and tymol were the materials which were used in the skeletal staining process. Euthanasia equipments such as scalpel; forceps and scissors, size dependent on age of fetus; falcon tubes, 50 ml and dissecting microscope were the required equipments.

Staining Procedure

First eviscerated embryos as possible as we could then fixed them in 90% or absolute ethanol for at least one weak.

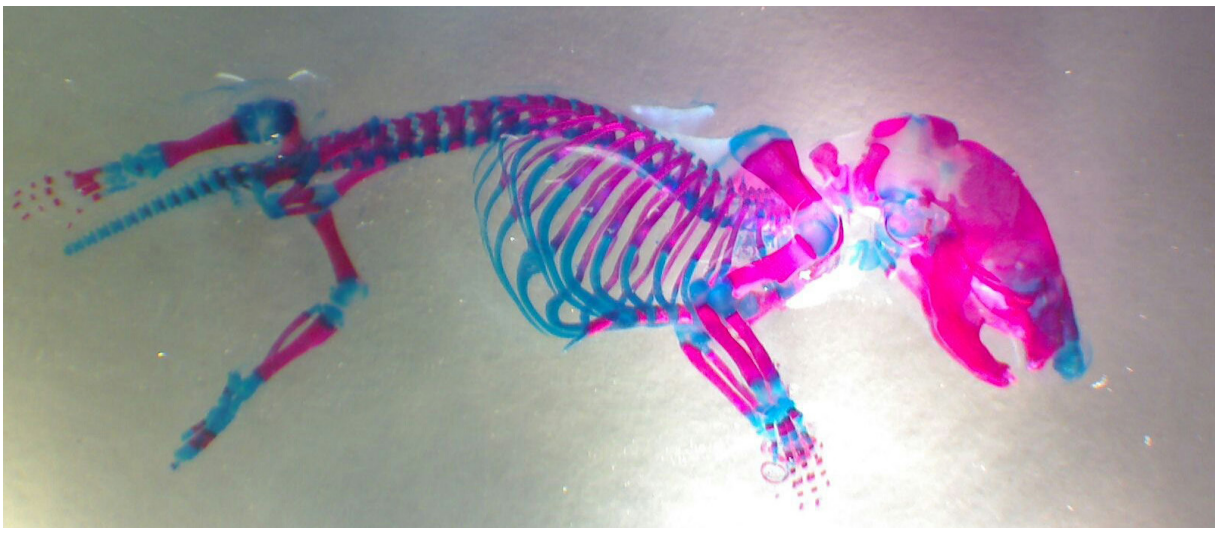

Figure 3. Stained 1st day mouse by mentioned double skeletal staining technique.
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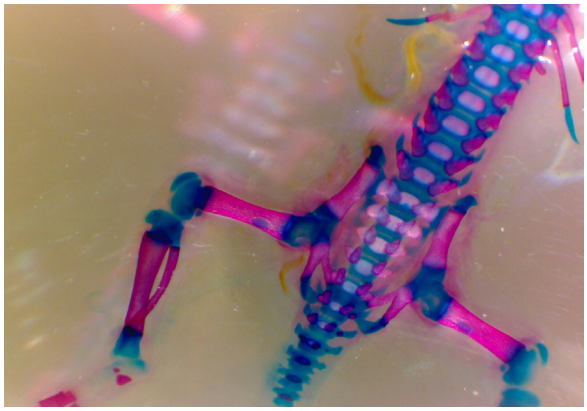

Figure 4. Showing the details of skeletal sys. in 1st day mouse hind limbs "the third trochanter start its formation as a cartilaginous protuberance in blue color". ANATOMICAL SCIENCES

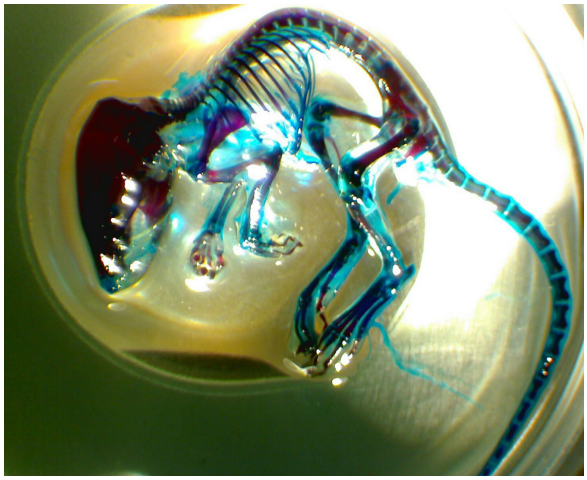

Figure 5. Stained 30th day mouse by mentioned double skeletal staining technique for adults. ANATOMICAL SCIENCES

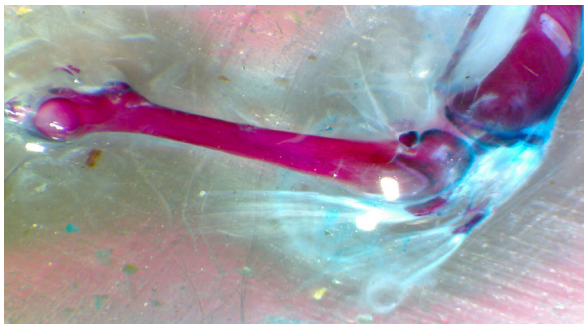

Figure 6. Showing the details of femur bone in 30th day mouse "the distal extremity growth palate is apparent". ANATOMICAL SCIENCES

Stained cartilages with 0.01% alcian blue prepared in ethanol and acid acetic glacial. Rehydration was performed with descending degree of ethanol, and finally distilled water 2 to 3 hours for each step. In order to clear the samples, we left them in 1% potassium hydroxide until the skeletal systems of the embryos were exposed.

Boney parts of skeleton were stained with 0.001% alizarin red and then the samples were rinsed with 1% potassium hydroxide three times, several hours each time.

Then the samples were treated with ascending series of glycerol in 1% potassium hydroxide, 24 hours for each step and as the last stage we left the stained embryos in pure glycerol which a crystal of tymol was added to it.

2.2. Staining adults

Alcian blue and Alizarin Red stain, acid acetic glacial, distilled water, ethanol, glycerol, neutral buffered formalin 10%, potassium hydroxide, saturated sodium borrate "Na2B4O7- 10H2O", trypsin and Tymol were reagents which were used in adult's skeletal staining procedure.

Euthanasia equipments such as scalpel; forceps and scissors; staining jars and dissecting microscope were the required equipments. Adult's skeletal staining procedures was as follows:

First we fixed mouse skeleton in 10% neutral formalin buffered for at least 24 hours, then the fixed samples were washed with distilled water for 24 hours and after that again the samples were fixed with 70% ethanol.

Skinning the samples and removing internal organs were done carefully. We Stained cartilaginous parts of the skeletal system with 0.02% alcian blue prepared in ethanol and acid acetic glacial for about 48 hours. After that the samples were washed in ethanol and acid acetic glacial solution.

As the next step the samples were soaked in absolute ethanol and then were treated in distilled water respectively for 24 and 48 hours. Clearing stage was done with a solution which was made of 1% trypsin and 30% saturated sodium borate and samples were remained in it until the skeletal parts particularly stained cartilages were exposed.

A little amount of alizarin red was added to 0.5 % potassium hydroxide until the color of solution became red purple and for about 36 hours the cleared samples were remained in it, then the samples were treated with gradient series of glycerol which was added to

0.5% potassium hydroxide several times, 48 hours for each step.

Finally the stained and cleared samples were stored in pure glycerol in which a crystal of tymol was added.

3. Results

Bones and cartilages as skeletal parts of a stained sample became red and blue respectively. Samples became so clear that the skeletal parts were seen through them. The absorbed colors were stable, so it wouldn't vanish by passing the time. At the end of staining process, other tissues and muscles didn't absorb any color. Samples kept their rigidity during staining processes. All of the details about cartilage and bones became obvious, so all the studies about skeletal parts became possible.

4. Discussion

Osteogenesis study in laboratory animals requires differential staining which is done by using two colors, alcian blue for cartilages and alizarin red for bones.

It is recommended to use colors in separate stages in order to give optimal stain. Our study shows that mixing two colors cause high color absorption by samples, so the next concern will be decolarization that is the problem of many investigators.

It is better to solve Alcian blue which is used for cartilage staining in ethanol and glacial acetic acid instead of water. Some kind of the alcian blue are insoluble in water and sedimentated, so the cartilages can't gain any color. Our experiment shows that it is the best way for alcian blue to solve and also for cartilaginous parts to gain the color.

The important point of clearing stage is that embryos and early postnatal samples don't require enzyme digestion and the best clearing process can be done by use of potassium hydroxide. The concentration of KOH is another problem because high amount of it cause fragility and end the staining process. Because the samples which lost their rigidity can't gain enough alizarin red and also can't clear again.

We came to this conclusion that 1% KOH is the best clearing agent for these samples. On the other hand, the adult samples require enzyme digestion and can't be cleared by using potassium hydroxide only. The important problem during enzyme digestion is tempreture. Some papers indicated to use incubator and specific temperature during this stage but we came to this conclusion that the best way is to remain the samples in room temperature and allow them digest by passing the time. High temperature can destroy the joints and make jelly – like samples.

Another point is using saturated sodium borate in which trypsin is solved. It supports the most rapid enzyme activity while maintaining a relatively stable and desirable pH over a long period of time, and it also prevents bacterial growth that lead to disagreeable odors.

If the samples remain in fixative solution longer, the best result will be gained in clearing stage. So you can lengthen the fixation step in ethanol and have the more transparent stained samples at last.

Alizarin red absorption by bones affected by the amount of Ca in bone structure. Embryos bones in which calcification is started newly and have a little amount of calcium, fixing in formalin solution such as formalin acid or neutral formalin result in bone decalcification so it will reduce the stain affinity.

The evidence from this study suggests that it is better to use ethanol as fixation, 1% KOH as clearing agents in embryos and early postnatal staining.

Acknowledgement

The author appreciated from Dr. Salar Amoli (Associated professor of toxicology department of Tehran University) as her Professional thesis supervisor, acknowledged the support of Toxicology center of Tehran university and all of its members and Miss Esfahani because of her truly assistance.

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