Modified double skeletal staining protocols with Alizarinred and Alcian blue in laboratory animals

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ABSTRACT

Purpose: Skeletal staining is a way to study the effect of many chemical or herbal substances on development of bones and cartilages in order to record the level of probable deformity. This article aimed to present a modified protocol to make all skeletal studies on laboratory animals possible.

Materials and Methods: The important notes about skeletal staining were fixing by ethanol and clearing by potassium hydroxide in embryonic and newborn samples. In adults it was fixing by neutral formalin buffer, then ethanol after washing the samples in ddH2O, and clearing the samples by trypsin and potassium hydroxide in separated stages. The amount of colors used for cartilages and bones was different in animals with different ages for a good stained sample.

Results: The mentioned procedures resulted incompletely clear stained samples whose skeletal parts, i.e. cartilages and bones, were stained blue and red, respectively.

Conclusion: Although most reviewed investigations have used the same protocol in different laboratory animals with different ages, the used materials and also their concentrations for skeletal staining procedures differ in embryos and adults.

Keywords: Alizarin red; Alcian blue; double skeletal staining.

INTRODUCTION

Staining of embryonic and fetal skeleton in laboratory animals is an important step in teratological investigations. Alizarin red as a color indicator for skeletal bony parts has a high affinity for binding with calcium ions and has been used for many years in order to stain bones. Also, staining with alizarin red which is followed by potassium hydroxide clearing has been used for many years as a simple and reliable technique.¹⁻³

Toluidine blue as a color for staining cartilaginous parts of the skeletal system was introduced in 1941 and it is used with alizarin red in order to cause differential staining.⁴⁻⁵ Alcian blue as another color for staining cartilage parts of skeletal system was suggested in 1970.⁶ After that all the double skeletal staining procedures have been done by Alizarin red and Alcian blue as bone and cartilage indicators.

Different protocols have been proposed since 1897 for staining skeletons and cartilages. Each of them had its own limitations. In most surveys a same protocol was used in laboratory animals with different ages. However, this study tried to explain two modified protocols and the difference of staining in adult and embryo of laboratory animals.

MATERIALS AND METHODS

All the laboratory animals which were planned to be stained were divided into two groups: 1) embryos and...
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2) infants and 2) adults. The method of staining for each group was as follows:

**Embryos and infants**

- **Preservation and fixation:** The first step was eviscerating. Since skinning was impossible in some embryos, it was ignored. In other cases, it was done and then the thorax and abdomen cavities were removed. The samples were left in 90% or absolute ethanol for at least seven days.

- **Cartilage staining:** The specimen was completely immersed in a solution of 0.01% Alcian blue which was prepared in 70 CC pure ethanol and 30 cc acid acetic glacial (7:3).

- **Rehydration:** The specimen was placed in a bath of 95% ethyl alcohol for two hours. This was repeated for another two hours in a new bath. Each specimen was placed in baths of successively decreasing concentrations of 75%, 40%, and 15% ethyl alcohol, two hours for each concentration.

- **Washing:** To eliminate any excess ethanol, the specimen was immersed and rinsed in several changes of distilled water for two to three hours.

- **Clearing:** The samples were left in 1% potassium hydroxide until the skeletal system of the embryo was exposed.

- **Bone staining:** The specimen was in 0.001% aqueous Alizarin red for three days in order to stain bony parts.

- **Washing:** To eliminate any excess alizarin red color, the specimen was immersed and rinsed in 1% potassium hydroxide three times, several hours each time.

- **Clearing and dehydration:** The samples were treated with ascending series of glycerol in 1% potassium hydroxide, 24 hours for each step.

- **Storage:** The specimen was placed in pure glycerin for permanent storage. A crystal of tymol was added to storage solution in order to prevent mold growth.

**Adults**

- **Fixation:** Neutral 10% buffered formalin was used for primary fixation for at least 24 hours.

- **Washing:** The fixed sample was washed with distilled water to remove the used fixation solution and was left in it for 24 hours

- **Fixation:** The sample was fixed with 70% ethanol. The samples were left in this solution for a long period of time.

- **Samples treatment:** The sample was skinned and its internal organs were carefully removed.

- **Cartilage staining:** Cartilaginous part of the skeletal system was stained with 0.02% Alcian blue prepared in ethanol and acid acetic glacial for about two days.

- **Washing:** The samples were washed in a solution by 70 cc ethanol and 30 cc acid acetic glacial and then soaked in absolute ethanol and treated in distilled water two and three days, respectively.

- **Muscle digestion:** The specimen was immersed in a saturated solution of 30 ml of sodium borate, 70 ml of distilled water, and 1g trypsin enzyme “trypsin from porcine pancreas”. The sample remained in digestive solution until the skeletal parts particularly stained cartilages were exposed.

- **Bone staining:** The specimen was transferred to 0.5% aqueous potassium hydroxide. Alizarin red S was added in powder until the solution became deep purple in color. The specimen was left in this solution for 36 hours.

- **Clearing and dehydration:** The sample was treated with ascending series of glycerol in 1% potassium hydroxide “sequential series: 3:1, 1:1, and 1:3”, 48 hours for each step.

- **Storage:** The specimen was placed in pure glycerin in which a crystal of tymol was added for permanent storage.

**RESULTS**

The protocols were successfully followed for staining laboratory animals such as mice, rat fetuses and adults. The specimens showed red staining of the bones and blue staining of the cartilages, so that anomalies of both the cartilaginous and the bony skeleton were examined. The defects in the cartilaginous part of the ribs, Sterne brae, tarsi, metatarsi, carpi, metacarpi and phalanges were not observable in single skeletal staining technique with Alizarin red. So Alcian blue was added for staining cartilages and bones simultaneously.

The results of this modified protocol were as follows:

1. As skeletal parts of a stained sample, the bones and cartilages became red and blue, respectively.

2. Samples became so clear that the skeletal parts were seen through them.

3. The absorbed color was stable so it was not vanished in time.

4. At the end of staining process, other tissues and muscles did not absorb any color.

5. Samples kept their rigidity during staining processes.

6. All details about cartilage and bones became obvious, making study of all skeletal parts possible (Figures 1...
Skeletal staining has been used for many years and it is employed in many anatomical and teratological investigations nowadays. A list of most researches on skeletal staining procedures has been given in Table 1. The main concern of all of them is the difference between the used materials and their concentrations. For example in the 1st article about single skeletal staining published in 1897, Schultze used only Alizarin red plus ethanol 95% and potassium hydroxide 3% for fixing and clearing respectively. Mall in 1906 used formaldehyde and potassium hydroxide 10%. In 1983, Kelly and Bryden claimed that formol-acetic-alcohol was the best fixative and superior to alcohol or formalin alone because it markedly decreased the time needed for enzyme clearing in skeletal staining procedures. In this article the skeletal staining technique in all the ages was done same and all the samples (embryos and adults) were cleared by enzyme digestion. Another study used formaldehyde which is buffered by magnesium carbonate as fixative. In our suggested protocol the fixation process is done by ethanol in young samples and buffer formaldehyde and ethanol in adults. It showed that the best and safest way for clearing embryos and also newborns is using potassium hydroxide.

Using a suitable clearing agent in one step and ascending degrees of glycerin resulted in clearing samples through which their skeletal parts can be seen. But many studies have suggested different chemical materials to clear samples. For instance, Hollister has recommended ultra-violet light in the clearing process. This had effect in bleaching and hastening the clearing stage. In 1939 a study had proposed toluol, toluol saturated with naphthalene, and anise oil saturated with naphthalene to clear the samples. In 1941 methyl salicylate was used as a clearing agent instead of glycerin. But using methyl salicylate for clearing tissue (first suggested by Spalteholz in 1914) had two slight disadvantages. First, the cleared tissue had a tendency to turn brown over a period of years. This was remedied by transferring the specimen to a fresh lot of the oil and re-clearing. Second, the tissues undergo a slight amount of shrinkage, although not enough to affect the relations of the skeletal parts in any appreciable manner. In another method the embryos were bleached in hydrogen peroxide and cleared in benzol and synthetic oil of winter-green. Some of the disadvantages of this technique were:

1. hydrogen peroxide caused the tissue to become filled with bubbles which were often difficult to remove,
2. hydrogen peroxide was not as efficient as a bleaching agent as are some other compounds,
3. the tissue tended to turn brown with age, and
4. oil of wintergreen caused the tissues to shrink.

In 1948 methyl benzoate was used for clearing as supplementary to the method mentioned above. Beautifully clear specimens were obtained, but the amount of shrinking was so great that they became unsuitable where it was necessary to retain, as nearly as possible, the natural shape. The specimen should appear
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<tr>
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translucent with the skeletal structure showing red and blue. If the amount of used colors is high or both colors are used in one step, the muscular tissues retain some stain. So the specimen should be further treated with acetic acid or other decolorization agents. The best way for clearing the embryos and infants samples is using potassium hydroxide and glycerol. Enzyme digestion, potassium hydroxide and glycerol are the best materials for suitable clearing in adult samples.

CONCLUSION

During the skeletal staining process it was concluded that fixing embryos in ethanol is better than fixing by formaldehyde. This is because formalin causes bone decalcifying, reducing the bones absorbance and making the stained samples pallid. Using colors in separate stages gives the best stain results compared to the time when two colors are mixed. So in this way and using appropriate concentrations, there is no need for decolorization agents. Laboratory animals which are in embryonic period or are newly born have a delicate body. Clearing them using enzyme digestion or high concentration of potassium hydroxide will make them lose their rigidity.

The appropriate clearing agent is 1% potassium hydroxide so the samples will become so clear that all the stained parts can be seen through. The best solvent for Alcian blue as cartilages marker is a solution of ethanol and glacial acetic acid. Thus, all the used color will be solved and cartilage parts will be stained properly. As long as you keep the specimen in ethanol as fixation solution, sharper stained samples will be achieved.

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