한국환경보건학회지, 제36권 제4호(2010)
J. Env. Hlth. Sci., Vol. 36, No. 4, pp 337~341(2010)

Determining the Proportions of Bone and Cartilage Growth in the Crucian Carp (*carassius auratus*) Using the Modified Simultaneous Differential Staining Technique

Jin Heon Lee†

Department of Environmental Education, Kongju National University, Chungnam 314-701, Korea

(Received April 25, 2010/Revised June 30, 2010/Accepted August 20, 2010)

ABSTRACT

The modified simultaneous differential staining technique, which enables double staining of cartilage and bones, needs to be improved to prevent soft tissues from being damaged during the staining process. Key factors influencing the extent to which soft tissues are damaged include the fixative used, macerating time, potassium hydroxide concentration, incubation temperature and the removal of skin from specimens. Here we describe a protocol that enables the hardening of tissues during bleaching and maceration. We also describe a method for objectively measuring rates of cartilage and bone growth. The use of formalin as a fixative rendered soft tissues more rigid due to the resulting chemical bonds formed between proteins. Blotted specimens were immersed in 1% potassium hydroxide (KOH) and incubated at 37°C for 1 day (smaller specimens) or 2-3 days (larger specimens). The 1% KOH solution was also used as the diluent solution for the subsequent immersion in a graded series of 30%, 50%, 70%, 90%, 100% glycerol solutions, a procedure that made soft tissues even more transparent and hardened. It was not necessary to remove the skin of specimens shorter than 2 cm, since the macerating solution could easily penetrate their thin skin layer and continuously remove those pigments hindering visibility. Since excessive osmosis is another factor that can damage soft tissues in the macerating process by causing the rupture of those cells not able to withstand the osmotic pressure, here it was minimized by balancing the salt concentration between the interior and exterior of cells with the addition of 0.9% sodium chloride (NaCl) in the macerating solution. Finally, to determine the proportions of cartilage and bone growth, photographs of the stained specimens were taken with a dissecting microscope and sections corresponding to the cartilage and bones were cut out from the printed pictures and weighed. Our results show that this method is suitable for the objective evaluation of bone and cartilage growth.

Keywords: staining, alcian blue, alizarin red, cartilage, bone, skeleton

I. Introduction

The development of techniques enabling double staining of cartilage and bone cleared from soft tissues markedly facilitated the study of skeletal formation and the interpretation of its alterations, with such procedures being now widely used in studies ranging from bone growth to embryogenesis and morphogenesis.

Earlier methods,[1-4] which used alizarin red S for staining mature, ossified bones, were modified to enable the differential staining of cartilage and bones by combining alizarin red S with alcian blue for cartilage staining.[5-11] The simultaneous differential staining method introduced by Inouye[5] and the rapid skinning procedure described by Kimmel and Trammell[10] enabled the examination of specimens in a period as short as 48 h.[12]

The most important aspect of the differential staining method was the possibility to visualize both the cartilage and bone through the cleared soft tissue. To this end, many studies[5-9] recommended that the pigments in the connective tissue hindering visibility should be removed during maceration. Kimmel and Trammell[10] also described

†Corresponding author : Department of Environmental Education, Kongju National University
Tel: 82-41-850-8814, Fax: 82-41-850-8816
E-mail: ejhl@kongju.ac.kr

a rapid skinning procedure, whereby specimens were immersed for approximately 30 seconds in a 70°C water bath before skinning. One drawback of this method, however, was that the soft tissue of skinned specimens was easily damaged during bleaching and maceration. Indeed, Wassersug[6] recommended that maceration should be carefully controlled to prevent damaging specimens.

Here we describe a new procedure to harden a specimen's soft tissues during bleaching and maceration, simultaneously enabling the skin to be effectively removed. In addition to the morphological examination of specimens, as required for instance in developmental toxicology and growth studies,[13] information about the dimensions of specimens is often needed. To our knowledge, however, no methodology has been developed to date that enables the direct measure ment of stained specimens. In the present study we additionally describe a method for measuring the cartilage and bones of crucian carp (C*arassius auratus*) specimens of different lengths.

II. Materials and Methods

1. Preparation of Specimens

Crucian carp specimens with body length ranging from 1 to 4 cm were captured from a stream near Gongju city during winter (January and February 2008) and transferred to a tank with clean aerated water, where they remained for 2 weeks before fixation. Each size group make to be included more 5 specimens.

2. Fixation

Specimens were immersed in 4% phosphate buffered formalin (the volume of the solution was 10 times that of specimens) until they sunk to the bottom as a result of enough formalin penetration into their tissues (a process that took approximately 3 to 5 days).

3. Skinning

Two simple procedures for skinning the specimens were developed. Since formalin is a volatile toxic and soluble material, specimens were washed with trickling tap water for approximately 6 h to rinse the formalin off their surface. The skin was then easily stripped off with a pincette to remove pigments in the connective tissue hindering transparency. It was not necessary to remove the skin of smaller specimens, with body length shorter than 2.5 cm.

4. Dehydration

To ensure absolute dehydration, specimens were sequentially immersed in 50%, 70%, 90%, 99% absolute ethanol for 24 h. Since complete dehydration is essential to alizarin red S staining, the number of ethanol changes and length of time in each step was adjusted for the larger specimens.

5. Simultaneous double staining

We used 0.3% alcian blue (Sigma, St. Louis), 0.12% alizarin red S (Sigma), 70% ethanol and glacial acetic acid. Alcian blue and alizarin red S were dissolved in 70% and 95% ethanol, respectively, and then filtered. Each of the four solutions was added to containers with the specimens at a rate of 1:1:17:1, respectively. The volume of the mixed solution should be enough for appropriate staining, about five times the volume of specimens. The specimens were incubated at 37°C in a sealed container for 3 days.

6. Maceration and bleaching of soft tissues

The blotted specimens were placed in a macerating solution composed of 1% potassium hydroxide (KOH) and 0.9% sodium chloride (NaCl). They were incubated at 37°C for one day, and then immersed in a new solution for 2 to 3 days at room temperature until the solution was purple.

7. Clearing, Hardening and Storage

Double stained specimens were placed in graded concentrations of glycerol solutions, e.g. 30%, 50%, 70%, 90%, 100% glycerol for 2 to 3 days in each case. The 1% KOH solution was used as the diluent solution for the graded series. They were then stored in vials containing fresh 100% glycerol and a small crystal of phenol to retard spoilage.

8. Measuring cartilage and bones

Specimens' photos were taken with a dissecting microscope (SZ-PT, Olympus) and printed. Sections corresponding to the cartilage and bones were cut out from the printed pictures and weighed. The

values are averages calculated from the printed paper weight of bone (or cartilage) images divided by weight of the specimen's body, and then multiplied by 100, with five specimens in each group. The error bars show standard deviation.

III. Results and Discussion

Although potassium hydroxide has been used to macerate and bleach blotted specimens, it could damage their soft tissues due to its corrosive properties. The maceration of soft tissues is however essential for the staining process, so a procedure must be employed to prevent their destruction. One possibility is the use of formalin as a fixative, which creates chemical bonds between tissue proteins, particularly between lysine residues, making them more rigid.[14] Fresh formalin must therefore

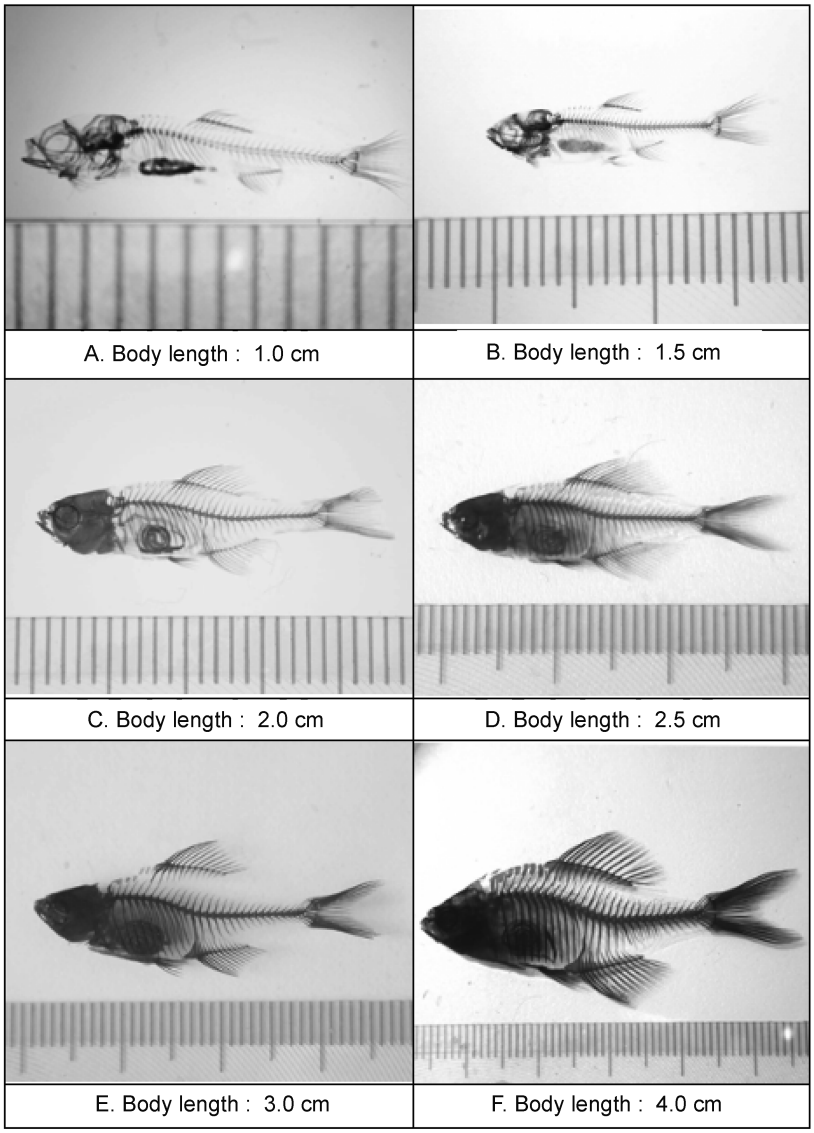

Fig. 1. Cleared, whole-mount specimens of crucian carp (*Carassius auratus*) stained using a modified alcian blue-alizarin red S double staining technique for cartilage and bone. Transparency was marked and equally achieved in small (A, B, C) and large specimens (D, E, F). Small scale marks correspond to 1 mm.

penetrate specimens for formalin fixation. However, the long term use of formalin as a fixative, such as in the case of museum specimens, renders soft tissues semitransparent.[15] Formalin penetrates tissue relatively slowly,[14] so we assessed whether fresh formalin had appropriately penetrated the specimens' tissues or not, by waiting for the specimens to sink to the bottom (formalin density: 1.09 kg/*l*, 20°C).

Ethanol is also a fixative, but it fixes proteins by dehydration and precipitation and depends on the water content and solubility of the materials in the mixture.[16] We therefore do not recommend the use of ethanol as a fixative, because ethanol-fixed specimens are easily destroyed during maceration and bleaching.

Other factors that affected the degree to which soft tissues were damaged were the macerating time, KOH concentration and incubation temperature. Webb and Byrd[13,20] noted that the optimal maceration time was approximately 24-48 h with 0.75% KOH at room temperature. Here we used 1% KOH for maceration, but incubated smaller specimens (less than 2.0 cm of body length) at 37°C for 1 day, or incubated larger specimens(more than 2.0 cm of body length) at 37°C for 2-3 days. Under these conditions, which varied depending on the size and/or age of specimens, all specimens macerated well. The 1% KOH solution was also used as the diluent solution for the graded series of 30%, 50%, 70%, 90%, and 100% glycerol solutions in which specimens were placed, and which enabled soft tissue to become more transparent during clearing and hardening. Skin removal was also a key factor in the destruction of soft tissues during maceration, making it important to preserve the skin as much as possible. For specimens of smaller sizes and/or ages, such as those shorter than 2 cm, pigments did not hinder the transparency of soft tissues. We believe that the macerating solution could easily penetrate the thin skin layer of smaller specimens and continuously remove pigments during the clearing and hardening process. Indeed, in Fig. 1(A, B, C) it is possible to see that transparency in these small specimens was satisfactory even though their skin was not removed.

Osmosis was another factor influencing the destruction of soft tissues during maceration. Cytolysis is caused by excessive osmosis, or the infiltration of water into a cell, which becomes swollen or 'hyper-hydrated'.[17,18] As the cell membrane cannot withstand the osmotic pressure of the water inside, it explodes. This phenomenon occurs in hypotonic solutions, which are characterized by a lower salt concentration than the cytoplasm.[17,18] Staining requires placing specimens in hypotonic solutions for a long time, thus it is necessary to prevent cytolysis by employing some procedures, such as rapidly pumping water out of cells, or balancing the salt concentration between the exterior and interior of cells. Here we tried balancing the salt concentration of cells by adding 0.9% NaCl in the macerating solution As shown in Fig. 1(A, B, C, D, E, F), this modified method positively affected the outcome of the maceration and bleaching procedures.

The study of bone growth, embryogenesis and morphogenesis depends both on the possibility of evaluating the morphology of specimens as well as of measuring their skeletal and cartilage size.[13] Here we propose that the cartilage and bones of stained specimens could be measured in the same way as that used in analytical chromatography of analytes in a mixture, where a variety of paper picks, e.g. chromatograms, are obtained and

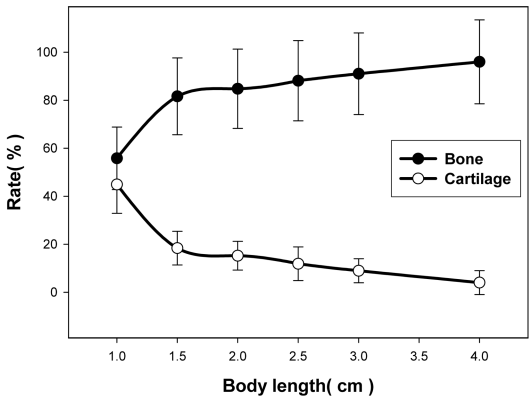

Fig. 2. Proportions of bone and cartilage in crucian carp (*carassius auratus*) specimens of different sizes (five specimens in each group) were measured by weighing the printed sections of bones and cartilages cut out from photographs of stained skeletons. The values are averages calculated from the printed paper weight of bone (or cartilage) images divided by the weight of specimen's body, and multiplied by 100. The error bars show the standard deviation.

subsequently weighed.[19] Similarly, we photographed the archived specimens with a dissecting microscope, cut out the sections corresponding to the cartilage and bones from the printed pictures and weighed them. Fig. 2 shows the percentage of bone and cartilage in crucian carp specimens of various sizes/ages. As expected, the percentage of bone increases with the size of the fish. More importantly, the results show the suitability of the method for the objective evaluation of bone and cartilage growth from morphological photographs.

References

1. Al-Saraj, A. : Use of saturated sodium chloride solution as a tissue fixative. *Iraqi Journal of Veterinary Sciences*, **24**(1), 53-58, 2010.
2. Baker, J. R. : Principles of biological microtechnique. London: Methuen. 1-368, 1958.
3. Borg, F. G. : What is osmosis? Explanation and understanding of a physical phenomenon: General Physics, Jyvaskyla University, Chydenius Institute, Karleby, Finland, 1-39, 2003.
4. Crary, D. D. : Modified benzyl alcohol clearing of alizarin stained specimens without loss of flexibility. *Stain Technology*, **37**, 124-125, 1962.
5. Dawson, A. B. : A note on the staining of the skeleton of cleared specimens with alizarin red S. *Stain Technology*, **1**, 123-124, 1926.
6. Dingerkus, G. and Uhler, L. D. : Enzyme clearing of alcian blue stained whole small vertebrates for demonstration of cartilage. *Stain Technolology*, **51**, 229-232, 1977.
7. Green, M. C. : A rapid method for clearing and staining specimens for the demonstration of bone. *Ohio Journal of Science*, **52**, 21-33, 1952.
8. Inouye, M. : Differential staining of cartilage and bone in fetal mouse skeleton by alcian blue and alizarin red S. *Congenital Anomalies*, **16**, 171-173, 1976.
9. Kelly, W. L. and Bryden, M. M. : A modified differential stain for cartilage and bone in whole mount preparations of mammalian fetus and small vertebrates. *Stain Technology*, **58**, 131-134, 1983.
10. Kimmel, C. A. and Trammell, C. : A rapid procedure for routine double staining of cartilage and bone in fetal and adult animals. *Stain Technology*, **56**(5), 271-273, 1981.
11. Lee, J. H., Park, K. L. and Kim, D. S. : Protective effects of Korean garlic juice against the toxicity of methyl mercuric chloride in relation to fetal ossification in pregnant fischer-344 rats. *Korea Journal of Environmental Health Sciences*, **34**(2), 161-169, 2008.
12. Mcleod, M. J. : Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red S. *Teratology* **22**, 299-301, 1980.
13. Park, E. H. and Kim, D. S. : A procedure for staining cartilage and bone of whole vertebrate larvae while rendering all other tissues transparent. *Stain Technology*, **59**(5), 269-172, 1984.
14. Skoog, D. A. : Chromatography: Principles of instrumental analysis, 3rd. Saunders College publishing, 653-679, 1991.
15. Staples, R. E. and Schnell, V. L. : Refinements in rapid clearing technique in KOH-alizarine red S method for fetal bone. *Stain Technology*, **39**, 61-63, 1964.
16. Wassersug, R. J. : A procedure for differential staining of cartilage and bone in whole formalin-fixed verterates. *Stain Technolology*, **51**(2), 131-134, 1976.
17. Webb, G. N. and Byrd, R. A. : Simultaneous differential staining of cartilage and bone in rodent fetuses: an alcian blue and alizarin red S procedure without glacial acetic acid. *Biotechnic and Histochemistry*, **69**(4), 181-185, 1994.
18. Whitaker, J. and Dix, K. M. : Double staining techniques for rat fetus skeletons in teratological studies. *Lab Animals*, **13**, 309-310, 1979.
19. Wikipedia : Cytolysis, the free encyclopedia, http://en.wikipedia.org, 2010.