Published in final edited form as:

Growth Horm IGF Res. 2010 April ; 20(2): 171–173. doi:10.1016/j.ghir.2009.10.004.

A Rapid, Valid and Inexpensive Assay for Measuring Epiphyseal Plates in Mouse Tibia

Jillian P. Interlichia^{1,*}, Nolann G. Williams^{2,*}, and Buel D. Rodgers^{1,2}

¹Department of Animal Sciences, Washington State University, Pullman WA 99164

²School of Molecular Biosciences, Washington State University, Pullman WA 99164

Abstract

One of the most accurate indices of changes in somatic tissue growth rate in rodents is the width of tibial epiphyseal plates as unlike most mammals, rodent growth plates never ossify. Unfortunately, the original procedure to measure tibial epiphyseal plate width (TEPW) was developed for rats and yields poor results with mice. This paper demonstrates a simple method for silver staining growth plates that can be used to inexpensively and quickly measure the TEPW of mice. Poor visualization due to overstaining and the shattering of growth plates necessitated several revisions to the original protocol. These include exposing the growth plate prior to acetone dehydration, reducing the silver nitrate concentration from 2% to 1.5% and staining time from 2 minutes to 10 seconds and finally, the use of reflective light rather than transmissive light when imaging. The optimized protocol was then validated by generating an age-dependent TEPW growth curve that matched changes in tibia length. A total of 120 tibias were processed in a combined time of less than one day and for less than \$30. By contrast, histological processing in the university's core facility would have cost \$1440 and taken approximately three weeks. Thus, the revised protocol is vastly more cost effective, reliable and can be performed considerably quicker with minimal training.

Keywords

tibial epiphyseal growth plate; growth hormone; IGF-I

Introduction

Quantifying the growth rate of long bones is an aspect of many biomedical studies that utilize rodent models and is often directly related to global changes in organismal growth. Excluding mineralization disorders, an accurate cross-sectional measurement of both long-bone and organismal growth rate in rodents is width of the tibial epiphyseal plate (1,2). Growth plates in general are zones of ossifying cartilage found between the metaphysis and epiphysis and are responsible for longitudinal growth of long bones. The width of these plates, therefore, is an accurate indicator of their growth rate at sacrifice as it can either increase or decrease and is controlled by factors (e.g. growth hormone, insulin-like growth factors, glucocorticoids) that

© 2009 Elsevier Ltd. All rights reserved.

Please address all correspondence and reprint request to the following: Buel D. Rodgers, Ph.D., 124 ASLB, Dept. of Animal Sciences, Washington State University, Pullman, WA 99164-6351, (509) 335-2991 (offc.), -4246 (fax), danrogers@wsu.edu.

*These authors contributed equally to this manuscript.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

similarly influence changes in organismal growth rate (3,4). Rodents are good models for these studies as their growth plates never completely ossify and can vary in size depending upon the presence or absence of regulating factors. This is in contrast to tail or tibia length, which reflect long-term aggregate growth over time, as these lengths are incapable of receding.

Measurements of tibial epiphyseal plate width (TEPW) are particularly useful in assessing growth plate status among rodent populations as they help extrapolate changes in linear growth in rodents (e.g. length) to humans (e.g. height). These assays are commonly performed in rats without the histological processing of tibia as they are simple, inexpensive and accurate. The original protocol was in fact used for many years in a bioassay for measuring growth hormone (5) and included three basic steps: the dehydration of bone with acetone, cutting the epiphyseal region to expose the growth plate and staining the growth plate with silver nitrate (Fig. 1A). This results in a strong brown/black staining of ossified bone, but not the growth plate as it is largely composed of glycosaminoglycans and collagen. This protocol was used successfully in rats, the most prevalent model organism between 1940 and 1980 (6,7), but is not used with mice due to difficulties in processing the tibia, mostly TEPW fracturing and overstaining. The relatively recent establishment of mice as the primary biomedical model has resulted in a need to revise the assay especially as current protocols require histological processing and staining with hematoxylin and eosin (8). These techniques are particularly useful for immunocytochemistry, but they are also time consuming and comparatively expensive. This paper, however, demonstrates a simple, inexpensive and rapid method for measuring TEPWs in mice that is based on the rat protocol of Evans *et al.* (5).

Materials and Methods

Animals

C57 Bl6/J mice were housed in environmentally controlled rooms with 12 h daily light and their use was performed according to protocols pre-approved by the Institutional Animal Care and Use Committees (IACUC) at Washington State University. Tibias were extracted from mice aged 1–10 months of age, measured and placed immediately in ddH₂O for at least one hour. Tail lengths were also measured from the top of the anus to the tip of the tail. Tibias from 1–5 week old mice were cut with #11 scalpel blades as the smaller, less ossified bones required a sharper blade. Fresh razor blades were used for older mice.

Original Protocol

Following ddH₂O immersion, the first cut was made as shown in Figure 1A across the femoral condyle opposite the fibula, using a quick sliding motion, as with a chef's knife, to avoid fracturing the epiphysis. Bones were then immersed in acetone for 45–60 minutes to dehydrate. This was followed by immersion in ddH₂O for two minutes, freshly made 2% AgNO₃ for two minutes and ddH₂O for two minutes with concurrent exposure to strong light (Alpha-1501 dissecting microscope light, LW Scientific, Inc., www.LWScientific.com). The second cut was then made as shown in Figure 1A across the second femoral condyle separating the fibula and tibia. Tibias were stored in 70% ethanol for 5–10 minutes to remove excess AgNO₃, nicked in the middle of the diaphysis with a razor blade and broken in half for easier visualization as this enabled the bone to lie flat on the microscope stage. Stained growth plates were then imaged using transmissive light and a Zeiss inverted microscope at 100×. Following visualization, bones were kept in 70% ethanol for extended storage periods.

Revised protocol

After soaking in ddH₂O, both the first and second cuts were made (Fig. 1B, C) followed by the one hour dehydration in acetone. This was followed by immersion in ddH₂O for 2 minutes, 1.5% AgNO₃ for two minutes, H₂O for two minutes and then a ten second exposure to strong

light. The final protocol reduced AgNO₃ exposure to 1 minute (Fig. 1C). After five to ten minutes in 70% ethanol, bones were first visualized with transmissive light (Fig. 1B) and later with reflective light (Fig. 1C). The final protocol was then used to measure the TEPW of mice of various ages including those as young as 14 days and as old as 300 days. Distance measurements were manually performed on images of TEPW using Photoshop by orienting the cursor at right angles from the growth plate borders. A total of 10–15 measurements were taken for each growth plate and an average distance was then assigned to each animal. Tibia and tail lengths were also measured for comparison.

Results

Protocol optimization

Performing the second cut on dehydrated tibias more often than not shattered the growth plate. Additionally, the two-minute immersion in AgNO₃ and two minute exposure to light resulted in overstaining that prevented visualization with transmissive light (Fig. 1A). Indeed, the brightest light penetrating the growth plate came through a break that occurred during the second cut. With the first revision, however, performing both cuts before the acetone dehydration step and reducing the AgNO₃ concentration to 1.5% and the light exposure time to 10 sec kept the growth plate intact and reduced the problem of overstaining. It was noted that longer light exposures significantly increased both background and specific staining. However, the use of transmissive light still resulted in a blurry image and the TEPW was difficult to quantify (Fig. 1B). Shortening the AgNO₃ exposure to one minute and imaging growth plates with reflective light substantially improved the staining and image quality as cartilaginous (light) and ossified (dark) tissues were clearly distinguished (Fig. 1C).

Assay validation

The improved protocol was used to measure the TEPWs of 60 mice of various ages and to compare these growth curves to those of tibia and tail lengths. These assays were performed by different people and resulted in no appreciable variability between observers and no differences between tibia of the same mice. TEPW declined rapidly between 14 and 70 days and held steady through 300 days of age (Fig. 2A). Changes in tibia and tail lengths were similar, increasing in size quickly between 14 and 70 days followed by little or no appreciable growth thereafter (Fig. 2BC).

Discussion

Our revisions to the rat protocol described by Evans *et al.* (5) optimized growth plate staining in mouse tibias without compromising growth plate integrity. These revisions include completely exposing the growth plate with both cuts before dehydrating, optimizing the staining procedure and imaging with reflective light. The new protocol (Fig. 2C) enabled us to successfully visualize and measure the growth plates of mice aged two weeks through ten months. The TEPW growth curve generated from these data mirrored changes in tibia length, as the window of rapid growth (day 0–70) was identical in both graphs (Fig. 2A&B), which validates the assay. The TEPW of older mice are still capable of widening with appropriate stimuli (9,10) suggesting that this assay could be used on mice of various ages including those older than 70 days.

Using our revised protocol, we were able to process the tibia and stain the growth plates of 60 mice for only the cost of silver nitrate and acetone, which can easily be obtained for under \$30. This represents significant savings as processing this many samples, for example, at our university's histology core facility (http://www.vetmed.wsu.edu/depts_WADDL/), would have required paraffin imbedding, sectioning and staining, would have cost \$1440 and would

have taken an estimated three weeks. This is in contrast to our processing of each sample in just a few minutes and in a combined time of less than a single day. However, the two assays, silver nitrate and histological processing, are not qualitatively the same. Silver nitrate stains only ossified bone whereas hematoxylin and eosin differentially stain cartilage and bone. The different zones within the cartilagenous growth plate can therefore only be distinguished using the latter procedure. We have noted from personal experience that growth plates stored in ethanol can often be reliably remeasured after 1 year, although tissue integrity of some samples is compromised. Nevertheless, our revised protocol produces an accurate measurement of mouse TEPW, in far less time and for much less money. Marking individual bones (e.g. by nicking) would also allow one to process many samples simultaneously and would further reduce assay time. In addition, our assay can be easily performed with very little training and is therefore a viable alternative to histological processing.

Acknowledgments

This work was supported by grants from the National Institutes of Health (AR051917) and the National Science Foundation (0840644) to Buel D. Rodgers. We also wish to thank Melissa Jackson for assistance in processing the tibia.

References

1. Greenspan FS, Li CH, Simpson ME, Evans HM. Bioassay of hypophyseal growth hormone; the tibia test. *Endocrinology* 1949;45:455–463. illust. [PubMed: 15396704]
2. Smeets T, van Buul-Offers S. A morphological study of the development of the tibial proximal epiphysis and growth plate of normal and dwarfed Snell mice. *Growth* 1983;47:145–159. [PubMed: 6618258]
3. Rodgers BD, Lau AO, Nicoll CS. Hypophysectomy or adrenalectomy of rats with insulin-dependent diabetes mellitus partially restores their responsiveness to growth hormone. *Proc Soc Exp Biol Med* 1994;207:220–226. [PubMed: 7938053]
4. Rodgers BD, Strack AM, Dallman MF, Hwa L, Nicoll CS. Corticosterone regulation of insulin-like growth factor I, IGF-binding proteins, and growth in streptozotocin-induced diabetic rats. *Diabetes* 1995;44:1420–1425. [PubMed: 7589849]
5. Evans MM, Simpson ME, Marx W, Kibrick E. Bioassay of pituitary growth hormone. Width of proximal epiphysial cartilage of tibia in hypophysectomized rats. *Endocrinology* 1943;32:13–17.
6. Birke L. Who--or what--are the rats (and mice) in the laboratory. *Soc Anim* 2003;11:207–224. [PubMed: 15146850]
7. Smith TP. Mouse work. How a small animal made it big in research. *Am Herit Invent Technol* 2007;23:22–27. [PubMed: 17992863]
8. Sakamoto A, Chen M, Kobayashi T, Kronenberg HM, Weinstein LS. Chondrocyte-specific knockout of the G protein G(s)alpha leads to epiphyseal and growth plate abnormalities and ectopic chondrocyte formation. *J Bone Miner Res* 2005;20:663–671. [PubMed: 15765186]
9. Oberbauer AM, Currier TA, Nancarrow CD, Ward KA, Murray JD. Linear bone growth of oMT1a-oGH transgenic male mice. *Am J Physiol* 1992;262:E936–E942. [PubMed: 1616026]
10. Oberbauer AM, Pomp D, Murray JD. Dependence of increased linear bone growth on age at oMT1a-oGH transgene expression in mice. *Growth Dev Aging* 1994;58:83–93. [PubMed: 7928023]

A. Original Protocol for Rats

- broken TEPW
- poor staining & resolution

B. First revision for mice

- intact TEPW
- improved staining

C. Final mouse protocol

- optimized staining
- optimized resolution

Figure 1. Revised protocol for measuring the tibial epiphyseal plate widths of mice

(A) The traditional protocol for processing and staining rat epiphyseal plates (1). (B) Revised protocol that prevents breaking of the epiphyseal plate and improves staining. (C) Optimized protocol that includes a shorter staining period and visualization from above with reflective light. Changes from previous protocols are indicated by asterisks.

Figure 2. Growth Rate of Mice Over Time

(A) Growth rate of 60 C57 BL6/J mice aged 14 to 300 days was quantified by measuring the tibia epiphyseal plate width (TEPW) according to our revised protocol (Fig. 1C). (B) Aggregate growth was also determined by measuring tibia length prior to performing the TEPW staining procedure and by measuring tail length (C) immediately following sacrifice.