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# A TECHNIQUE FOR THE *IN VITRO* INCUBATION OF DEER ANTLER TISSUE

V. RAMIREZ* and R. D. BROWN†
Caesar Kleberg Wildlife Research Institute, Texas A&I University, Kingsville, TX 78363, USA.
Telephone: (512) 595-3922

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**Abstract**—1. A procedure for the *in vitro* incubation of velvet deer antler tissue was developed. Biopsy samples were collected in June with a trephine from 2 adult white-tailed deer and incubated in modified BGJb medium up to 48 hr. Calcium (Ca) and hydroxyproline (OH-proline) concentrations in the tissue were determined.

2. A significant increase ($P < 0.05$) in Ca was exhibited at 4 and 8 hr of incubation, and, after replenishment of media, at 48 hr.

3. Hydroxyproline concentrations continued to rise throughout the duration of the incubation period and were significantly higher than controls ($P < 0.05$) at 16, 24, and 48 hr.

4. Results suggest antler tissue can be incubated *in vitro* with the protocol described, although length of incubation may vary with parameter measured.

## INTRODUCTION

Male deer have several characteristics that make them potential models for the study of bone. During antler growth, deer reabsorb minerals from their skeletons creating a "physiological osteoporosis", from which they recover annually (Banks *et al.*, 1968a, b). In addition, growing antler is physiologically bone without a marrow, with a full compliment of chondrocytes, osteoblasts, osteocytes, and osteoclasts (Goss, 1983).

Several *in vivo* studies of antler growth have been conducted (Brown, 1983), but if antler tissue could be grown *in vitro*, more rapid and direct observations could be made. This study, which utilizes procedures adopted from *in vitro* studies of embryonic bone, is the first attempt at developing a method whereby antler tissue can be incubated *in vitro* and its metabolism measured.

## MATERIALS AND METHODS

### *Antler biopsy*

The deer for this study were maintained at the Caesar Kleberg Wildlife Research Institute facilities located 8 km south of Kingsville, Texas, USA. On 21 June 1984, 2 adult (>3.5 years old) male white-tailed deer (*Odocoileus virginianus*) were sedated with xylazine hydrochloride. The collection sites on each antler were washed thoroughly with Betadine (1% iodine) and rinsed with distilled water and isopropanol. The antlers measured approximately 33 cm in length. Six horizontal core samples (3 from each animal) were collected through the girth of main beams along the middle axis with a 7.94 mm Michele Trephine bone biopsy corer (Kirshner, Aberdine, MD, USA). Samples were immediately placed in sterile whirl-pacs containing 5 ml of modified BGJb medium and kept in a thermos filled with sterile distilled water (37°C) until transferred to the lab.

### *Medium*

The BGJb medium (Fitton–Jackson Modification, Irving Scientific, Santa Ana, CA, USA) is similar to that used in earlier studies (Bingham and Raisz, 1973) and is available in a preformulated powder which is made up to volume with sterile distilled water, L-glutamine (200 mg/l), sodium bicarbonate (3.5 g/l) and pen-strep (100 IU-100 $\mu$g/ml). The solution was adjusted for pH (7.3) with 1 M HCl and 1 M NaOH, sterilized by filtration through a 0.22 $\mu$m millipore membrane, and stored at 4°C. The medium was made on the day preceding tissue collection.

### *Incubation*

The samples were sliced longitudinally into 8 sections and weighed. Dissections were conducted in a laminar air-flow hood over the surface of sterile autoclave wraps. Each slice was placed on a droplet of pen-strep (10,000 IU–10,000 $\mu$g/ml, 37°C) arranged along the border of a 15 × 150 mm Petri dish for 20 min at room temperature.

One slice from each original sample was incubated for 2, 4, 8, 16, 24 or 48 hr in 6-well culture plates (Falcon, Dickinson Labware, Oxnard, CA, USA) filled with 2 ml of modified BGJb medium (37°C). The remaining samples (2 from each original core sample) were used as 0 time controls. The medium was changed after 24 hr of incubation by transfering the tissue to new plates.

### *Calcium analysis*

The 0 time controls and incubated slices were rinsed with 0.9% saline, and the wet weight of the tissue was determined. Each sample was then placed in plastic 5 ml vials and decalcified with 4 ml of 5% trichloracetic acid (TCA) overnight (4°C). The extractants were diluted 4 to 16 times with distilled water, and the Ca concentrations were determined in quadruplicate with a Calcette Automatic Calcium Analyzer model 4008 (Precision Systems, Sudbury, MA, USA) set at sample size 40 $\mu$l.

### *Hydroxyproline assay*

The TCA extracted samples from the Ca assay were defatted for 5 min each in acetone and ethyl ether at room

*Present address: Department of Periodontics, Dental School, University of Texas Health Science Center at San Antonio, TX 78284, USA.
†Author to whom correspondence should be addressed. Present address: Department of Wildlife and Fisheries, P.O. Drawer LW, Mississippi State, MS 39762, USA.

temperature. Samples were then dried (110°C, 48 hr), desiccated for 30 min, and weighed. Samples were hydrolized with 3 ml of 4 N HCl (110°C, 22 hr) in 16 × 125 mm Teflon-coated, screw-capped tubes. The hydrolate was brought to volume with distilled water in 25 ml volumetric flasks, filtered through No. 4 Watman filter paper (0.7 $\mu$m), and diluted 2 to 5 times. Hydroxyproline concentrations were measured using an overnight method of Bergman and Loxley (1963), which was essential for the number of samples run simultaneously.

*Statistical analysis*

The means ± standard error (SE) were determined for Ca and OH-proline concentrations at each interval of incubation time. A randomized complete-block analysis of variance (ANOVA) followed by Fisher's Least Significant Difference Test was used to test the differences between incubation times. In addition, concentrations at each interval were tested by paired $t$-tests (SAS Inst., 1985).

## RESULTS AND DISCUSSION

*Calcium concentrations*

The data in Table 1 represents the Ca concentrations for 6 core samples at their respective incubation periods. Results indicate a significant rise in Ca ($P < 0.05$) at 4 hr (9.7%) and 8 hr (14.2%), a decrease at 16 hr and 24 hr, and an increase at 48 hr (9%, $P < 0.05$, from 0 hr). The decrease after 8 hr and later rise after 24 hr may have been due to a depletion of nutrients from the medium which were replenished when the medium was changed after 24 hr of incubation. One must assume that the 16 and 24 hr samples increased in Ca concentration, then released Ca back into the medium. Future investigations simultaneously measuring Ca concentrations of both the tissue and the medium could confirm this. Comparison of the Ca concentrations in this study to those previously conducted is difficult since most previous studies used a number of bones to standardize Ca values (Bingham and Raisz, 1973; Chen and Raisz, 1975; Rabadjija and Goldhaber, 1982). Nonetheless, Ca was apparently absorbed from the media in our study at least up to 8 hr of incubation.

*Hydroxyproline concentrations*

During the mineralization of bone, proline is converted to OH-proline (Stryer, 1981). Because the amino acid is specifically found in collagen, OH-proline is an indicator of bone development. Measuring OH-proline has been used as a method for studying the *in vitro* growth of fetal long bones (Bingham and Raisz, 1973; Chen and Raisz, 1975). In our study, OH-proline increased throughout the 48 hr incubation period (Table 1). Due to large standard errors, a significant increase (27%, $P < 0.05$) was not evident until 16 hr of incubation. Other significant increases ($P < 0.05$) were found at 24 (30.5%) and 48 hr (30.7%) of incubation.

Table 1. Calcium and hydroxyproline concentrations of antler tissue from two adult white-tailed deer incubated *in vitro*

| Incubation time (hr) | Calcium (mg/g wet wt) $\bar{x}$ | SE | Hydroxyproline (M/g dry wt) $\bar{x}$ | SE |
|---|---|---|---|---|
| 0 | 74.4 A | 4.4 | 406.8 A | 31.4 |
| 2 | 75.4 A | 5.9 | 427.6 A | 44.1 |
| 4 | 81.6 B | 4.3 | 444.5 A | 30.5 |
| 8 | 85.0 B | 6.1 | 505.5 A | 64.0 |
| 16 | 77.6 A | 2.7 | 515.5 B | 53.5 |
| 24 | 74.9 A | 1.7 | 531.0 B | 37.0 |
| 48 | 81.1 B | 4.2 | 531.6 B | 36.8 |

Note: each value is the mean ± SE of 6 samples. Calcium values with different letters are significantly different ($P < 0.05$) from each other by ANOVA and from 0 time values by repeated $t$-tests. Hydroxyproline values with different letters are different from 0 time values by repeated $t$-tests only.

Our results concur with previous *in vivo* studies conducted on the OH-proline concentrations of growing white-tailed deer antler in which the greatest amounts of the amino acid were detected in highly mineralized tissue (Newbry *et al.*, 1983). The fact that OH-proline increased in the tissues throughout the incubation while Ca did not may indicate collagen formation in lieu of mineralization. Thus, results indicate that as antler tissue grows, both *in vivo* and *in vitro*, OH-proline concentrations increase, a pattern noted to occur in bone tissue of other species (Biggers *et al.*, 1961; Raisz and Bingham, 1972; Miyahara *et al.*, 1984).

The OH-proline analysis used by Newbrey *et al.* (1983) tested antler tissue samples collected from the center of main beams and tine tips. Our samples were collected cross-sectionally and at lower sites along main beams. Thus, the present study offers the OH-proline concentrations at perhaps greater levels of mineralization.

## CONCLUSIONS

Biopsy samples collected from the main beams of growing antler tissue can be incubated *in vitro*. Results demonstrate that significant increases ($P < 0.05$) in Ca occur after 8 hr and OH-proline after 16 hr of incubation. Similar results occur *in vivo* (Stryer, 1981), suggesting that tissue incubated *in vitro* for this period mimics the natural growth and mineralization of the tissue. Further research into optimum incubation times and methods of measuring growth metabolism is needed. This procedure may increase the research potential of deer antlers for the study of human and animal bone morphology and disease.

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