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# Bone regeneration strategy inspired by the study of calcification behavior in deer antler

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ABSTRACT

Bone regeneration has attracted much attention from various researchers and inspired numerous strategies for bone formation. In this study, rapid calcification of deer antlers was studied to unravel bone biology by investigating mineral composition, morphology and microstructure. Calcification model was hypothesized and preliminarily established by *in vitro* experiments. In our model, mineral deposition and phase conversions in the gel matrix were mimicked. Results revealed that mineral metabolism including deposition and phase conversion plays key roles in calcification *in vivo*, which inspired the bone regeneration strategy with three main components, *i.e.* enhanced mineral nucleation, mineral ions sources and crystals habits. Rapid mineral metabolism of implant apatite biomaterials was supposed as the critical aspect of bone regeneration. This study will provide a relatively ideal model for peer bone regeneration studies.



## 1. Introduction

Recently, many successful methods have been quested for bone regeneration, such as selecting suitable cell sources and tissue scaffolds, creating appropriate biomechanical tissues and integrating into native tissues. Meanwhile, lots of researchers are attracted by the functional and structural replication of bone tissues. The main challenge lies on better understanding of tissue/cell-material interaction which is of great importance for positive bone regeneration [1]. Nevertheless, further investigations of biomineralization including mineral deposition and phase conversions within tissue matrix are equally critical. Adaptive biomaterials, which are favorable for biomineralization, have been proved to promote the growth of bone tissues [2,3]. Therefore, the application of bioactive apatites has been increasingly a focus of attention for positive bone regeneration [4,5].

Antlers are the cranial appendages of deer, and regenerate each year. Deer antler is one of the fastest growing organs among all animal species [6]. Both endochondral and intramembranous ossification are involved in the formation of antler hard tissue. Thus, the annual renewal of antlers can provide a relatively ideal model to explore the mammalian organ regeneration [6,7]. It is currently accepted that antlers are similar to bones in regard to chemical composition and physiological structure. The calcification of antlers is also very similar to the biomineralization of bones, including mineral metabolism within cartilaginous matrix and bone diseases caused by fast mineral deposition [6]. Hence, antlers could be regarded as a simple extension of mammalian bones [8,9], and used as an ideal model for studying bone formation.

In this study, spotted-deer antler specimens were collected from their branches. Chemical composition, morphology, and microstructure were characterized. Possible mechanisms of antler calcification were discussed. Biomineralization hypothesis model for the calcification behaviors was then proposed, which was further confirmed by the *in vitro* experimental model of bioactive mineral system. Inspired by these studies, positive bone regeneration strategy was proposed from the perspective of mineralogy aimed at rapid and adaptive bone formation. With the understanding of the bone-like calcification system of antlers, the possible mechanisms of tissue–biomaterial interactions could be clearer. More promising bone regeneration strategies would be applied for further investigations.

## 2. Experimental

### 2.1. Preparation and characterization of antler tissue samples

Spotted-deer (Cervus Nippon Temminck) antlers were selected as experimental bodies in this study. Six four-year-old adult spotted-deer were breed in open-air environment of Northeastern China. Antler tissue collection was performed under the Stock-breeding Law of the People's Republic of China (2006). Three specimens were cut from the top, the middle and the bottom of each antler tissue, respectively (Fig. 1A). All specimens were then cut into slices with thickness of

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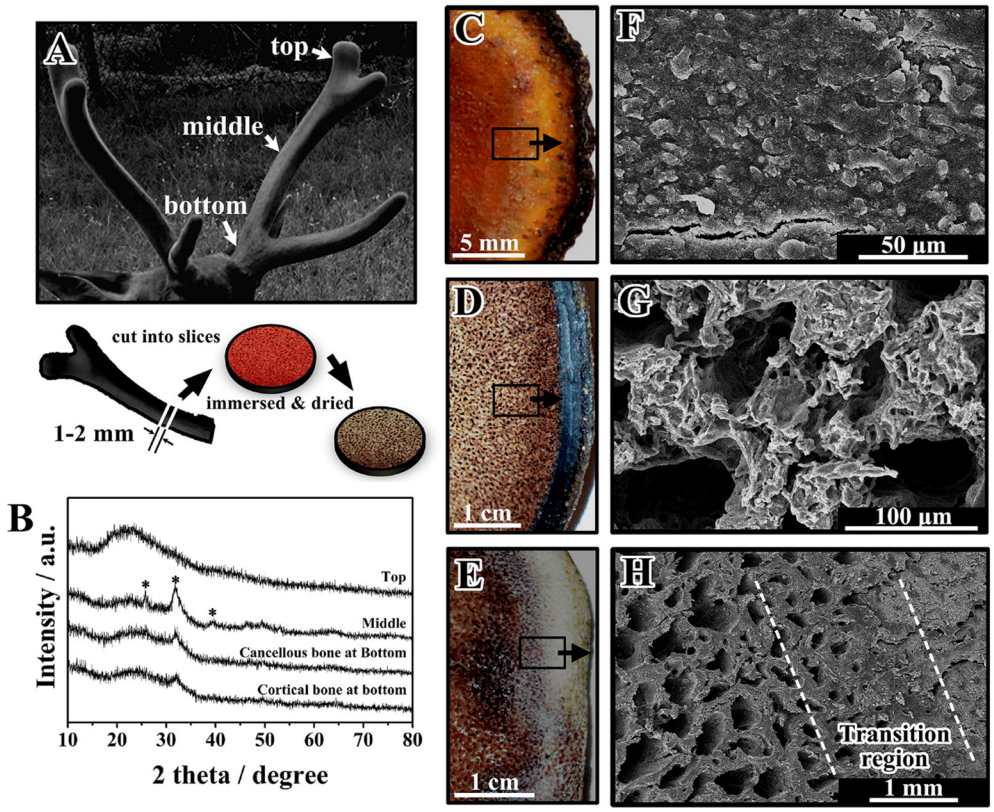


**Fig. 1.** Biological specimens of S1, S2 and S3 were taken from the top, the middle and the bottom of spotted-deer antler branches, respectively (A). These specimens were cut into round slices, immersed in absolute alcohol and air dried. Also shown are XRD patterns (B), optical photographs and FESEM images of cross-sections of these specimens (C, F: S1; D, G: S2; E, H: S3).

1–2 mm, immersed in absolute alcohol for 1 h and finally air dried at room temperature for characterization. Specimens of the top, middle and bottom were labeled as S1, S2 and S3, respectively. In addition, the cancellous bone and cortical bone of S3 were marked by S3-a and S3-b, respectively. Crystallographic features were investigated using X-ray powder diffraction (XRD, X'Pert PRO, PANalytical, Netherlands) with CuKα radiation (λ = 1.5418 Å). Content of each chemical component of these specimens were identified by thermogravimetry (TG) analysis using a simultaneous thermal analyzer (STA449C, NETZSCH, Germany). The microstructure and morphology of specimens were observed through field emission scanning electron microscope (FE-SEM, JSM-6490, JEOL, USA) equipped with EDS (Inca Energy, Oxford Instruments Analytical, UK). To preserve the original appearance of the microstructure, these specimens were not treated with any coatings. The microstructure and crystalline states of specimens were characterized by high resolution transmission electron microscope (HRTEM, JEM-2100 F, JEOL, Japan). Before TEM analysis, these specimens were embedded in an epoxy resin and their ultra-thin (~70 nm) sections were obtained using an ultramicrotome (UCT, Leica, Germany).

### 2.2. Model of in vitro biomineralization

The brief *in vitro* experimental model is described in Fig. S1. All chemical reactants used are summarized in Table S1. It is reported that the role of collagens on the biomineralization in bone tissues has already been illustrated as mineral deposition within organic matrix [10]. Other non-collagenous organics, for instance chondroitin sulfate (CS), in antler matrix or bone are also of importance for *in vivo* biomineralization, acting as cation-exchanging calcium reservoirs [11,12]. Therefore gelatin gel was employed for our study because of its biochemical similarity to collagen. The formation and phase conversions of calcium phosphates were investigated using gelatin gel matrix and $Ca^{2+}$ solutions. Solutions containing calcium and phosphate ions were incubated on both sides of gelation gel matrix in long tube at 37 °C for 2 weeks, respectively. By contrast with the control group of pure gelatin gel matrix, different concentrations of CS were introduced into the gelatin gel system. White precipitates were formed within the gel matrix, obtained by rinse and separation. Followed by, these precipitates were immersed in solutions containing different concentrations of $Ca^{2+}$ at 37 °C for 2 weeks. After immersion, all immersed samples were rinsed and lyophilized for further characterization. Phase components of final products were identified by XRD with CuKα radiation (λ = 1.5418 Å). Raman spectral analysis was performed by Smart Raman Spectrometer (LabRAM Aramis, HORIBA Jobin Yvon, France) using Nd:YAG laser light at wavelength of 532.8 nm. Surface morphology of these samples was observed using FE-SEM (Nova NanoSEM 430, FEI, USA) equipped with EDS (Inca Energy, Oxford Instruments Analytical, UK).

## 3. Results and discussion

### 3.1. Morphology and microstructure of antlers

Structural observations of antler samples are demonstrated in Fig. 1. Optical microscopy of slices revealed different porosity and pore structures of antlers tissues (Fig. 1C–E). Amorphous grains were uniformly dispersed in the relatively dense structure of S1 (Fig. 1C, F). Obvious interconnected macro porous structure of S2 was demonstrated (Fig. 1D, G). The gradually calcified tissues in S3 were clearly seen, where a transition region between cortical bone and cancellous bone could be found (Fig. 1E, H).

XRD results are shown in Fig. 1B. Broad diffraction peaks at 26°, 32° and 39° were detected in all antler specimens except S1. These diffraction patterns indicate a weak crystallization, which are similar to that in bone [13–15]. The phase components of S1 were mainly amorphous organics. The crystallinity of S2 was relatively higher than that of others.