Three-Dimensional *in situ* Morphology of Viable Porcine Growth Plate Chondrocytes: A Confocal Microscopy Study

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INTRODUCTION: The longitudinal growth of bones takes place within the growth plates located at their ends. The growth plate has heterogeneous morphology and composition and is histologically subdivided in three zones: the reserve, proliferative and hypertrophic zones [1,3], which correspond to specific stages in the growth process [2,3]. Conventional histology, light microscopy and electron microscopy have been used to characterize cell arrangement, cell size as well as cell/matrix ratios from the three zones [3,4,5]. Recently, quantitative three-dimensional imaging of isolated chondrons was performed using confocal microscopy in articular cartilage [6]. However, intact tissue visualization and live three-dimensional morphological analysis of chondrocyte structure and its zonal variations in the growth plate has not been reported. The objective of this study was to quantify the three-dimensional morphology of *in situ* chondrocytes using fluorescence labeling of cell cytoplasm coupled with three-dimensional reconstruction of serial confocal sections.

METHODS: Full depth explants of epiphyseal/growth plate/metaphyseal bone were harvested from distal ulnae of 4-week old pigs using 4 mm diameter biopsy punches. Animals were obtained within 3 hours of slaughter from a local abattoir. For each sample, upper and lower surfaces of the disks were trimmed using a Vibratome (Vibratome 1500 Sectioning System) to obtain two parallel surfaces and to provide the thickness of each growth plate sample. Using the same system, each cylindrical specimen was cut into halves perpendicular to the longitudinal axis of the sample to expose the growth plate on the stage of the inverted microscope. The specimen was then immersed in a solution of 0.5 µM Calcein AM (Molecular Probes) and incubated for 30 min at 37°C in an atmosphere of 95% O_2 and 5% CO_2 to fluorescently label the live chondrocyte cytoplasm. The optimal stain concentration was previously determined using viability testing.
For each sample (twelve porcine ulnae), images of two randomly chosen fields were analyzed in each histological zone. In each field, a total of 1-5 chondrocytes were randomly selected for analysis, for a total of 264 analyzed chondrocytes. For each selected zone, three-dimensional volume images were recorded using a laser scanning confocal microscope (LSM 510, Zeiss). Serial sections of 512×512 pixels were taken using two objective lens for cell and tissue levels: 40×: 0.6 NA, 120-140 sections, 0.99-1.15 µm interval; 10×: 0.3 NA, 20-40 sections, 5.7 µm interval. Prior to three-dimensional reconstruction, optical sections were deconvolved using Huygens software (Huygens Essential, Scientific Volume Imaging BV). Three-dimensional reconstruction and quantitative morphological analysis at both tissue and cell levels were performed using IMARIS software (Bitplane) (experimentally calibrated with TetraSpeck™ microspheres prior to experiments). Data was processed using a statistical analysis software (Statistica 8.0, Statsoft Inc.) with a p-value ≤ 0.05 considered statistically significant. A one-way ANOVA (Tukey) was carried out to verify difference between the mean values obtained for each of morphometric parameters both at cell and tissue level for three zones of growth plate.

RESULTS: Figure 1 presents a 3D iso-view of porcine growth plate with 10X objective, showing the spatial distribution of the reserve (R), proliferative (P) and hypertrophic (H) chondrocytes within the tissue.

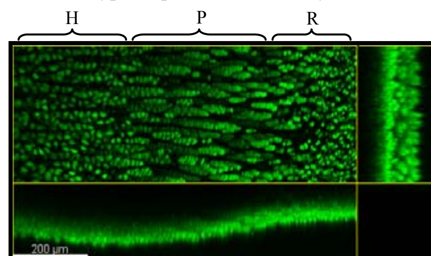

Figure 1: 3D image of porcine growth plate

Cell and tissue level morphometric data are summarized in Table 1 and Table 2. Statistical differences were found between the three zones for each investigated parameter.

Table 1: Tissue level morphometric analysis

Zone	reserve (n=12)	proliferative (n=12)	hypertrophic (n=12)
overall growth plate thickness (µm)	3983.33 ± 9%		
cell/matrix volume ratio (%)	10.95 ± 20.1%	16.83 ± 17.96%	14.8 ± 19.11%
Young's modulus (MPa) [7]	0.48 ± 25%	0.25 ± 48%	0.32 ± 37.5%

Table 2: Cell level morphometric analysis

chondrocyte type	Reserve (n=86)	Proliferative (n=80)	Hypertrophic (n=98)
volume (μm^3)	1242.138 ± 23.4%	2767.475 ± 24.9%	5331.590 ± 30.9%
surface area (μm^2)	603.016 ± 16.2%	1194.483 ± 18.2%	1613.083 ± 21.9%
sphericity	0.922 ± 4.3%	0.811 ± 7.1%	0.913 ± 5.02%
major diameter (µm)	8.896 ± 12.5%	13.062 ± 10.6%	13.484 ± 14.5%
minor diameter (µm)	5.061 ± 10.4%	5.327 ± 17.6%	8.861 ± 13.3%

DISCUSSION: This is the first *in situ* true three-dimensional morphological analysis of growth plate chondrocytes. Obtained results are consistent with published studies. Proliferative chondrocytes were flat compared to reserve chondrocytes. Their minor diameter increased by 5% while their major diameter increased by 46% when compared to reserve chondrocytes. Proliferative chondrocytes then hypertrophied mainly along the growth direction, with their minor diameter increasing by 67% from proliferative to hypertrophic zones. Data from previous studies, combined to the current results, suggest that larger animals have smaller proliferative and hypertrophic cell volumes as well as thicker growth plates [4,5]. This trend is presented for hypertrophic chondrocyte cell volume (Figure 2).

Figure 2: Hypertrophic chondrocyte volume in different animal models

Cell volume increased by a factor 2 in the direction of growth from proliferative to hypertrophic zones, compared to a previous study in rats [5], which showed a 10 fold cell volume increase. Animal model could contribute to this difference. As shown in Table 1, the lowest cell/matrix volume ratio was found in the reserve zone whereas the highest was in the proliferative zone. These observations, combined to a mechanical study on the same growth plate model [7], suggest that the extracellular matrix (ECM) might have a greater contribution to mechanical properties as compared to chondrocytes. However, other factors, such as intrinsic mechanical properties of the ECM, chondrocyte alignment, collagen fiber arrangement in each histological zone, …etc might influence the overall mechanical properties of the growth plate.

FUTURE WORK: The long-term objective of this research is to characterize the mechanical behavior of the growth plate, its constitutive cells and extracellular matrix. Mechanical tests will be performed on growth plate samples using a single assembly loading apparatus combined with the inverted microscope.

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