IGF-1 TMJ Injections Enhance Mandibular Growth and Bone Quality in Juvenile Rats

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Running Title: IGF-1 increases mandibular dimensions and condylar bone quality

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Declarations

Authors 1, 2 and 5: Contributed to conception, design, data acquisition and interpretation, drafted and critically revised the manuscript. Author 3: Contributed to design, data acquisition, assisted in statistical analyses and drafted the manuscript. Author 4: Contributed to design, data analyses and critical review and revision of the manuscript. None of the authors have any conflicts of interest. Data related to this manuscript will be available on request.

Abstract

Objectives: Dentofacial orthopedic treatment of mandibular hypoplasia has unpredictable skeletal outcomes. Although several biomodulators including insulin-like growth factor 1 (IGF-1) are known to contribute to chondrocyte proliferation, their efficacy in modulating mandibular growth has not been validated. The aim of this study was to determine the effect of locally delivered IGF-1 on mandibular growth and condylar bone quality/quantity in juvenile rats. ***Setting and Sample Population***: Institutional vivarium using twenty-four 35-day-old male Sprague-Dawley rats***. Methods***: PBS or 40 µg/kg (low-dose) IGF-1 or 80 µg/kg (high-dose) IGF-1 was injected bilaterally into the temporomandibular joints of the rats at weekly intervals for four weeks. Cephalometric and micro-computed tomography measurements were used to determine mandibular dimensions. Bone and tissue mineral density, volume fraction, and mineral content were determined, and serum IGF-1 concentrations assayed. ***Results:*** Intra-articular administration of high-dose IGF-1 contributed to a significant 6-12% increase in mandibular body and condylar length compared to control and low-dose IGF-1 treated animals. Additionally, IGF-1 treatment resulted in a significant decrease in the angulation of the lower incisors to mandibular plane. Condylar bone volume, bone volume fraction, mineral content, and mineral density were significantly increased with high-dose IGF-1 relative to control and low-dose IGF-1 groups. Serum IGF-1 levels were similar between all groups confirming limited systemic exposure to the locally administered IGF-1. ***Conclusion:*** Local administration of high-

dose 80 μg/kg IGF-1 enhances mandibular growth and condylar bone quality and quantity in growing rats. The findings have implications for modulating mandibular growth and potentially enhancing condylar bone health and integrity.

Key Words: mandibular growth, mandibular condyle, insulin-like growth factor-1, bone quality, temporomandibular joint

Introduction

Diminished mandibular growth and size results in mandibular retrusion that can range from mild to severe. Although the milder forms of mandibular retrognathia are often managed with orthodontics alone, the more severe forms require "growth modification" using functional appliances or surgical intervention.[1] For individuals with moderate mandibular hypoplasia, various removable and fixed functional appliances, which purportedly enhance mandibular growth by holding it in a protruded position are frequently used in growing patients.[2–5] Despite decades of use of these appliances, these treatment modalities remain controversial due to the lack of consensus regarding their efficacy in truly enhancing condylar and mandibular growth, as opposed to restricting maxillary growth and/or enhancing dentoalveolar compensations to correct the skeletal discrepancy.[6–8] Correction of mandibular skeletal deficiency by dentoalveolar compensations or maxillary changes can result in adverse outcomes such as poor esthetics, proclination of lower incisors, loss of periodontal bone support and lack of long-term stability.[6–8] Finally, individuals with severe mandibular hypoplasia such as those with acquired or congenital unilateral or bilateral mandibular deficiencies typically require surgical mandibular advancement or distraction osteogenesis[1,9] that are invasive with associated morbidity, can have unpredictable outcomes, and are expensive.

The identification of biological factors that control cartilage growth has generated substantial interest for their potential use in more predictably enhancing mandibular growth.[10,11] Prior studies point to numerous factors that regulate chondrogenesis and may be useful in modulating cartilage growth, including fibroblast growth factor-2, vascular endothelial growth factor, parathyroid hormone, parathyroid hormone related peptide, indian hedgehog, insulin-like growth factor-1 (IGF-1) and transforming growth factor-b1.[12–14] Of these factors, IGF-1 appears to hold particular promise because of its known paracrine and autocrine effects in enhancing chondrocyte proliferation and survival and enhancing extracellular matrix synthesis.[15–17] The role of IGF-1 in cartilage growth was further validated in mice with deletion of Igf1, which led to

defects in chondrocyte maturation and reduced long bone lengths.[18,19] Furthermore, IGF-1 has anabolic effects on trabecular and cortical bone by promoting survival and stimulating proliferation of osteoblasts, enhancing synthesis of bone matrix, and healing of bone fractures.[18,19] Accordingly, mice with global or conditional deletion of the IGF-1 or its receptor showed decreased osteoblast number and function along with reductions in bone mineral density, trabecular bone volume, trabecular thickness, trabecular number, mineral apposition rate, and bone formation rate.[19–21] Also, systemic delivery of recombinant IGF-1 has been shown to be successful in correcting growth retardation and short stature resulting from IGF-1 signaling defects in mice and humans.[22,23]

Despite substantial knowledge on the molecular regulation of the epiphyseal growth plate in long bones,[11,14,15,18,22,23] less is known about the regulation of mandibular growth that primarily occurs in condylar cartilage. Since condylar cartilage differs from the epiphyseal growth plate in its embryonic origins (neural crest vs. mesoderm derivation), organization, composition, and function,[24] the identification of optimal approaches to achieving targeted condylar growth is essential for clinical interventions in mandibular hypoplasia. Evidence that IGF-1 could potentially be of therapeutic value for modulating condylar growth and enhancing bone quality include specific expression of IGF-1 receptor (IGF-1R) in condylar cartilage,[25] increased expression of condylar IGF-1 by anterior displacement of the rat mandible,[26] and significant increases in condylar endochondral ossification and growth with continuous, systemic infusion of very high dose IGF-1 (640 μg/day) in a mature rat model of acromegaly.[27] Moreover, local administration of IGF-1 into the rat temporomandibular joint (TMJ) at a 50 μg/kg dose increased the condylar cartilaginous tissue layer.[28,29] In spite of these encouraging histologic findings on the effects of local administration of IGF-1 on cartilage, no study to date has examined whether local IGF-1 delivery causes an increase in mandibular dimensions. Therefore, the purpose of this study was to determine whether local intra-articular administration of IGF-1 into the TMJ enhances mandibular growth in peri-pubertal rats. Furthermore, because of the known bone anabolic effects of IGF-1,[15–17] we also evaluated its effects on condylar bone quality. Our findings demonstrate that local delivery of IGF-1 significantly increases the dimensions of the mandible and condyle, as well as enhancing bone indices. Thus, IGF-1 holds great potential as a therapeutic agent for enhancing mandibular growth and condylar bone quality.

Materials and Methods

Experimental Protocol

Twenty-four Sprague-Dawley male rats (35 days old) were divided into three groups of 8 rats each of a sham control and two experimental groups. Sample size determinations were made from data obtained in pilot studies. Animals in the sham control group were administered intra-articular phosphate buffered saline (PBS), whereas those in the experimental groups received either low dose (40 µg/kg) or high dose (80 µg/kg) recombinant IGF-1 (R&D Systems, Inc. Minneapolis, MN) on days 1, 7, 14 and 21, followed by euthanasia on day 28. This dosage range of IGF-1 was selected from prior rodent studies showing its histologic effects in enhancing condylar chondrogenesis.[27–29] Blood was collected from the tail lateral vein at the beginning of the experiment and at days 14 and 28 for serum IGF-1 assays. Lateral cephalometric radiographs and micro-computed tomographic (micro-CT) scans were taken following euthanasia. All aspects of animal care and experiments were approved by the University of Michigan Committee on Use and Care of Animals, complied with the ARRIVE guidelines, and in accordance with the National Institutes of Health guide for the care and use of Laboratory Animals. No animals were excluded from the analyses, and thus no inclusion/exclusion criteria were used. Although the animals were not randomized, they were assigned arbitrarily to each group. One investigator (TH) was aware of group allocation, while all data was collected and analyzed by a blinded observer (ASJ).

Injection Procedure

PBS or IGF-1 were injected bilaterally in each rat in a manner similar to that described previously.[29] The skin above the TMJ was shaved and a reference line joining the outer canthus of the eye with the tragus of the ear was drawn. Anatomical landmarks including the zygomatic ridge and buccinator muscle were used to locate the TMJ space. The location of the mandibular condyle was confirmed by palpation during forced mandibular movements. A 30-gauge needle was directed anterosuperiorly and inserted into the joint area until it hit bone. The lower jaw was gently manipulated to confirm the position of the needle and the equivalent volumes of PBS or IGF-1 were slowly injected into the joint capsule. The intra-articular injection technique was first optimized through pilot studies by injecting a fluorescent dye, DiI (1,1-Dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate) into the TMJ and confirming its location by resecting

the TMJ until the above repeatable and consistent approach was defined.

Cephalometric Analysis

Immediately after euthanasia, lateral cephalograms with a magnification factor 1.6-fold were taken with the Faxitron Radiography System MX-20 (Faxitron X-ray Corp. Wheeling, IL). Scanned radiographs were analyzed using *Image J software* (National Institutes of Health, Bethesda, MD) using previously established cephalometric landmarks and measurements (Fig. 1A).[30] To minimize any potential effects of individual variability in the size of each animal, mandibular linear measurements were standardized to the cranial base dimension (So-E).

Micro-computed Tomography

Mandibles were hemisected and mandibular halves were analyzed by micro-CT using the explore Locus SP system (GE Healthcare Pre-Clinical Imaging, London ON, CA). Specimens were immersed in water and scanned over 200 degrees of rotation at 80 kVp and 80 µA. Calibrated densitometry was performed by scanning inside an acrylic beam flattener with added filtration in the form of a 0.02-inch aluminum filter, and comparing values to a calibration phantom containing water, air, and a hydroxyapatite-mimicking bone substitute. Scanned images were reconstructed at an effective voxel size of 24 μm^3 and linear measurements made using Microview 2.2 software (GE Healthcare, Waukesha, WI) using landmarks described in Figure 1. In addition, the height of the condyle was measured by constructing a tangent joining the deepest curvature between the coronoid process and condyle, and the condyle and gonion to which a perpendicular line was drawn to condylion (Fig. 1C, D). Micro-CT linear measurements for each animal were standardized to the cranial base dimension (So-E).

To assess bone quantity and quality in the trabecular bone of the mandibular condyle, a region of interest sized 1.5022 mm x 0.5007 mm x 1.5022 mm was centered at a line joining the widest part of the condyle from the sagittal perspective (Fig. 1E and F). For each specimen, a gray voxel value histogram was generated to determine an optimal threshold value to distinguish mineralized from non-mineralized tissue. An average of all the threshold values was used to conduct the bone and trabecular analysis. Measurements of bone volume, bone mineral content, bone mineral density, tissue mineral content, tissue mineral density, bone volume fraction, trabecular thickness and trabecular spacing were made using *Microview 2.2* software and previously established algorithms.

Enzyme-linked Immunosorbent Assay (ELISA)

Circulating IGF-1 levels on days 1, 14, and 28 were determined by ELISA (Quantikine® E immunoassay, R&D Systems, Minneapolis, MN) according to manufacturer instructions on serum from five animals randomly selected from each of the three groups.

Statistical Analysis

Descriptive statistics were calculated for all parameters. A repeated measures analysis of variance was used to compare linear data between the three groups (PASW Statistics 18.0, IBM Corporation). Micro-CT bone quality data were analyzed using a one-way analysis of variance. A Tukey posthoc test was used to analyze pairwise differences between groups and times. Intraoperator reliability was determined by repeating linear cephalometric and micro-CT measurements on 10 randomly selected samples at two different time points, one month apart, followed by paired t-tests and correlation coefficients. These demonstrated a high level of intra-operator reliability ($p < 0.05$ and r values >0.93 for all measures). One-way ANOVA was performed for systemic levels of IGF-1 and statistical significance was set at $p<0.05$.

Results

Animal Status

The animals from all groups showed similar weight gains, which were not significantly different between the groups (data not shown).

Morphometric Analysis

Both cephalometric and micro-CT morphometric analyses showed significant differences between groups, particularly the high dose IGF-1 treatment. Cephalometrically, the mandibular corpus length (Go-Me) and the total craniomandibular length (Po-Me) were significantly larger with high dose IGF-1 than control or low-dose IGF-1 (Fig. 2A and C). In contrast, total mandibular length (So-Me), was not significantly different between groups (Fig. 2B). Notably,

lower incisor inclination (Go-Me-L1º) showed dose-dependent decreases with low and high dose IGF-1 relative to control (Fig. 2D). Consistent with cephalometric findings, micro-CT measurements revealed significant effects to mandibular dimensions with IGF-1 injections (Fig. 3). The mandibular corpus length (Go-Me), total mandibular length (Co-Me), and ramus length (Co-Go) were significantly larger with high dose IGF-1 (Fig. 3A-C). Condylar length (Co-Tangent) was also significantly larger with high dose IGF-1 compared to low dose IGF-1, however no significant difference in this variable were noted between high dose IGF-1 and control groups (Fig. 3D). In all cephalometric and micro-CT measurements, no significant differences in mandibular morphology between control and low dose IGF-1 were observed except for Go-Me-L1º measurements.

Bone Quality and Quantity

High dose IGF-1 enhanced mandibular condylar bone quality and quantity (Fig. 4). Specifically, high dose IGF-1 resulted in significant increases in condylar bone volume (Fig. 4A), bone volume fraction (bone volume/total volume; Fig. 4B), bone mineral density (a measure of the density of bone and non-bone tissues; Fig. 4C), and tissue mineral content (Fig. 4F) relative to rats receiving low dose IGF-1 or PBS. However, neither bone mineral content (Fig. 4D) or tissue mineral content (Fig. 4E) were significantly modulated by high dose and low dose IGF-1. Low dose IGF-1 did not have any significant effects on all measures of bone quantity or quality relative to sham controls.

Systemic IGF-1 Levels

While circulating IGF-1 levels significantly increased over time during the course of the experiment, no significant differences were found between groups, at any of the three time-points (Fig. 5).

Discussion

Growth at the condyle, the primary growth site in the mandible, occurs via endochondral ossification whereby undifferentiated cells undergo proliferation, followed by chondrocytic

differentiation, and cartilage matrix deposition, which is eventually replaced by bone.[31] Although attempts have been made to modify condylar growth in individuals with small mandibles by orthopedic appliances, this approach does not result in consistent desired skeletal responses.[6] Also, more severe types of mandibular hypoplasia require surgical intervention with associated morbidity and costs. Growth factors that are known to modulate chondrogenesis may serve as potential therapeutic agents for correcting such dysplasias. Here we focused on determining whether locally administered IGF-1, a known chondrogenic and osteogenic agent,[15] enhances mandibular growth and condylar bone quality to potentially serve as a therapy for growth modification of retrognathia and management of osteolytic or degenerative condyles, respectively. Our findings show that local, non-surgical administration of IGF-1 in the TMJ of growing rats results in significant increases in mandibular dimensions (Figs. 2, 3) and enhances several measures of bone quality (Fig. 4). Specifically, we found that high dose IGF-1 significantly increased the length of mandibular corpus and ramus relative to controls. Low dose IGF-1 did not contribute to any increases in mandibular dimensions. Concordant with the enhanced growth of the mandible, the inclination of lower incisor was decreased in rats administered IGF-1. Our findings provide the first evidence of mandibular dimensional changes mediated by locally administered IGF-1 and complement previous histologic observations on the effects of IGF-1 on chondrogenic responses during endochondral growth,[11,28,29] thereby extending upon the known cellular effects of local IGF-1 in growing rats to demonstrate actual dimensional changes in the mandible.

Despite the unique dual articular and growth functions of condylar cartilage that distinguish it from long bones, where the articular surface is distinct from and spatially distant from the epiphyseal growth plate, our findings on IGF-1's modulation of condylar and mandibular growth are similar to its known effects on long bones.[15,21,32] IGF-1 effects on long bones are evident from findings in global and conditional IGF-1 inactivated mice, which exhibit impaired chondrocyte maturation, severe growth retardation, reduced tibial length and width, and overall body length.[21,32] Furthermore, systemic administration of IGF-1 in global and conditional IGF-1 inactivated mice and growth hormone-deficient mice rescued the bone and cartilage phenotypes and result in normalization of endochondral bone growth.[23] Finally, local administration of IGF-1 to long bones contributes to significant increases in bone length.[11] Evidence that the TMJ may potentially be similarly responsive to IGF-1 is provided by studies localizing IGF-1R to TMJ cartilage,[25] the proliferative responses of cartilage progenitor cells in fetal and neonatal rat mandibular condyle explants,[33] and the increase in the thickness of cartilage layer of the mandibular condyle in response to local injections of IGF-1 in the TMJ in

rats.[28,29] Despite these observations, the clinically translatable effects of IGF-1 to mandibular growth had been lacking until now. Our findings showing that local administration of IGF-1 causes a 6-12% increase in various mandibular dimensions (Figs. 2, 3), that if mimicked in humans would translate into substantial enough increases in mandibular dimensions in growing individuals to alleviate moderate to severe mandibular hypoplasia.

Our findings also show that high dose IGF-1 contributed to a significant increase in bone volume, bone mineral density, and tissue mineral content (Fig. 4). Moreover, bone volume fraction or the proportion of space occupied by trabecular bone was significantly higher with low and high dose IGF-1 compared to controls. Similar responses to IGF-1 have been reported previously in long bones,[34,35] and an increase in the thickness of the subchondral bone area in TMJ condyles of 3-week-old rats subjected to local administration of IGF-1,[29] showing that IGF-1 has similar effects on the condylar bone as that in other bones and pointing to its potential efficacy in restoring bone integrity in degenerative and osteolytic joint conditions. This change probably results from the known effects of IGF-1 in stimulating osteoblast proliferation and synthesis of bone matrix, as well as promoting cell survival in preosteoblasts and osteoblasts.[36,37] *In vivo* evidence for this mechanism of action of IGF-1 is provided by mice with deletion of *Igf-1*r gene that exhibited decreased osteoblast number and function, resulting in reduced bone formation and trabecular bone volume.[21] These findings together with ours show that the dual role of IGF-1 as systemic and local mediators of cartilage growth and bone anabolism manifest as measurable changes in dimensions and bone quality in the mandibular condyle.

We also found that while the serum concentration of IGF-1 almost doubled in all three groups of rats over the duration of the experiment, the local administration of IGF-1 did not contribute to any significant change in serum IGF-1 concentrations (Fig. 5). These findings suggest that the increase in serum IGF-1 levels over the experimental period is likely due to physiologic changes in systemic IGF-1 known to occur during periods of rapid growth.[38] Thus, the local administration of IGF-1 at the doses that increase mandibular dimensions and bone quality did not result in increased systemic IGF-1 levels thereby demonstrating its desirable effects without systemic exposure. Besides the likely therapeutic benefits of such an approach in enhancing mandibular growth bilaterally in subjects with micrognathia, this localized effect of IGF-1 could be therapeutically useful in specific craniofacial anomalies with unilateral mandibular hypoplasia for which unilateral administration on the underdeveloped side could be used to enhance mandibular growth of the affected side provided adequate condylar cartilage is present to respond to this therapy.

The clinical translation of these findings to humans could be facilitated by the fact that rHIGF-1 is currently approved by the US Food and Drug Administration and European Medicines Agency for short stature due to growth hormone insensitivity or IGF-1 deficiency.[39,40] Nevertheless, due to the substantial side effects of systemic administration of IGF-1 therapy, risks associated with IGF-1 treatment of mandibular growth or TMJ bone regenerative therapies would need to be minimized through methods that curtail systemic exposure, such as those we achieved with low but effective doses of IGF-1 administered intraarticularly in our studies or via sustained low dose localized sustained release delivery systems. Such future studies on novel sustained drug delivery systems No interventional clinical trials testing the use of IGF-1 for growth and bone anabolic therapies of the mandible have been performed, thereby providing opportunities for future clinical trials. Also, building on our studies, future investigations on animals should aim at elucidating the cellular and tissue changes in the condylar cartilage to supplement our observations on mandibular and condylar growth modifications, and those of previous investigations on the effects of IGF-1 on histologic changes condylar cartilage.[28,29] Finally, mechanistic molecular and cell biology studies would be valuable in providing a comprehensive understanding for our observations.

Conclusions

This study demonstrates an effective non-surgical method for localized administration of IGF-1 into the TMJs of rats, which at a dose of 80 μg/kg delivered weekly over four weeks in growing rats led to a significant increase in mandibular size and enhanced mandibular condylar bone quality. These desirable outcomes occurred in the absence of any increases in systemic exposure to intra-articular administration of IGF-1. Our findings suggest that IGF-1 may be a potential therapeutic agent for enhancing chondrogenesis, growth, and enhancing bone quality of the mandibular condyle with implications to its uses in treating mandibular hypoplasia and osteo-degenerative disorders of the condyle.

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Figure Legends

Figure 1. Landmarks and measurements made from lateral cephalograms and micro-computed tomograms. (A) The cephalometric landmarks used were: Po, the most posterior point on cranial vault; So, intersection of tympanic bulla with posterior border of basisphenoid; Go, the most posteroinferior point on the mandibular body where ramus intersects the corpus; Me, the most anterior-inferior point on lower border of mandible; E, point at the intersection of frontal bone and the most superior anterior point of the posterior limit of the ethmoid bone; L1, the tip of the mandibular incisor; Me-L1, inclination of the mandibular incisor. Linear and angular measurements made include cranial base length (So-E), mandibular corpus length (Go-Me), total mandibular length (So-Me), total craniomandibular length (Po-Me) all in μm and lower incisor inclination angle (Go-Me-L1). (B) Micro-computed tomography image to show mandibular landmarks and measurements of corpus length (Go-Me), total mandibular length (Co-Me) and ramus height (Co-Go). The definitions for Go and Me are the same as used in the cephalometric landmarks, while Co is the most posterior-superior point on the condyle. (C and D) Condyle dimensions measured from Co to tangent formed by joining the deepest curvatures between coronoid process and condyle and gonion and condyle. (E and F) Region of interest (ROI) from lateral (E) and axial (F) views used for determining bone quality. A standard ROI measuring 1.5022mm x 0.5007mm x 1.5022mm was generated and localized within the cancellous bone of the mandibular condyle.

Figure 2. Local administration of IGF-1 modulates changes in mandibular cephalometric dimensions. Mean (+/- standard error; N=8 animals in each group) of linear and angular cephalometric measurements demonstrate that high dose IGF-1 increases the standardized mandibular corpus length (Go-Me; A) and total craniomandibular length (Po-Me; C) relative to PBS and low-dose IGF-1, while both high and low dose IGF-1 decrease lower incisor inclination angle (Go-Me-L1; D) relative to PBS controls. Neither dose of IGF-1 affects total mandibular length (So-Me; B). Linear measurements were standardized to cranial base dimension (So-E) to account for the individual variability in size of each animal.

Figure 3. Local administration of IGF-1 modulates changes in mandibular micro-

computed tomographic morphometric measurements. Mean (+/- standard error; N=8 animals in each group) of standardized linear micro-CT measurements demonstrate that high dose IGF-1 significantly increases mandibular corpus length (Go-Me; A), and total mandibular length (Co-Me; B) relative to low-dose IGF-1 and PBS controls. Additionally, high-dose IGF-1 causes a significant increase in ramus height (Co-Go; C) and condylar length (Co-Tangent; D) relative to PBS controls. Linear measurements were standardized to cranial base dimension (So-E) to account for the individual variability in size of each animal.

Figure 4. Local administration of IGF-1 enhances measures of condylar bone quality and quantity. Mean (+/- standard error; N=8 animals in each group) of micro-CT bone variables demonstrate that high dose IGF-1 increases condylar bone volume (A), bone mineral density (C), and tissue mineral content (F) relative to low-dose IGF-1 and PBS controls, while both high and low dose IGF-1 increases bone volume fraction relative to PBS controls (B). Neither of the two IGF-1 doses affect bone mineral content (D) or tissue mineral density (E).

Figure 5. Intra-articular administration of IGF-1 does not result in changes in systemic IGF-1. Mean (+/- standard error) of serum IGF-1 assessed by ELISA at days 1, 14 and 28 of the experimental period (A), and fold-changes relative to day 1 (B) showed age-related significant increases in serum IGF-1 ($p<0.05$) within each of IGF-1- and PBS-administered groups, but no significant differences in systemic IGF-1 exposure between the three groups at any of the time points (N=5 at each time-point).

Fig. 1

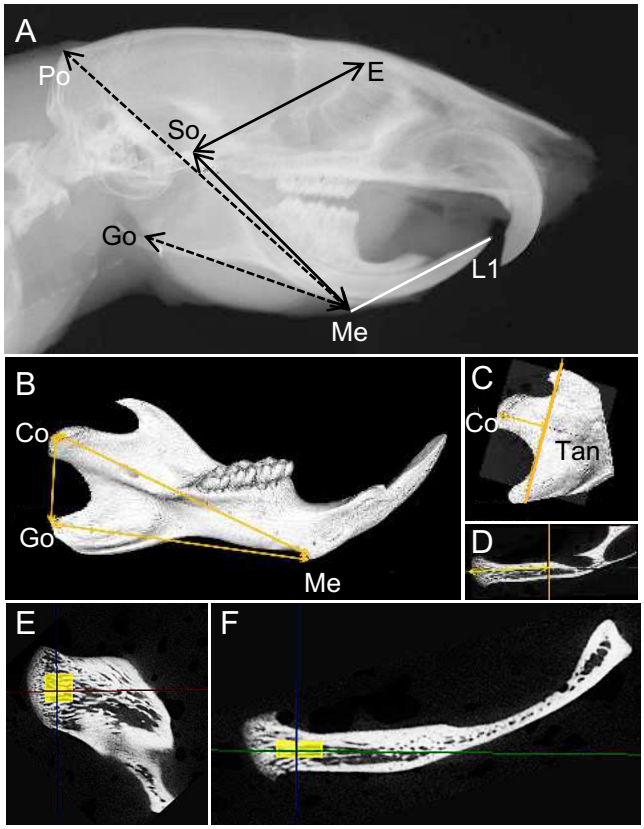

Fig. 2

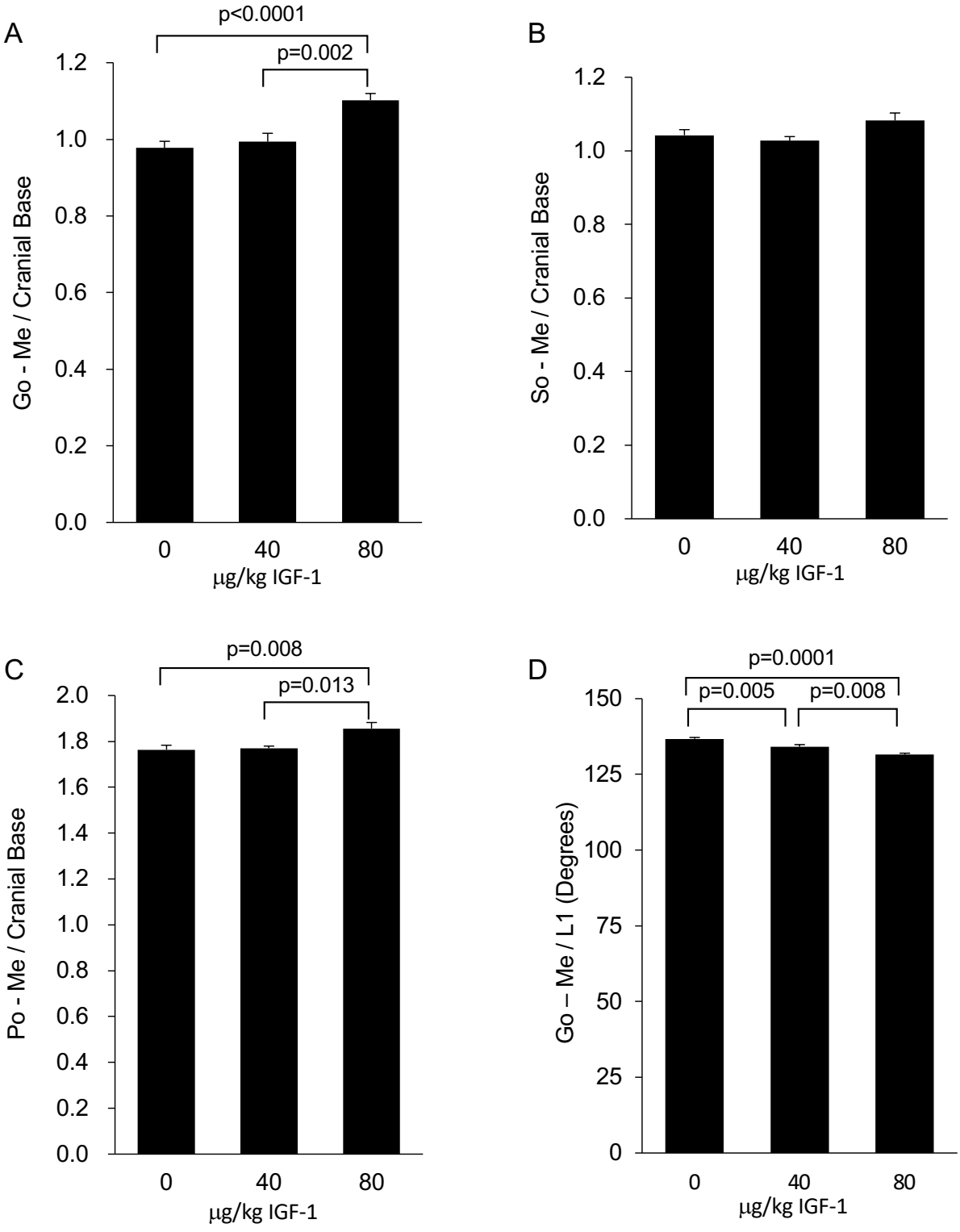

Fig. 3

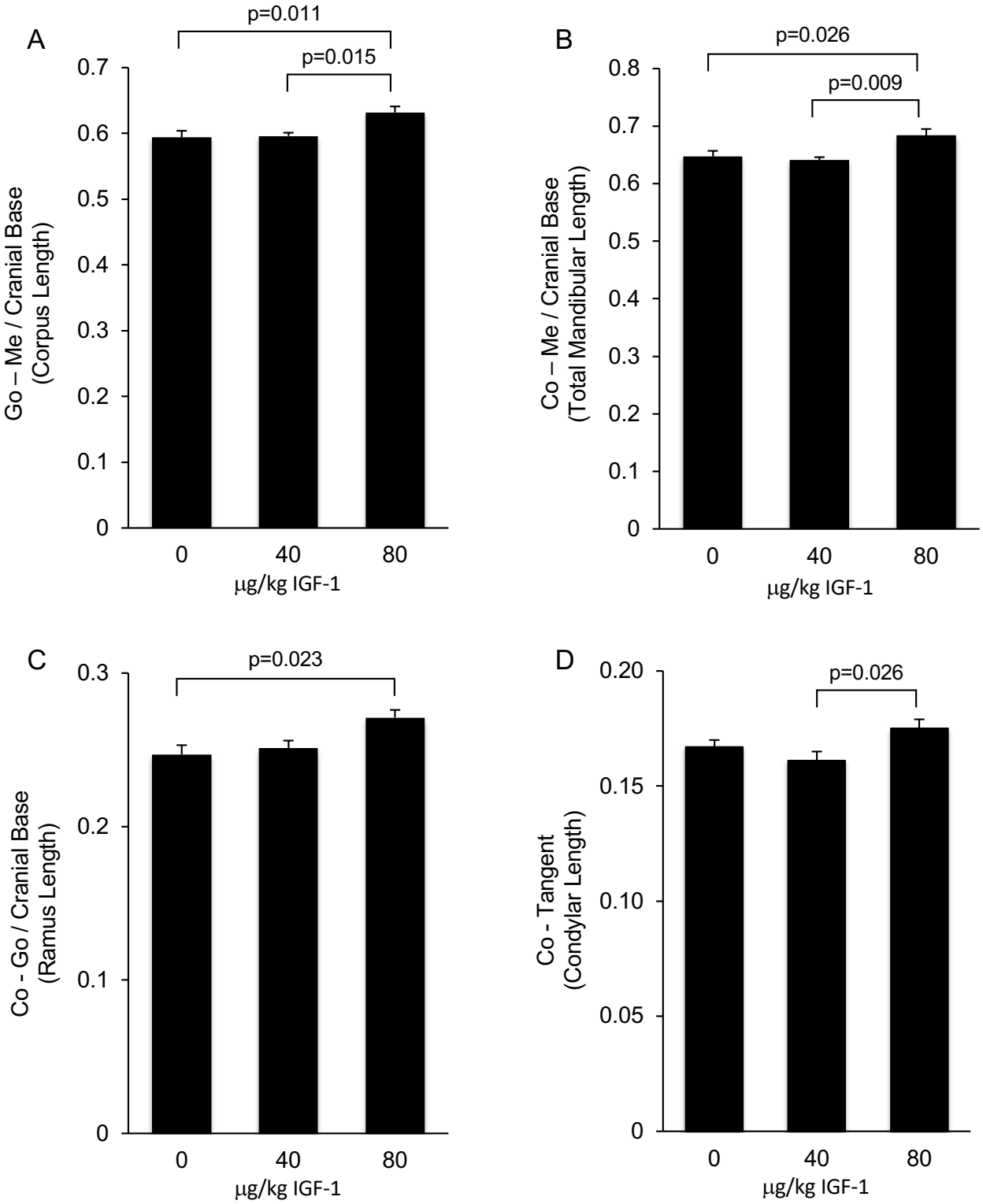

Fig. 4

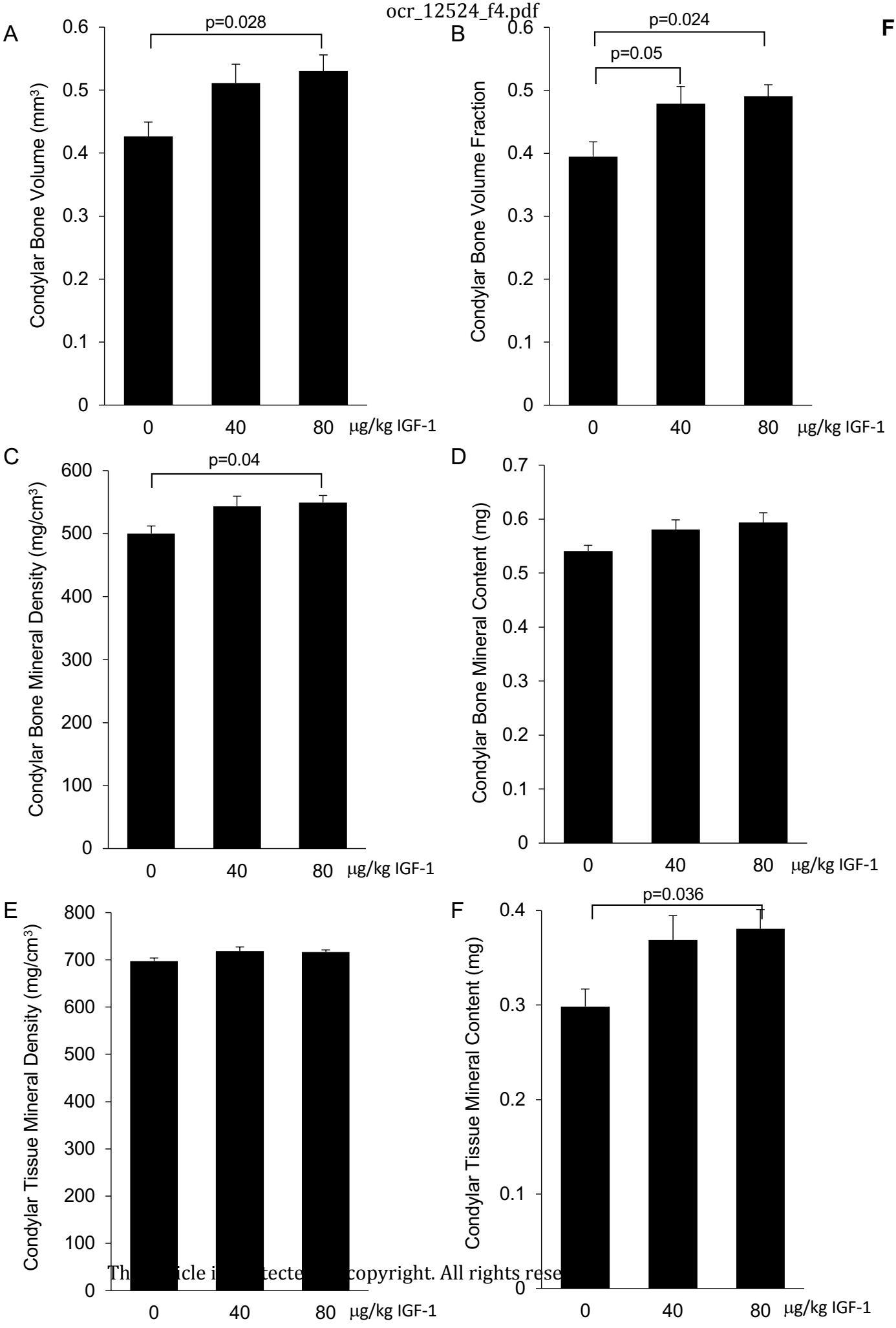

Fig. 5