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Repeated Mechanical Loading Enhances the Expression of Indian Hedgehog in Condylar Cartilage

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TABLE OF CONTENTS

1. ABSTRACT

Indian hedgehog (Ihh) acts as a mechanotransduction mediator that converts mechanical strain into cellular proliferation and cartilage formation in mandibular condylar cartilage. The aim of this study was to examine the effect of repeated mechanical strain on the level of expression of Ihh and type II collagen mRNA in condylar growth. Two hundred and eighty 35 days old Sprague-Dawley rats were divided into 10 experimental and 10 control groups. Repeated mechanical loading was applied by advancing the mandible in a stepwise manner by fitting a stepwise bite jumping appliance. Animals were sacrificed together with their matched controls on 10 different time points. Total RNA was extracted from condylar cartilage immediately after dissection. Ihh and type II collagen mRNA was quantified using real-time PCR. In the experimental group, Ihh mRNA increased significantly on experimental day 7. Upon the second advancement, another peak was elicited 7 days later. Type II collagen showed a significant increase on days 21 and 44 of advancement. This indicated that mechanical loading in a repeated manner, triggers the expression of Ihh which in turn increases the number of replicating mesenchymal cells as well as the amount of the cartilage formed. Taken together these events increase condylar growth.

2. INTRODUCTION

Indian Hedgehog (Ihh) was reported to be a critical mediator transducing mechanical signals to stimulate chondrocyte proliferation (1, 2). Mechanical loading at moderate levels is considered to be essential for maintenance and adaptation of the growing as well as adult skeleton (3, 4).

Rabie and Co-workers examined the effect of mechanical loading on mandibular condylar growth (5-8) and reported that Ihh is the mechanotransduction mediator in the condyle (2). Mandibular forward positioning led to changes in the biophysical environment that caused deformation of the mesenchymal cells and other cells in the extra-cellular matrix (9). Thus create strain alignment that causes deformation of the cytoskeleton of these cells and trigger the expression of Ihh (2). The expression of Ihh elicited by mechanical loading of the mandibular joint promoted mesenchymal cell proliferation (10) and initiated a cascade of cellular and molecular responses that led to condylar growth (7).

Indian Hedgehog, a member of the vertebrate hedgehog morphogen family, was reported to be key morphogen during skeletal development and regeneration

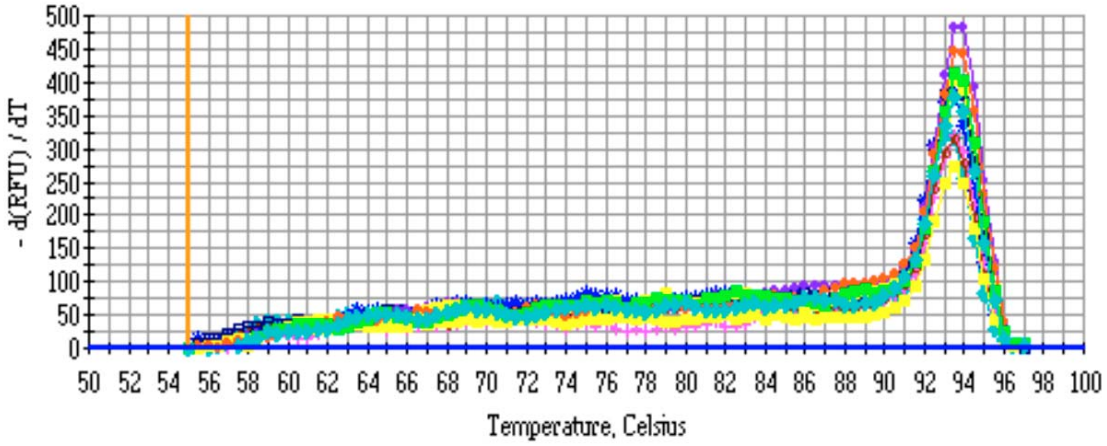

Figure 1. Melting curve analysis in RT-PCR. A sharp peak showing specific Ihh amplicon (melting temperature of 93.5ºC) was generated.

(11). Mechanical stress stimulated Ihh expression in chondrocytes *in vitro* by 18-fold (1). Over expression of Ihh in transgenic mice led to an increase in the number of replicating chondrocytes in the growth plate (12). Earlier we reported that the number of replicating mesenchymal cells in the condyle directly impacts the growth potential of the condyle (10). Furthermore, the number of replicating chondrocytes impacts the amount of cartilage to be formed (13) and subsequently influences condylar growth because cartilage acts as the template onto which bone will form (14). Therefore, over expression of Ihh in the condylar cartilage in response to mechanical loading could result in enhanced condylar growth.

It is conceivable, then, that upon repeated mechanical loading as a result of repeated mandibular advancements, over expression of Ihh is achieved with its ultimate effects of enhancing mandibular growth,

The objectives of this study were:

1. To carry out a quantitative assessment of the expression of Ihh mRNA in mandibular condyles under repeated mechanical loading.

2. To carry out a quantitative analysis of the expression of type II collagen mRNA in condylar cartilage under the same conditions.

3. To correlate the level of expression of Ihh and type II collagen to bone formation under the same conditions.

3. MATERIAL AND METHODS

Animal experiment was approved by the committee on the Use of live Animals in Teaching and Research of the University of Hong Kong.

3.1. Experimental Animals

Two hundred and eighty female Sprague-Dawley rats, 35 days old, were randomly divided into 10 control groups and 10 experimental groups (n=14). Ten groups of experimental rats wore stepwise bite-jumping appliances, which produced an initial advancement of 2 mm. After 30 days, acrylic veneers were added to the appliances, for a total advancement of 4.0 mm (15). The appliances were worn 24 hours producing a continuous forward and downward positioning of the mandible. The animals were sacrificed on days 3, 7, 14, 21, 30, 33, 37, 44, 51, and 60, respectively by an intraperitoneal injection of 20% pentobarbital sodium.

3.2. Total RNA Extraction

Mandibular condylar cartilage was collected and frozen in liquid nitrogen immediately after dissection under light microscope. The tissue was homogenized with Mikro-dismembrator U (B. Braun Biotech International). Total RNA was extracted by RNeasy® Fibrous Tissue Midi Kit (Qiagen) according to manufacturer's specification.

3.3. Reverse Transcription and Real-time PCR

Reverse transcription of extracted RNA into cDNA was carried out with the SuperScript First Strand synthesis System (Invitrogen).

Real-time PCR and data analysis were performed in the iCycler iQ™ real-time detection system (Bio-Rad Laboratories). PCR-asssays were carried out in 25µl reaction mixture with 2.5µl 10X PCR buffer, 1.6µl 50mM $MgCl_2$, 1.0µl dNTP, 0.4 µl forward primer, 0.4µl backward primer (Table 1), 0.1µl Taq Polymerase (Invitrogen), 1.25 µl 10X SYBR® Green I (Molecular Probes) and 1µg cDNA of the sample. Cycling conditions were optimised to 45 cycles of denaturation at 94°C for 30s, annealing at 60°C for 30s and extension at 72°C for 45s. Specificity of the reaction product was checked by the melting curve analysis (Figure 1) and electrophoresis on 1.0% (w/v) agarose gel (Figure 2).

3.4. Primer sequence

Primers for amplification using SYBR Green I were based on the published sequences (16) for Indian

Table 1. Primer sequence, length of amplicaon (bp) used in the RT-PCR

Primer	Primer/Probe	Sequence	Length (bp)
Ihh	Forward Reverse	GCTTCGACTGGGTGTATTACG GCTCACGGTCCAGGAAAAT	231
Type II collagen	Forward Reverse	GATTGTCCGCAATGAAGAGC TTGGGGTTGAGGGTTTTACA	448
GAPDH	Forward Reverse	CATGTTCCAGTATGACTCTACCC AGCATCACCCCATTTGATGT	136

hedgehog, type II collagen and Glyceraldehyde-triphosphate dehydrogenase (Gapdh).

3.5. Standard PCR

PCR products of each gene were prepared under the same conditions as mentioned above with Thermal Cycler (Takara) except SYBR Green I was not added. Ihh and collagen II amplicons were extracted from the agarose gels and purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Purified amplicons were sequenced with the 3730 DNA Analyzer (Applied Biosystem) to determine the identity.

3.6. Quantification of mRNA

Quantification was performed by comparing the target threshold cycle directly with the absolute standard curve for each individual amplicon. The copy numbers of the experimental mRNA was calculated from the linear regression of that standard curve using iCycler iQ^{TM} Real-Time PCR Detection System Software (Bio-Rad). The target copy numbers were normalized with the copy numbers of Gapdh.

3.7. Statistical Analysis

The data was input and processed using the SPSS version 11.0 for windows. Paired t-test was performed to compare the mean differences of mRNA expression between the control group and the experimental group at each time point.

4. RESULTS

4.1. Ihh expression in mandibular condylar cartilage

Ihh mRNA expression showed a decrease with age during natural growth (Figure 3). Forward mandibular positioning led to a significant increase of Ihh mRNA, with the peak identified on day 7 and dropped to control level after 14 days of appliance wearing. The peak increment corresponded to a fold change of 5 in the stepwise advancement group.

Upon the second advancement on experimental day 30, Ihh mRNA showed a significant increase on experimental day 33 and reached another peak on experimental day 37, which has a corresponding fold change of 12.5. After the peak, the level of expression started to decrease again to the normal level.

4.2. Type II collagen Expression in mandibular condylar cartilage

The amount of type II collagen mRNA in the control group was gradually reduced to a very low level till the end of the experiment (Figure 4).

In the experimental group, collagen II mRNA started to increase on days 14. Although the critical values were statistically insignificant when compared with the control, a greater amount of mRNA was detected on experimental days 14 and 21. The fold change identified was 2.5 and 2.8 respectively.

The second advancement on days 30 led to a significant increase in type II collagen mRNA, which reached the peak on days 51 with a fold change of 21.8. The amounts of mRNA detected on experimental days 44 and 51 were significant when compared with natural growth.

5. DISCUSSION

Results of the current study demonstrated that repeated mechanical loading of the temporomandibular joint *in vivo* led to a significant increase in the level of expression of Ihh (Figure 3). The first load application, created by advancing the jaw forward by 2mm, resulted in a 5 fold increase in the level of Ihh mRNA on day 7 of advancement when compared to untreated matched controls. Indian hedgehog is known to induce cellular replication and to enhance cartilage development (11, 17, 18). Recently, we reported that increase in Ihh expression in the mandibular condyle was closely related to enhanced cellular turnover and cell renewal capacity in the condyles (2). Cellular turnover and cell renewal capacity control tissue expansion and subsequently affect growth. Therefore, an increase in cellular replication in response to significant increase in the level of expression of Ihh could explain enhanced condylar growth that was observed under mechanical loading. Chayanupatkul *et al.* reported a significant increase in new bone formation in the mandibular condyle in response to mechanical strain produced as a result of mandibular advancement (7).

Rabie *et al.* demonstrated a close correlation between replicating cells and osteogenesis in the condyle (10). Therefore, the current data point to a direct role of Ihh in transducing the “mechanical strain” into cellular responses that impact growth of the condyle. Most interestingly the second load application, that resulted from a second advancement of 2mm adding up to a total of 4 mm mandibular advancement, has resulted in a significant increase in the expression of Ihh mRNA 3 days after the second advancement followed by a peak 7 days after the advancement (day 37). The corresponding fold change was 12.5 when compared to untreated matched controls (Figure 3). Such a significant increase in the level of expression of Ihh to the second load application explains our earlier results where the second advancement produced further

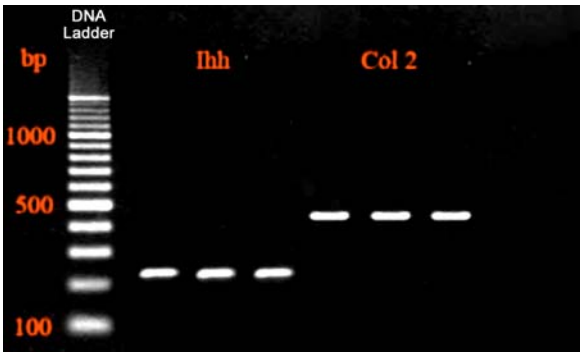

Figure 2. Analysis of final PCR products using electrophoresis with 100bp DNA ladder. Ihh and type II collagen with amplicon product size of 231bp and 448bp respectively showing high specificity.

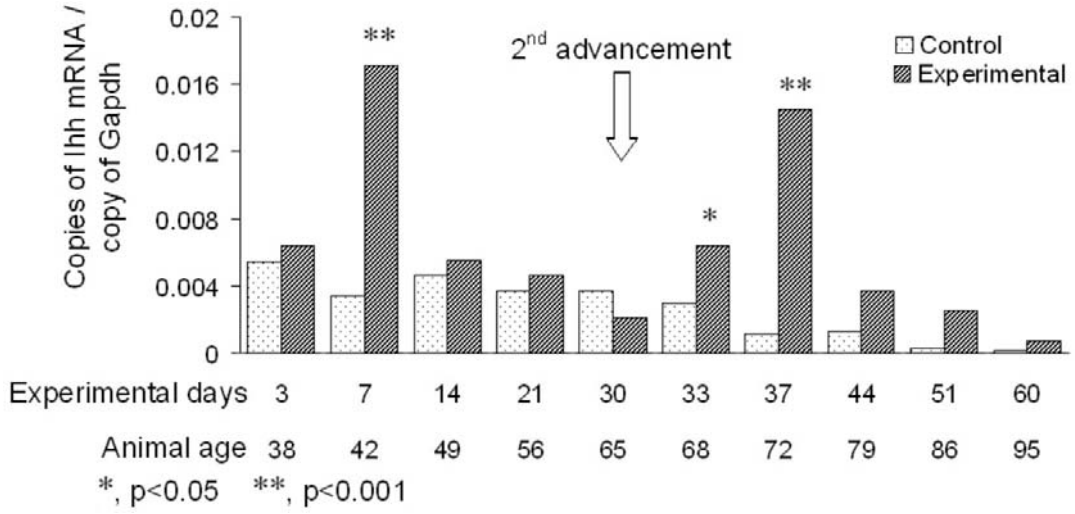

Figure 3. Indian Hedgehog mRNA expression in condylar cartilage during natural growth (control) and repeated mechanical loading of the mandible (experimental group). *p<0.05, **p<0.001.

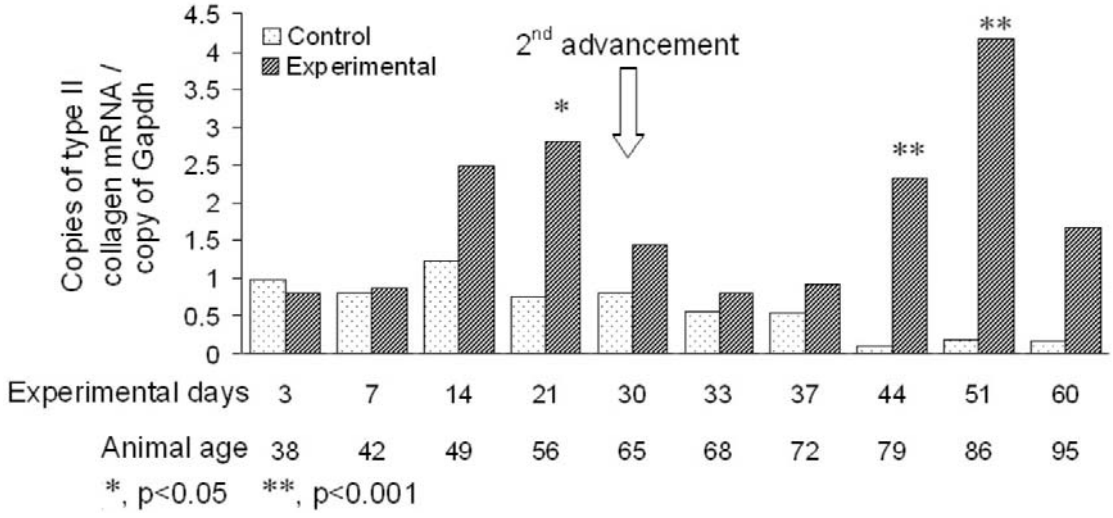

Figure 4. Type II collagen mRNA expression in condylar cartilage during natural growth (control) and repeated mechanical loading of the mandible (experimental group). *p<0.05, **p<0.001.

bone formation in the condyle (6, 19) thus leading to further condylar growth.

Another known function of Ihh is to regulate cartilage development (11), therefore, we decided to identify the level of expression of type II collagen, the major component of the cartilage extracellular matrix and correlate that to the pattern of expression of Ihh. The first load application resulted in a significant increase in collagen II mRNA expression on days 14 and 21 of load application (Figure 4). The maximum level of expression occurred on day 21 and amounted to 2.8 fold change when compared to untreated controls. The second load application resulted in a significant increase in type II collagen mRNA which reached a peak 21 days after the second advancement with a fold change of 21.8 (Figure 4). The significant increase in collagen II is due to the significant increase in Ihh which was shown to regulate cartilage development. Cartilage is the template onto which bone forms in the condyle (20). Therefore, the amount of cartilage formed ultimately affects the amount of bone formed.

It is important to elucidate the mechanism by which Ihh ultimately affects cartilage development and subsequently condylar development and in turn condylar growth. Karp *et al.*, 2000 reported that activation of Ihh signaling upregulates PTHrP expression in the articular surface in long bones (21). Recently, we reported that, PTHrP level of expression significantly increased (5 fold) in response to mechanical loading in mandibular condyle (22) on day 7 of advancement. Cellular kinetics demonstrated in the same study that PTHrP expression was associated with increase in new chondrocyte population (21).

6. CONCLUSION

In conclusion, mechanical loading in a repeated manner, triggers the expression of Ihh, in the mandibular condylar cartilage. In response to increase in the levels of expression of Ihh, the number of replicating mesenchymal cells as well as the amount of the cartilage formed increases thus leading to condylar growth. It is important to note that repeated mechanical load applications triggered a repeated series of events that enhanced growth. Therefore, we point out to a direct coupling between mechanical loading and specific gene expression affecting growth without participation of circulating systemic regulatory factors.

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7. REFERENCES

1. Wu, Q., Y. Zhang & Q. Chen: Indian hedgehog is an essential component of mechanotransduction complex to stimulate chondrocyte proliferation. *J Biol Chem*, 276, 35290-6 (2001)

2. Tang, G. H., A. B. Rabie & U. Hagg: Indian hedgehog: a mechanotransduction mediator in condylar cartilage. *J Dent Res*, 83, 434-8 (2004)

3. Lanyon, L. E.: Using functional loading to influence bone mass and architecture: objectives, mechanisms, and relationship with estrogen of the mechanically adaptive process in bone. *Bone*, 18, 37S-43S (1996)

4. Huiskes, R., R. Ruimerman, G. H. van Lenthe & J. D. Janssen: Effects of mechanical forces on maintenance and adaptation of form in trabecular bone. *Nature*, 405, 704-6 (2000)

5. Rabie, A. B., M. J. Tsai, U. Hagg, X. Du & B. W. Chou: The correlation of replicating cells and osteogenesis in the condyle during stepwise advancement. *Angle Orthod*, 73, 457-65 (2003)

6. Rabie, A., A. Chayanupatkul & U. Hagg: Stepwise advancement using fixed functional appliances: Experiamental perspective. *Seminars in Orthodontics*, 9, 41-46 (2003)

7. Chayanupatkul, A., A. B. Rabie & U. Hagg: Temporomandibular response to early and late removal of bite-jumping devices. *Eur J Orthod*, 25, 465-70 (2003)

8. Rabie, A. B., F. Y. Leung, A. Chayanupatkul & U. Hagg: The correlation between neovascularization and bone formation in the condyle during forward mandibular positioning. *Angle Orthod*, 72, 431-8 (2002)

9. Rabie, A. B., Z. Zhao, G. Shen, E. U. Hagg, O. Dr & W. Robinson: Osteogenesis in the glenoid fossa in response to mandibular advancement. *Am J Orthod Dentofacial Orthop*, 119, 390-400 (2001)

10. Rabie, A. B., L. Wong & M. Tsai: Replicating mesenchymal cells in the condyle and the glenoid fossa during mandibular forward positioning. *Am J Orthod Dentofacial Orthop*, 123, 49-57 (2003)

11. McMahon, A. P.: More surprises in the Hedgehog signaling pathway. *Cell*, 100, 185-8 (2000)

12. Long, F., X. M. Zhang, S. Karp, Y. Yang & A. P. McMahon: Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. *Development*, 128, 5099-108 (2001)

13. Rabie, A. B., T. T. She & U. Hagg: Functional appliance therapy accelerates and enhances condylar growth. *Am J Orthod Dentofacial Orthop*, 123, 40-8 (2003)

14. Rabie, A. B., T. T. She & V. R. Harley: Forward mandibular positioning up-regulates SOX9 and type II collagen expression in the glenoid fossa. *J Dent Res*, 82, 725-30 (2003)

15. Xiong, H., U. Hagg, G. H. Tang, A. B. Rabie & W. Robinson: The effect of continuous bite-jumping in adult rats: a morphological study. *Angle Orthod*, 74, 86-92 (2004)

16. Wang, X. & B. Seed: A PCR primer bank for quantitative gene expression analysis. *Nucleic Acids Res*, 31, e154 (2003)

17. Vortkamp, A., K. Lee, B. Lanske, G. V. Segre, H. M. Kronenberg & C. J. Tabin: Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science*, 273, 613-22 (1996)

18. Pathi, S., J. B. Rutenberg, R. L. Johnson & A. Vortkamp: Interaction of Ihh and BMP/Noggin signaling during cartilage differentiation. *Dev Biol*, 209, 239-53 (1999)

19. Rabie, A. B., L. Wong & U. Hagg: Correlation of replicating cells and osteogenesis in the glenoid fossa during stepwise advancement. *Am J Orthod Dentofacial Orthop*, 123, 521-6 (2003)
20. Rabie, A. B. & U. Hagg: Factors regulating mandibular condylar growth. *Am J Orthod Dentofacial Orthop*, 122, 401-9 (2002)
21. Karp, S. J., Schipani, E., St-Jacques, B., Hunzelman, J., Kronenberg, H., McMahon, A. P. Indian hedgehog coordinates endochondral bone growth and morphogenesis via parathyroid hormone related-protein-dependent and -independent pathways. *Development* 127, 543-8 (2000)
22. Rabie, A. B., G. H. Tang, H. Xiong & U. Hagg: PTHrP regulates chondrocyte maturation in condylar cartilage. *J Dent Res*, 82, 627-31 (2003)

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