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Alterations in diet consistency and variation in Hypoxia Inducible Factor-1α expression in condylar chondrocytes

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Summary. Objective. To investigate the expression of HIF-1α during the postnatal development of the mandibular condyle under normal and soft consistency diet conditions. Materials and Methods. Forty eight Wistar-Furth rats, aged 21 days, were divided into two groups, each being fed with either normal or soft consistency diet. Three animals from each group were sacrificed after 3, 7, 10 days (initial period), 14, 17 days (intermediate period), and 21, 24 and 28 days (final period) after the start of the experiment. Immediately after sacrifice, the mandible was excised surgically, fixed, demineralised and embedded in paraffin. Six μm thick sections were obtained, processed for conventional and immunohistochemical staining and observed in the optical microscope. HIF-1α expression was assessed semiquantitavely and graded separately for the nucleus stained cells and the cells stained exclusively in the cytoplasm. Differences in HIF-1α expression in the experimental groups were evaluated statistically. Results. HIF-1α expression was evident in the proliferative and chondroblast layers. No differences were observed between the anterior, the intermediate and the posterior parts of the condylar cartilage. In the normal consistency diet group, nuclear HIF-1α expression increased gradually until the end of the experiment. On the contrary, in the soft diet animals, nuclear HIF-1α expression increased only at the final period of the experiment. The normal diet fed animals exhibited more intense nuclear HIF-1α expression compared to cytoplasmic expression. Conclusions. HIF-1α expression in condylar chondrocytes varies under altered conditions of diet consistency.

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Introduction

Masticatory muscle activity has been regarded as a primary factor in the osteogenetic processes in the craniofacial region and research data has shown that relevant changes can affect general growth in this area (Kiliaridis, 2006). In the case of the mandibular condyle, the differential loads exerted during muscle function are essential for normal endochondral osteogenesis. Restricted fetal temporomandibular joint (TMJ) movement influences the process of bone formation in the condylar cartilage and results in reduced cartilage volume, total number of cells, and number of 5-bromo-2'-deoxyuridine-positive cells (Habib et al., 2005). In post-natal animals variations in the condyle loading by mandible propulsion have been shown to affect the differentiation and maturation of chondroprogenitor cells (Rabie et al., 2003a,b).

The biomechanical environment in the craniofacial skeleton is altered when food consistency is changed to soft. Under such conditions the condyle is significantly smaller in the soft diet fed animals (Barber et al., 1963; Bouvier and Hylander, 1984; McFadden et al., 1986; Bouvier and Zimny, 1987; Endo et al., 1998; Kiliaridis et al, 1999). Moreover, in soft consistency diet conditions the rate of differentiation and maturation of mesenchymal cells into chondrocytes is affected (Kantomaa et al., 1994). Thus, the biomechanical changes from the lowered masticatory function and the decreased loading of the TMJ in the event of soft diet affect condylar metabolism and produce changes in condylar homeostasis and osteogenesis.

Hypoxia Inducible Factor-1α (HIF-1α), a

transcription factor that mediates adaptive responses to reduced oxygen availability (Schipani et al., 2001), has been shown to exert a regulatory role in chondrogenesis and endochondral osteogenesis (Takahashi et al., 2000; Pfander et al., 2003, 2004; Robins et al., 2005; Provot et al., 2007; Kanichai et al., 2008), processes that are carried out under hypoxic conditions (Provot and Schipani, 2007). However, research focused on the role of HIF-1α in the cartilage of the mandibular condyle has been limited.

The aim of the present study was to investigate the expression of HIF-1α in condylar chondrocytes during the postnatal development of the mandibular condyle under normal and soft diet conditions.

Materials and methods

Tissues

Forty eight Wistar-Furth rats aged 21 days were used in the experiment. They were housed in well-ventilated stainless cages with 12h light-dark cycle and a maximum of three rats per cage. Food and water were provided *ad libitum*. Protocols were reviewed by the appropriate University committee with respect to the humane care and treatment of animals used in the study.

The rats were divided into two groups of 24 animals, each being fed with either normal (hard pellet) diet or soft consistency (ground pellet) diet. Three animals from each group were sacrificed after 3, 7, 10 days (initial period), 14, 17 (intermediate period) and 21, 24 and 28 days (final period) after the start of the experimental period. The division into three experimental periods was carried out in a way that the intermediate period coincides with the period of peak velocity for physical growth in the rat (Luder, 1996).

Immediately after sacrifice the mandible was excised surgically. The excised tissues were fixed in 4% paraformaldehyde for 24 hours and demineralised in 5% formic acid for 10 days. After dehydration the tissues were embedded in paraffin.

Conventional and immunohistochemical staining

Six μm thick sections starting from the middle of the mandibular condyle and parallel to the midsagittal plane were obtained and processed. Conventional staining (hematoxylin-eosin and alcian blue - PAS) was used to aid in determining condylar cartilage layers and immunohistochemical staining (mouse monoclonal (H1alpha67) to HIF1 alpha α (ab1) (Abcam, Cambridge, UK)) to investigate HIF-1α expression. Sections processed with PBS substituted for the antibody served as negative control (Fig. 1).

Assessment of HIF-1α expression

The stained sections were observed in an optical microscope Zeiss Primo Star (Carl Zeiss Ltd., UK) and photographed with a digital camera Canon Power Shot A 640 (Canon Europe Ltd.).

The condylar cartilage was divided into 3 equal parts, the anterior, the intermediate, and the posterior and stain intensity was evaluated in the cartilage layers. Since staining of the nucleus is indicative of the cellular reaction to the reduced oxygen tension, HIF-1α expression in the nuclei of cartilaginous cells was primarily recorded. However, as accumulation of HIF-1α in the cytoplasm may reflect a state of cellular preparedness, so that tissues can more readily react in a case of further lowering of oxygen tension (Kanichai et al., 2008), the exclusive localisation of HIF-1α in the cytoplasm, without nuclear translocation, was also scored and recorded separately. All HIF-1α stained sections were evaluated in a semiquantitative fashion according to a modification of the method described by McCarty et al. (1985), which considers both the intensity and the percentage of cells stained. HIF-1α stain intensity was graded separately for the nucleus stained cells and the cells stained exclusively in the cytoplasm on a scale of 0 to 3+ (0: not detectable, +: mild staining ++: moderate staining, +++: intense staining), according to Kloen et al. 2003. For each stained section, HIF-1α expression was assessed with a score obtained by application of a modified method of McCarty et al. (1985). Results were assigned to four groups according to their overall scores: not detectable, mild, moderate and intense HIF-1α expression, following a modification of the method of Maw et al (2009).

Statistical analysis

Data of HIF-1α expression was evaluated in the

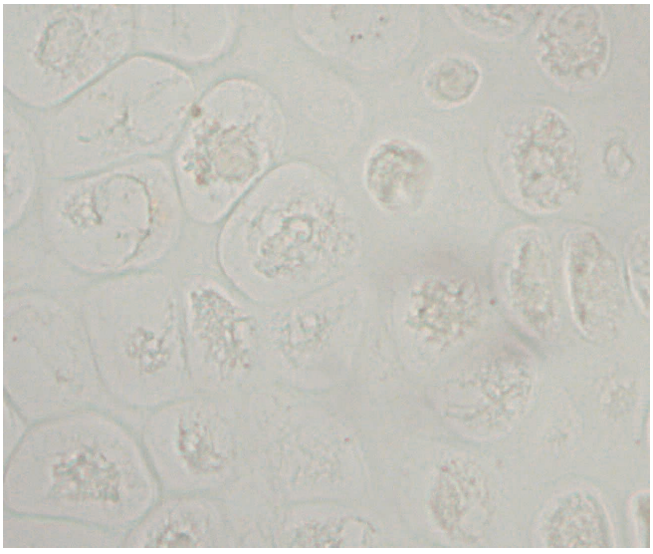

Fig. 1. Tissues processed with PBS instead of the antibody that served as negative control. x 300

groups of animals sacrificed during the initial, intermediate and final periods of the experiment, as initial data analysis showed no intra-group differences.

The differences in HIF-1α expression in the nucleus and the cytoplasm, between the animals of each diet group sacrificed during the initial, intermediate and final periods of the experiment were assessed with the Kruskal-Wallis test. HIF-1α expression differences between the nucleus and the cytoplasm in each food consistency group, at the initial, intermediate and final periods of the experiment were analyzed with the Wilcoxon signed ranks test. HIF-1α expression differences, regarding the nucleus and the cytoplasm, between the normal and the soft consistency diet groups, at the initial, intermediate and final periods of the experiment were analyzed with the Mann-Whitney U test.

All statistical analyses were performed with the PASW Statistics™ software (©SPSS Inc.). The statistical significance level α was set at 0.05.

Results

Body weight in both groups increased during the experimental period. No statistically significant differences were found between the normal and soft diet fed animals ($p<0.05$, t-test).

Morphological assessment of the retrieved condyles showed smaller condyles in the soft diet animals at each time period. As observed in the conventionally stained sections, the thickness of the cartilaginous layers decreased gradually as the experiment progressed (data not shown).

Observation of the sections processed for immumohistochemical staining showed no HIF-1α expression among the fibrous and the hypertrophic layers of the condyle. On the contrary, similar HIF-1α expression was noted among the cells of the proliferative and chondroblast layers in the mandibular condylar cartilage. HIF-1α stain was observed in both nuclei and cytoplasm (Fig. 2) and no differences were observed between the anterior, the intermediate and the posterior parts of the condylar cartilage during the course of the experimental period (data not shown).

At the initial period of the experiment, mild nuclear and mild cytoplasmic HIF-1α expression was prevalent in both animal groups. However, HIF-1α expression did not present the same distribution between the two experimental groups during the course of the experiment (Table 1).

In the normal consistency diet group (Fig. 3), nuclear HIF-1α expression increased gradually until the end of the experiment ($p=0.000$, Kruskal-Wallis test), whereas cytoplasmic HIF-1α expression decreased after exhibiting a peak at the intermediate period of the experiment ($p=0.000$, Kruskal-Wallis test). On the contrary, in the soft diet animals (Fig. 4), nuclear HIF-1α expression became prevalently moderate in most animals only at the final period of the experiment ($p=0.000$, Kruskal-Wallis test), whereas cytoplasmic HIF-1α

Table 1. Semiquantitative assessment of HIF-1α expression in condylar cartilage layers in normal and soft consistency diet groups, at the initial, intermediate and final periods of the experiment.

	Normal food diet		Soft food diet	
	Nuclear localization	Cytoplasmic localization	Nuclear localization	Cytoplasmic localization
Initial experimental period				
Not detectable	1 [11%]	0	2 [22%]	0
Mild	8 [89%]	9 [100%]	7 [78%]	9 [100%]
Moderate	0	0	0	0
Intense	0	0	0	0
Intermediate experimental period				
Not detectable	0	0	0	0
Mild	0	0	4 [67%]	0
Moderate	5 [83%]	5 [83%]	2 [33%]	5 [83%]
Intense	1 [17%]	1 [17%]	0	1 [17%]
Final experimental period				
Not detectable	0	0	0	0
Mild	0	7 [78%]	0	0
Moderate	2 [22%]	2 [22%]	8 [89%]	2 [22%]
Intense	7 [78%]	0	1 [11%]	7 [78%]

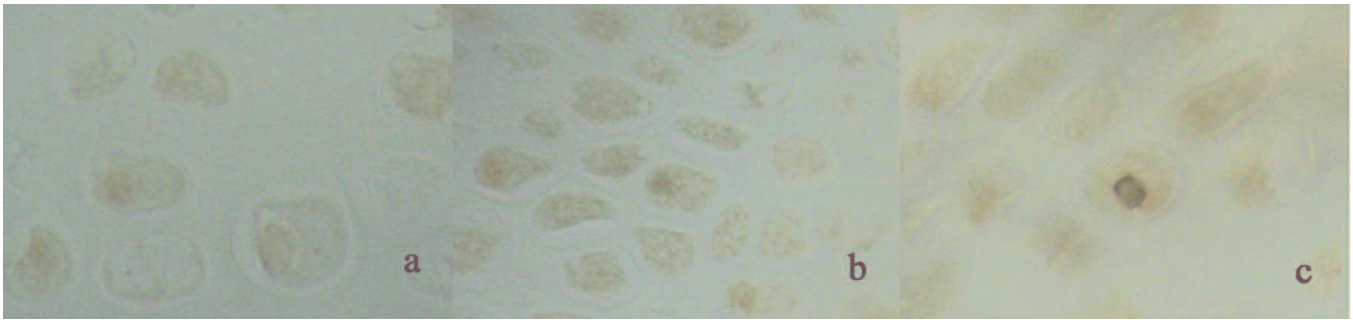

Fig. 2. Representative HIF-1α staining in condylar chondrocytes. **a.** Mild nucleus and cytoplasm staining. **b.** Moderate nucleus and cytoplasm staining. **c.** Intense nucleus and mild cytoplasm staining. x 360

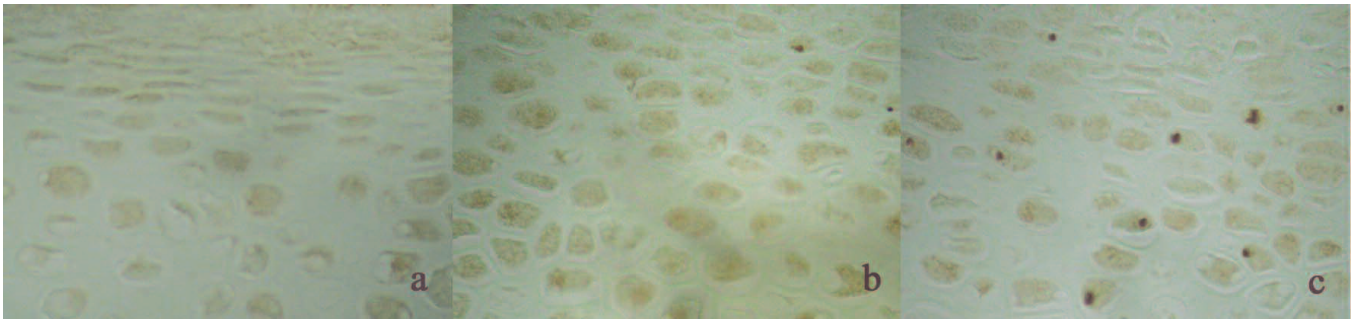

Fig. 3. HIF-1α expression in condylar cartilage in animals fed with normal consistency diet. Representative cartilaginous regions. **a.** Day 7. **b.** Day 17. **c.** Day 28. x 240

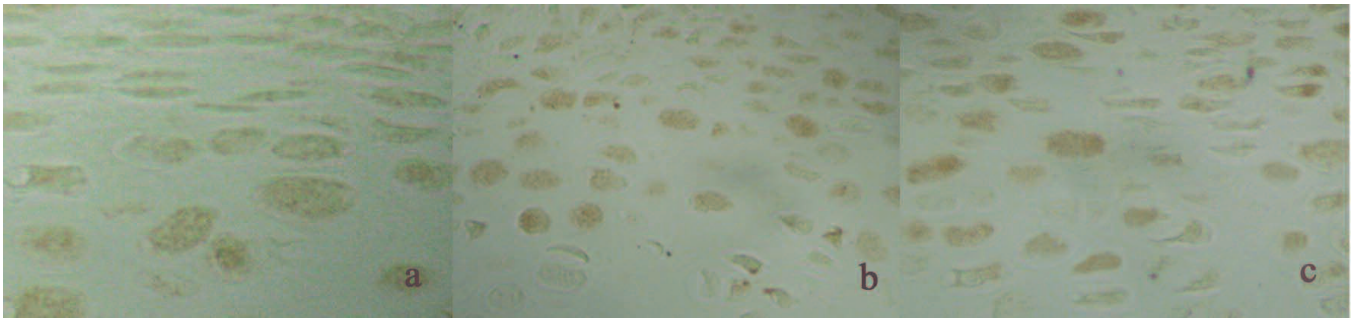

Fig. 4. HIF-1α expression in condylar cartilage in animals fed with soft consistency diet. Representative cartilaginous regions. **a.** Day 7. **b.** Day 14. **c.** Day 24. x 240

Table 2. Differences in the distribution of HIF-1α expression between the nucleus and the cytoplasm in each food consistency group, at the initial, intermediate and final periods of the experiment.

	Normal diet group	Soft diet group
Initial period	NS	NS
Intermediate period	NS	NS
Final period	0.008	0.014

NS: not significant

Table 3. Differences in the distribution of HIF-1α expression regarding the nucleus and the cytoplasm, between the normal and the soft consistency diet groups, at the initial, intermediate and final periods of the experiment.

	Nuclear localization	Cytoplasmic localization
Initial period	NS	NS
Intermediate period	0.018	NS
Final period	0.006	0.000

NS: not significant

expression exhibited a steady, gradual increase (p=0.000, Kruskal-Wallis test).

The differences in the distribution of HIF-1α expression between the nucleus and the cytoplasm, in each animal group at various time points is shown in Table 2. The normal diet fed animals exhibited more intense nuclear HIF-1α expression, whereas the soft diet fed animals presented more intense cytoplasmic HIF-1α expression at the end of the experiment.

The differences in the distribution of HIF-1α expression observed in the nucleus and the cytoplasm between the two animal groups at various time points is shown in Table 3. The normal diet fed animals exhibited more intense nuclear HIF-1α expression at the intermediate and the final period, whereas the soft diet fed animals presented more intense cytoplasmic HIF-1α expression at the end of the experiment.

Discussion

The biomechanical changes and the altered stress distribution in the TMJ under soft consistency diet conditions have been shown to affect condylar homeostasis and osteogenesis (Kantomaa et al., 1994). Recent studies have shown that HIF-1α exerts a

regulatory role in chondrogenesis and endochondral osteogenesis (Takahashi et al., 2000; Pfander et al., 2003, 2004; Robins et al., 2005; Provot et al., 2007; Kanichai et al., 2008). The immunohistochemical investigation of HIF-1α in condylar chondrocytes showed increased HIF-1α expression during the progress of the experiment in both diet groups. The normal diet fed animals exhibited more intense nuclear HIF-1α expression at the intermediate and the final period and less intense cytoplasmic HIF-1α expression than the soft diet fed animals at the end of the experiment.

During the progress of the experiment, increasing HIF-1α expression was evident in the proliferative and chondroblast layers. These layers are the most distant from oxygen sources and have been shown to be hypoxic in other cartilaginous tissues as well (Schipani et al., 2001). The increase of HIF-1α expression may be the physiologic result of changes in the size of the mandibular condyle. According to Harvey (1928) the oxygen tension needed for the survival of cells residing in the interior of a sphere is proportional to the quadrant of the radius of the sphere: $PO_2 = VO_2 r^2/6K$ (PO_2: the oxygen tension on the surface of the tissue, VO_2: oxygen consumption, r: the radius of the sphere, K: the diffusion constant). During development, the condyle grows in size following the general tendency for bodily growth. Consequently, the increase in the distance of the cells residing in the inner layers results in a decrease in oxygen availability and hypoxic conditions. The findings of the present study did not reveal differences in HIF-1α expression between the anterior, the intermediate and the posterior parts of the condyle, as have been documented for other characteristics regarding structure or tissue homeostasis in the condylar cartilage (Mizoguchi et al., 1993; Kantomaa et al., 1994).

The trend of increasing cellular staining evolved differently in the two diet groups during the course of the experimental period. At the final period of the experiment, HIF-1α expression was more intense in nucleus stained cells compared to cells stained exclusively in the cytoplasm, for the normal consistency diet group. Conversely, for the animals fed on soft diet, the HIF-1α expression of exclusively cytoplasm stained cells was greater compared to the nucleus stained cells. When the between groups differences were analyzed, more intense nuclear HIF-1α expression and less intense cytoplasmic HIF-1α expression were observed in the normal diet group compared to the soft diet fed animals. Staining of the nucleus is indicative of cellular reaction to reduced oxygen tension. Under low oxygen tension, HIF-1α concentrates in the cytoplasm and translocates to the nucleus where, in complex with its partner protein HIF-1ß, it binds to the promoter region of specific genes that respond to hypoxia (Déry et al., 2005; Bruick and McKnight, 2002). On the other hand, the accumulation of HIF-1α in the cytoplasm may reflect a state of cellular preparedness, so that tissues can more readily react in a case of further lowering of oxygen tension (Kanichai et al, 2008).

The differences in HIF-1α expression may reflect changes in oxygen availability that correspond to the different muscular activity and TMJ loading patterns among the two groups. During the masticatory function, large forces are exerted on the condyle on biting, whereas while chewing lower loading of the condyle occurs (Weijs and Dantuma, 1975). When low masticatory function is induced, as in the case of animals fed on a soft diet, biting on pellets is negated and functional loading in the condyle is decreased. It is possible that under these circumstances oxygen diffusion in the cartilaginous tissue is facilitated or that more oxygen is available in the region because of the lowering in functional demands. Oxygen diffusion is further facilitated in the animals fed on ground food, because of the smaller condyle size observed, in accordance with the notions of Harvey (1928). The finding of reduced condylar size is in agreement with the findings of previous investigations (Barber et al., 1963; Bouvier and Hylander, 1984; McFadden et al., 1986; Bouvier and Zimny, 1987; Endo et al., 1998; Kiliaridis et al., 1999).

The variation in HIF-1α expression influences the formation of cartilage and bone as endochondral ossification progresses under hypoxic conditions (Provot and Schipani, 2007). An increase in HIF-1α levels promotes mesenchymal cell differentiation to cartilage (Kanichai et al., 2008) and is involved in the process of hypertrophy of mature chondrocytes (Provot et al., 2007). HIF-1α expression also affects extracellular matrix synthesis as it is necessary for collagen synthesis (Takahashi et al., 2000; Pfander et al., 2003). When Von Hippel Lindau protein, which downregulates HIF-1α synthesis, is missing, matrix secretion is increased (Pfander et al., 2004). In addition, HIF-1α controls the expression of any genes related to cartilage and chondrogenesis, including the chondrogenetic transcriptional factor Sox-9 (Robins et al., 2005) which plays a pivotal role in the processes of chondrogenesis and the formation of the skeleton (Healy et al., 1999). In the case of endochondral ossification in the mandibular condyle Sox 9 promotes the differentiation of mesenchymal cells into chondroblasts and results in the formation of collagen type II (Rabie et al., 2003a).

In the present study greater nuclear HIF-1α expression was observed in the normal food fed animals compared to the soft diet group. The effects downstream this increase in HIF-1α expression, in the first group, could provide the condyle with a greater potential to form a cartilaginous scaffold that would by replaced by bone in the subsequent endochondral ossification process (Rabie et al., 2003b). This difference in HIF-1α expression may account for a part of the effect of the degree of loading in chondrogenesis and chondrocyte differentiation (Zuscik et al., 2008) and explain the positive effect of the increased activity of the craniofacial muscles in the growth of the skeletal parts of the region (Kiliaridis, 2006). From this perspective hypoxia constitutes not merely a noxious state for tissue homeostasis but potentially a necessary stimulus for normal growth, as has been shown at least for the case of

the fetal growth plate (Provot and Schipani, 2007).

In summary, during the present investigation HIF-1α was expressed in condylar chondrocytes of the growing rat. HIF-1α expression varied under conditions of normal or lowered loading in the condyle induced by changes in food consistency. Greater nuclear HIF-1α expression was observed in the normal diet fed animals compared to the soft diet group. It is possible that the alteration of muscular activity and patterns of TMJ loading is associated with changes in oxygen availability in the microenvironment of the mandibular condyle. Lowering of oxygen tension may constitute an important stimulus for normal growth of the region and a part in the sequence of events linking the changes in biomechanical activity and loading to chondrogenesis and chondrocyte differentiation.

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