Development of a Rat Model of Mechanically Induced Tunable Pain and Associated Temporomandibular Joint Responses

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Purpose: Although mechanical overloading of the temporomandibular joint (TMJ) is implicated in TMJ osteoarthritis (OA) and orofacial pain, most experimental models of TMJ-OA induce only acute and resolving pain, which do not meaningfully simulate the pathomechanisms of TMJ-OA in patients with chronic pain. The aim of this study was to adapt an existing rat model of mechanically induced TMJ-OA, to induce persistent orofacial pain by altering only the jaw-opening force, and to measure the expression of common proxies of TMJ-OA, including degradation and inflammatory proteins, in the joint.

Materials and Methods: TMJ-OA was mechanically induced in a randomized, prospective study using 2 magnitudes of opening loads in separate groups (ie, 2-N, 3.5-N and sham control [no load]). Steady mouth opening was imposed daily (60 minutes/day for 7 days) in female Holtzman rats, followed by 7 days of rest, and orofacial sensitivity was measured throughout the loading and rest periods. Joint structure and extent of degeneration were assessed at day 14 and expression of matrix metalloproteinase-13 (MMP-13), hypoxia-inducible factor-1α (HIF-1α), and tumor necrosis factor-α (TNF-α) in articular cartilage was evaluated by immunohistochemistry and quantitative densitometry methods at day 7 between the 2 loading and control groups. Statistical differences of orofacial sensitivity and chondrocyte expression between loading groups were computed and significance was set at a P value less than .05.

Results: Head-withdrawal thresholds for the 2 loading groups were significantly decreased during loading (P < .0001), but that decrease remained through day 14 only for the 3.5-N group (P < .00001). At day 14, TMJs from the 2-N and 3.5-N groups exhibited truncation of the condylar cartilage, typical of TMJ-OA. In addition, a 3.5-N loading force significantly upregulated MMP-13 (P < .0074), with nearly a 2-fold increase in HIF-1α (P < .001) and TNF-α (P < .0001) at day 7, in 3.5-N loaded joints over those loaded by 2 N.

Conclusion: Unlike a 2-N loading force, mechanical overloading of the TMJ using a 3.5-N loading force induced constant and nonresolving pain and the upregulation of inflammatory markers only in the 3.5-N group, suggesting that these markers could predict the maintenance of persistent orofacial pain. As such, Dr Winkelstein receives publishing royalties from Taylor and Francis. Dr Granquist is a paid consultant for Zimmer-Biomet Microfixation, including institutional research support, and receives publishing royalties from Wiley-Blackwell.

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Temporomandibular joint (TMJ) disorders are the second most common source of orofacial pain, with 33% of the adult population having at least 1 symptom of a TMJ disorder. Osteoarthritis (OA) is one of the most prevalent TMJ pathologies and can result in low-grade inflammation and joint degeneration. TMJ-OA is often associated with degeneration of the articular cartilage, subchondral bone loss, synovial inflammation, and persistent pain. Painful OA is believed to be due to the TMJ’s decreased adaptive capacity to manage external stress, which induces degeneration of the articulating tissues and condylar deformation. Sustained joint inflammation also can lead to persistent pain and eventual joint dysfunction. In most patients with TMJ-OA, pain and joint instability are short-lived and a favorable outcome is achieved by conservative care. Unfortunately, in up to 15% of patients, persistent disease and progressive joint degeneration and chronic pain develop. Currently, effective clinical management is challenging because some patients experience acute pain that abates over time, whereas in others pain never resolves. Despite the known association between TMJ-OA and orofacial pain, identifying patients with quiescent TMJ-OA from those who will develop persistent orofacial pain remains a clinical challenge owing to undefined pathomechanisms in this disease.

Animal models have been developed to understand the progression from normal adaptive remodeling to joint degeneration and orofacial pain. Most experimental models use surgical or chemical manipulation to disrupt the TMJ through disc perforation or intra-articular injection of inflammatory agents into the joint. Although these models induce degenerative changes by physical alteration, the artificial damage imposed to the TMJ recapitulates neither the characteristic OA lesions nor the clinical progression of persistent TMJ-OA pain. Mechanical overload is increasingly implicated in the progression of painful TMJ-OA. Functional overload can stress articular structures and induce degenerative and inflammatory cascades. Several noninvasive models simulate the functional overload of the TMJ by steady mouth opening, creating OA lesions, and thinning of the articular cartilage reminiscent of early OA pathology in the condyle. The authors previously found that although repeated daily mouth opening using a 2-N loading force produces signs of OA in the TMJ and immediate behavioral sensitivity (ie, pain), that pain resolves within days after the cessation of loading. Although mechanically induced TMJ-OA provides a useful platform to understand relations between TMJ pathology and the sequelae driving TMJ-OA and orofacial pain, current models are limited by not simultaneously modeling the pathology and pain symptoms.

TMJ-OA is characterized primarily by the deterioration of condylar cartilage, with changes in chondrocyte proliferation and activity. Hypoxia is believed to mediate the destructive processes associated with OA by the expression of hypoxia-inducible factor-1α (HIF-1α) in mature chondrocytes of overloaded rat TMJs. Activation of HIF-1α signals cartilage destruction through the production of vascular endothelial growth factor and subsequent activation of matrix metalloproteinases (MMPs), such as MMP-13. Moreover, inflammatory cytokines, including the interleukins and tumor necrosis factor-α (TNF-α), are involved in the activation of osteoclasts in osteoarthritic cartilage and TMJ-OA. Further, increased TNF-α has been reported in synovial samples of patients with TMJ dysfunction and pain, suggesting that inflammatory cytokines might be involved in TMJ-OA pain. Despite that speculated role, no study has investigated inflammation and degradation within the context of mechanically induced painful TMJ-OA or symptom presence or progression.

Because there are few clinically relevant animal models simulating the pathomechanisms of painful TMJ-OA and associated disorders, it remains a clinical challenge to define disease progression and understand which patients with TMJ-OA might develop chronic orofacial pain. The purpose of this study was to develop a noninvasive model of mechanically induced TMJ-OA with sustained orofacial pain in the rat by adapting the authors’ previous model that uses a repeated 2-N mouth-opening load to induce acute TMJ pain. The authors hypothesized that repeated steady mouth opening using a higher loading force of 3.5 N, which is below the load limit for dislocating the rat TMJ, would induce constant nonresolving orofacial pain. In addition, given their role in TMJ inflammation and pain, the authors further hypothesized that expression of degradation and inflammation proteins MMP-13, HIF-1α, and TNF-α in chondrocytes would differ in loaded joints for cases with acute versus persistent orofacial pain. The
specific aims of this study were to measure and compare 1) the development and maintenance of orofacial pain between the 2 loading conditions and 2) the condylar cartilage of loaded TMJs for signs of degeneration, including cartilage loss, using histologic staining and the expression of MMP-13, HIF-1α and TNF-α in chondrocytes of cartilage of loaded and sham TMJs. Collectively, these aims seek to better understand the mechanisms driving the development of acute and resolving versus constant and nonresolving TMJ-OA pain.

Materials and Methods

Experimental procedures were approved by the Institutional Animal Care and Use Committee and performed according to the Committee for Research and Ethical Issues of the International Association for the Study of Pain. Female Holtzman rats (weighing 245 ± 16.2 g) were housed with a 12-hour light and 12-hour dark cycle and free access to food and water.

STUDY DESIGN

Separate, randomized groups of rats were exposed to repeated daily mouth opening using a 2-N or 3.5-N load as the main predictor variable. All loading procedures were performed under isoflurane anesthesia (4% induction; 3% maintenance). Mouth opening was applied for 60 minutes daily for 7 consecutive days, and then rats were followed with no mouth opening for the next 7 days (days 7 to 14). An additional randomized group of age- and weight-matched rats served as a sham controls that received the same daily anesthesia regimen, but no applied mouth opening. Orofacial behavioral sensitivity, joint structure harvest day, rats were anesthetized with sodium pentobarbital (65 mg/kg) and perfused with phosphate buffer saline (PBS) 300 mL followed by 4% paraformaldehyde 250 mL in PBS and post-fixed in 4% paraformaldehyde overnight at 4°C. TMJs were harvested, stored in 30% sucrose in PBS at 4°C, and later decalcified using 10% ethylenediaminetetraacetic acid (pH, 7.2 to 7.4) for 2 weeks. Samples were embedded in OCT Compound (Sakura Finetek, Torrance, CA), sagittally sectioned (20 μm thickness), and thawn-mounted onto slides. Tissue sections harvested at day 14 were washed with distilled water and incubated for 15 minutes in hematoxylin Gill number 2 to visualize nuclei and then counterstained with eosin-Y alcoholic (Sigma, St Louis, MO) for an additional 5 minutes to highlight cellular organization. Slides were dehydrated in a graded ethanol series and mounted using Permount (Fisher, Pittsburgh, PA) and the mandibular condyle was imaged at ×20 using a Leica Widefield microscope (Leica, Allendale, NJ). At least 4 representative images of the articular cartilage of condyle were obtained for each rat and the cartilage layers were qualitatively evaluated as previously described.

Orofacial Behavioral Testing

To quantify the onset and maintenance of behavioral sensitivity, mechanical hyperalgesia was assessed in the region of the bilateral TMJs until the designated day of tissue harvest. Stimulation thresholds were measured before the start of the study (day 0) to define baseline responses for each rat, on days 1 to 6 during the loading phase, and on days 7, 9, 13, and 14. Head-withdrawal thresholds were measured as described previously, with response thresholds measured by stimulating the skin around each TMJ with a series of von Frey filaments of increasing strengths from 0.6 to 26 g (Stoelting, Wood Dale, IL). Each session consisted of 3 rounds of 5 stimulations to each TMJ, with a 10-minute rest period separating each round. The lowest-strength filament evoking a response was recorded as the threshold if the next higher filament also elicited a response, which was taken as an immediate pawing at the stimulated area or a sudden head withdrawal. Thresholds from the left and right sides were compared using a paired t test to test whether there were differences; because no differences were detected and the mouth opening symmetrically loads the bilateral TMJs, bilateral responses were averaged for each rat on each day. Withdrawal thresholds were compared between groups using repeated-measures analysis of variance with the Tukey post hoc HSD test, with time and group as the factors. All statistical analyses were performed using JMP 9 (SAS Institute, Cary, NC) with significance at a P value less than .05.

Tissue Harvest and Histologic Staining

After behavioral testing on each designated tissue harvest day, rats were anesthetized with sodium pentobarbital (65 mg/kg) and perfused with phosphate buffer saline (PBS) 300 mL followed by 4% paraformaldehyde 250 mL in PBS and post-fixed in 4% paraformaldehyde overnight at 4°C. TMJs were harvested, stored in 30% sucrose in PBS at 4°C, and later decalcified using 10% ethylenediaminetetraacetic acid (pH, 7.2 to 7.4) for 2 weeks. Samples were embedded in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA), sagittally sectioned (20 μm thickness), and thawn-mounted onto slides. Tissue sections harvested at day 14 were washed with distilled water and incubated for 15 minutes in hematoxylin Gill number 2 to visualize nuclei and then counterstained with eosin-Y alcoholic (Sigma, St Louis, MO) for an additional 5 minutes to highlight cellular organization. Slides were dehydrated in a graded ethanol series and mounted using Permount (Fisher, Pittsburgh, PA) and the mandibular condyle was imaged at ×20 using a Leica Widefield microscope (Leica, Allendale, NJ). At least 4 representative images of the articular cartilage of condyle were obtained for each rat and the cartilage layers were qualitatively evaluated as previously described.

TMJ Immunohistochemistry and Analysis

The mandibular cartilage was assessed at day 7 using immunohistochemistry for MMP-13, HIF-1α, and TNF-α.
TMJs were harvested as described earlier; tissue from naive rats (n = 2) was included as controls and for normalization. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in PBS 0.01 mol/L and antigen retrieval was performed by incubating slides in DeCal Antigen Retrieval (BioGenex, Fremont, CA) solution for 30 minutes. Slides were washed, blocked with normal horse serum (Vector, Burlingame, CA) for 90 minutes, and incubated in primary antibodies against MMP-13 (1:250; Abcam, Cambridge, MA), HIF-1α (1:500; Abcam), or TNF-α (1:500; ABD Serotec, Raleigh, NC) overnight at 4°C. After washing, sections were incubated with biotinylated donkey antirabbit secondary antibody (1:1,000; Vector) for 30 minutes, developed using 3,3-diaminobenzidine, and mounted using Permount. The articular cartilage of the condyle was imaged at ×40 (1,360 × 1,024 pixels) using a Leica Widefield microscope, with at least 6 representative images for each rat.

To quantify the expression of each protein, images were cropped (80 × 400 pixels) to include only the mature layer of the condylar cartilage and analyzed by image analysis with ImageJ software (National Institutes of Health, Bethesda, MD). Nuclear and cytoplasmic labeling for each protein was assessed in samples from naive rats to define a threshold for immunopositive labeling for each protein based on the mean signal intensity for representative cells. For each section in the study groups, 200 cells were evaluated and counted as immunopositive for each protein if the mean signal intensity was greater than or equal to the normal threshold for that protein. The total number of immunopositive cells for each protein was divided by the total number of mature chondrocytes assessed in each image to determine the percentage of cells positive for that protein and averaged for each group. Separate 1-way analysis of variance with the Tukey post hoc test compared the average percentage of positive cells between groups for each protein.

Results

All rats exhibited eating and grooming behaviors consistent with normal rats throughout the entire study period. The average weight gain for the 2-N and 3.5-N load groups was 3.02 ± 0.24 and 2.51 ± 0.48 g/day, respectively. Neither group was different from the daily weight gain of sham rats (3.28 ± 0.57 g/day).

Mouth opening by either load induced behavioral sensitivity, with the withdrawal threshold decreased immediately at day 1 from baseline levels (P < .001) and remaining considerably lower for the 2 groups during the loading period (Fig 1). In contrast, the withdrawal thresholds in the sham group did not differ from baseline.
on any day. The 2 magnitudes of load decreased the withdrawal threshold significantly from baseline \((P < .0001)\) and sham \((P < .0001)\) levels on days 1 to 7 and day 9 (Fig 1). However, on days 13 and 14, the withdrawal threshold for the 3.5-N group remained significantly lower than baseline \((P < .0001)\) and sham \((P < .0001)\) thresholds, whereas the response thresholds of the 2-N group resolved and returned to baseline and sham levels by day 15 (Fig 1). In addition, head-withdrawal thresholds of the 3.5-N loading group were significantly lower than the response thresholds for the 2-N loading group on days 13 and 14 \((P < .0001; \text{Fig 1})\).

The TMJ articular cartilage surface was assessed at day 14 in the sagittal view of the condyle (Fig 2A). All 4 distinct layers of articular cartilage were visible, including the fibrous, proliferative, mature, and hypertrophic layers (Fig 2B). Joints in the 2 loaded groups exhibited thinning in the condylar cartilage and were less thick than sham unloaded joints (Fig 2B). The condyles of the loaded rats exhibited decreased thickness in all 4 cartilage layers, particularly in the proliferative and mature layers. The 2-N and 3.5-N groups (Fig 2C, D) also displayed irregularities in chondrocyte organization in the hypertrophic layer of cartilage and cell-free areas that were not evident in the sham group (Fig 2B). Despite these differences from unloaded sham TMJs, the cartilage thickness and cellular organization were not different between the 2 magnitudes of applied loading.

MMP-13 expression in condylar cartilage of the 2-N (Fig 3A) and 3.5-N (Fig 3B) groups increased over sham expression (Fig 3C) at day 7, with greater MMP-13 expression in the pained 3.5-N group. MMP-13 expression in mature chondrocytes was significantly increased in the 2-N \((P < .0004)\) and 3.5-N \((P < .0001)\) groups over sham expression (Fig 3D). The increase in MMP-13 after 7 days of a 3.5-N load was nearly twice that of sham levels and significantly greater than the expression in the 2-N loading group \((P < .0074; \text{Fig 3D})\).

Unlike MMP-13 expression, HIF-1\(\alpha\) and TNF-\(\alpha\) were upregulated only in the joints exposed to the 3.5-N opening force. Chondrocyte expression of HIF-1\(\alpha\) after loading (Fig 4A, B) was not increased over sham (Fig 4C) levels after a 2-N load, but was significantly increased after the 3.5-N load over the sham \((P < .0001)\) and 2-N load \((P < .001)\) levels (Fig 4D). In fact, the increase in HIF-1\(\alpha\) expression in the 3.5-N group was approximately 50% greater than in each of the 2 other groups (Fig 4D). Paralleling HIF-1\(\alpha\), TNF-\(\alpha\) expression in differentiated chondrocytes was not increased in the 2-N loading group (Fig 5A) but was in the 3.5-N group (Fig 5B), with a significant increase \((P < .0001)\) in that group over the sham and the 2-N load groups (Fig 5D). However, there were no meaningful differences in TNF-\(\alpha\) expression between the 2-N and sham groups.

### Discussion

The purpose of this study was to develop an experimental model of TMJ-OA and persistent nonresolving pain to better study the pathomechanisms involved in the development of constant pain in patients with TMJ-OA. The authors modified an existing model of mechanically induced TMJ-OA to use a higher loading force of 3.5 N and hypothesized that such a load would induce sustained orofacial sensitivity and OA pathology in the joint. Moreover, the authors hypothesized that expression of MMP-13, HIF-1\(\alpha\), and TNF-\(\alpha\), proteins relevant to the development of painful TMJ-OA, would differ in the 2 loading conditions. To the authors’ knowledge, this is the first TMJ-OA model with tunable pain symptoms using noninvasive mechanical joint overloading (Figs 1, 2). By modulating only the applied joint load, pain resolved (2-N) or persisted (3.5-N; Fig 1), despite articular cartilage exhibiting similar extents of degeneration (Fig 2). MMP-13 expression at day 7 appeared to be sensitive to joint loading magnitude, with differences between groups and increasing with load (Fig 3). Interestingly, HIF-1\(\alpha\) and TNF-\(\alpha\) increased only in the 3.5-N group at day 7, which exhibited persistent pain (Figs 1, 4, 5). Together, these results suggest that a 3.5-N loading force induces sustained orofacial sensitivity and that such pain is accompanied by the early upregulation of inflammatory and hypoxic markers that might play an important role in the development and maintenance of persistent orofacial pain.

Steady mouth opening using the larger 3.5-N load induced sustained orofacial sensitivity after loading was stopped, which was not the case for the 2-N load (Fig 1), despite the 2 loads inducing similar extents of OA pathology (Fig 2). The cartilage thinning and regional chondrocyte loss in the 2 loaded groups (Fig 2) are consistent with condylar degradation observed clinically and in other mechanically induced TMJ-OA models. Condylar degradation has been reported within 5 to 7 days after the start of joint loading, suggesting that adaptive remodeling might be active during TMJ overloading. This is consistent with the load-dependent increase in MMP-13 observed at day 7 (Fig 3), especially because MMP-13 is a known extracellular matrix proteinase and key enzyme in joint degradation. Although MMP-13 has been reported in TMJ chondrocytes during active loading of that joint, it is absent 2 weeks after injury in mice models of TMJ-OA. Despite differential MMP-13 expression at day 7 (Fig 3), the 2 loaded groups displayed similar degeneration at day 14 (Fig 2). Because HIF-1\(\alpha\) increased at day 7 only in the group with persistent pain (Fig 4) and because hypoxia activates MMP-13, HIF-1\(\alpha\) might be an early regulator of pain-related destruction of the TMJ.
At day 7, HIF-1α and TNF-α increased in the mature chondrocytes of only the 3.5-N loaded joints in which pain persisted (Figs 1, 4, 5), supporting these early-acting proteins as possible drivers of constant OA pain. Furthermore, increased synovial TNF-α has been reported in patients with TMJ pain35 and synovial inflammation has been correlated with orofacial pain in clinical studies.26,27 HIF-1α also is involved in maintaining inflammatory processes through the production of proinflammatory cytokines such as TNF-α.36 Given their role in neuropathic pain37,38 and the apparent specificity relating to pain progression in the present study (Figs 1, 4, 5), HIF-1α and TNF-α might be promising predictors of constant pain development in TMJ-OA.

The authors selected 3.5 N as the maximum load below the biomechanical threshold for dislocating the rat jaw,28 but it might not be sufficiently greater than 2 N to alter the local condylar mechanical environment. Furthermore, it is not known how long that pain persists. Although the authors have shown sensitivity is present at 3 weeks after the termination of loading in pilot studies,28 they did not measure the long-term behavioral responses in the present study. However, the present results are similar to other inflammatory-based TMJ-OA models exhibiting sensitivity lasting for 2 to 3 weeks.16,39 Because of differences in the methods of these models, the behavioral sensitivity in the present model might come from the orofacial muscles or ligaments. Studies are needed to evaluate damage in the surrounding tissues and pharmacologic treatments could be used to isolate pain sources in this model. Moreover, expression of MMP-13, HIF-1α, and TNF-α was probed only in the articular cartilage of the condyle; contributions from other inflammatory mediators and in the surrounding tissue, including the synovial tissue, likely contribute to the development of persistent pain.26,27 In addition, expression of these degradation and inflammatory markers were evaluated only at day 7, immediately after the cessation of loading. Longitudinal studies with these early-acting markers and other elements of the OA and cartilage degeneration cascades are needed to further define relevant mechanisms in this model.

In summary, this noninvasive mechanically induced model of TMJ-OA can induce acute (resolving) or persistent pain by modulating the TMJ loading force. Together, the results of this study not only begin to

**FIGURE 2.** A, Three-dimensional reconstruction of computed tomograms of the temporomandibular joint in the sagittal plane and bone window inset of the central sagittal section showing the anatomy and specific location where the condylar cartilage was assayed. Representative images of the condylar surface from temporomandibular joints at day 14 show thinning of the articular cartilage layers in the C, 2-N and D, 3.5-N loading groups compared with the B, sham group, but with no difference between the 2 loading groups. Irregularities in cellular arrangement (arrows) are observed in the 2-N and 3.5-N temporomandibular joints but are absent in the sham joints. Scale bar = 100 μm in B-D.

FIGURE 3. Representative images and quantification of matrix metalloproteinase-13 immunoreactivity in mature chondrocytes of the condylar cartilage at day 7. Chondrocytes positive for matrix metalloproteinase-13 are displayed (arrows). Matrix metalloproteinase-13 expression after A, 2 N and B, 3.5 N loading was increased compared with the C, sham. D, Quantification of percentage of cells positive for matrix metalloproteinase-13 showed significantly more matrix metalloproteinase-13 after a 3.5-N load than after a 2-N load (*P < .0074) or sham (##P < .0001). A mouth opening of 2 N also induced greater matrix metalloproteinase-13 expression than in the sham group (##P < .0004). Scale bar = 50 μm in A-C.

identify potential regulators of persistent pain, but also suggest that the extent of joint overloading might be an important factor in this clinical pathology. Nevertheless, because the 2 load conditions produce clinically relevant pathology despite different pain outcomes and inflammatory responses in the joint, they are a useful platform to investigate issues related to patient management and prognosis. Studies using

**FIGURE 4.** Representative images for A, 2-N loaded, B, 3.5-N loaded, and C, sham groups and D, quantification of hypoxia-inducible factor-1α expression in the articular cartilage at day 7. Chondrocytes positive for hypoxia-inducible factor-1α are displayed (arrows). The percentage of cells positive for hypoxia-inducible factor-1α was significantly larger in the 3.5-N loading group than the 2-N (*P < .001) and sham (##P < .0001) groups. Scale bar = 50 μm in A-C.

FIGURE 5. Representative images for A, 2-N loaded, B, 3.5-N loaded, and C, sham groups and D, quantification of chondrocytes positive for tumor necrosis factor-α in articular cartilage at day 7. Chondrocytes positive for tumor necrosis factor-α are displayed (arrows). The percentage of cells positive for tumor necrosis factor-α increased significantly after mouth opening at the 3.5-N load compared with the 2-N load (*P < .0001) and sham exposure (##P < .0001). Scale bar = 50 μm in A–C.

novel molecular and functional imaging in this model, as used to diagnose TMJ-OA in patients, could provide useful insight into potential diagnostics or even help identify therapeutic targets for TMJ-OA.

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**References**