Structure-Function Relationships of the Temporomandibular Joint in Response to Altered Loading

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Aims: To elucidate the effects of decreased occlusal loading (DOL), with or without reloading (RL), on the structure and bite force function of the mandibular condylar fibrocartilage in skeletally mature male mice. **Methods:** At 13 weeks old, 30 wild type (WT) male mice were subjected to: *(1)* 6 weeks normal loading (NL); *(2)* 6 weeks DOL; or *(3)* 4 weeks DOL + 2 weeks RL. Histomorphometry, cell metabolic activity, gene expression of chondrogenic markers, and bite force tests were performed. **Results:** DOL resulted in a significant increase in apoptosis ($P < .0001$) and significant decreases in fibrocartilage thickness ($P < .05$) and hypertrophic chondrocyte markers indian hedgehog and collagen type X ($P < .05$). A corresponding decrease in bite force was also observed ($P < .05$). RL treatment resulted in a return to values comparable to NL of chondrogenic maturation markers ($P > .10$), apoptosis ($P > .999$), and bite force ($P > .90$), but not in mandibular condylar fibrocartilage thickness ($P > .05$). **Conclusions:** DOL in skeletally mature mice induces mandibular condylar fibrocartilage atrophy at the hypertrophic cell layer with a corresponding decrease in bite force. *J Oral Facial Pain Headache 2019;33:451–458. doi: 10.11607/ofph.2094*

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Temporomandibular disorders (TMD), which afflict roughly 10% of the world population,[1,2] refer to a wide group of disorders affecting the muscles of mastication and the temporomandibular joint (TMJ). Common signs and symptoms of TMJ disorders include orofacial pain and functional disturbances of the TMJ complex.[3,4] Impaired mechanical loading–induced TMJ remodeling has been implicated as one of the major factors in the development of TMD.[5,6] As such, it is imperative to understand the role of altered loading in mediating TMJ remodeling and function.

A physiologic loading regime is necessary to maintain a healthy and functional TMJ.[7,8] Specifically, sufficient loading is necessary to prevent mandibular condylar fibrocartilage atrophy,[9] while overloading enhances mechanical stress to the condylar chondrocytes and is implicated in TMJ degeneration.[10] In order to study the effects of altered TMJ loading, rodent models have been created by modifying their diets and/or occlusal function. It has been shown that variations in food properties, such as volume or hardness, initiate different levels of masticatory muscle contraction that lead to differences in TMJ loading.[11] In addition, altering occlusal loading via incisor trimming has been shown to reproducibly reduce masticatory function.[12] Previous studies have illustrated that incisor trimming and/or soft diets lead to decreased condylar fibrocartilage growth in skeletally immature rats[7,12–14] and mice.[15–17] Further, in these young rodents, growth is restored with restoration of normal loading.[15,18] However, these results were obtained from skeletally immature rodents that were still experiencing robust mandibular condylar growth. In adult rodents, both soft diet and incisor trimming studies have provided inconsistent results. For example, it was shown that incisor trimming increased mandibular condylar length in adult mice.[19] However, soft diet administration decreased the mandibular condylar fibrocartilage

thickness and condylar length in rats.[18,20] Thus, studies are needed to examine the combined effects of soft diet and incisor trimming using a decreased occlusal loading (DOL) model on TMJ remodeling in skeletally mature mice to translate the potential effects of altered loading in adult patients.

While changes in mandibular condylar fibrocartilage histomorphometry and gene expression provide vital information at the cellular and protein levels, models of TMJ functional change are required for clinical translation. Several models have been developed to evaluate functional changes to the craniofacial region of rodents. Head withdrawal threshold, as measured using von Frey filaments, is commonly utilized as a behavioral measurement of sensitivity and pain in the TMJ.[21–23] Also, digitized feeding modules have been utilized to precisely record meal patterns as a measure of behavioral changes to the function of the TMJ.[24] While both of these models provide evidence of changes in masticatory sensitivity and behavior, they fail to determine the mechanical forces that the TMJ can withstand in response to treatment. Bite force, which is generated from the jaw elevator muscles and mandibular biomechanics, has previously been utilized as an accurate measure of the functional state of the masticatory system,[25–28] but has been implicated in TMJ pain solely using inflammatory models rather than correlated with structural changes. It is known that a diminished capacity of masticatory muscle activity exists in patients with TMD.[29] Therefore, bite force is a promising method for characterizing structure-function changes that occur in response to altered loading.

Thus, the aim of this study was to elucidate the effects of DOL with or without reloading (RL) on the structure and bite force of the mandibular condylar fibrocartilage of skeletally mature male mice. These effects were assessed using histomorphometry, cell metabolic activity, and gene expression of chondrogenic markers. Further, these changes were correlated with alterations in bite force to provide evidence for structure-function changes to the TMJ. The results from this study provide a model to assess both structural and bite force changes that occur in response to DOL to the mandibular condylar fibrocartilage and highlight the ability of this unique fibrocartilage to remodel in skeletally mature mice.

Materials and Methods

DOL Model

All experiments were performed in accordance with animal welfare based on an approved Institutional Animal Care and Use Committee (IACUC) protocol (IACUC, protocol #AAAH9166) from Columbia University, New York, New York, USA. Wild-type (WT) male mice were purchased from the Jackson Laboratory. At 13 weeks of age, 40 male WT mice were randomly divided into three groups of 12 to 14 each: *(1)* normal loading (NL) for 6 weeks (n = 12); *(2)* DOL for 6 weeks (n = 14); and *(3)* DOL for 4 weeks + RL (return to hard diet and cessation of incisor trimming) for 2 weeks (n = 14). Based on the knowledge that, in laboratory mice, eruption of molars and occlusion are complete by 21 days of age[30] and the majority of the mandibular condylar fibrocartilage growth is complete around 60 to 86 days of age,[31–33] 13-week-old mice were deemed to have skeletally mature mandibular condyles. Cages were labeled with numbers rather than condition to ensure the administration of bite force assessment was done blindly. DOL was administered based on established protocols.[15] Briefly, the DOL groups were fed a soft-dough diet (Transgenic Dough Diet) and had their mandibular incisors trimmed approximately 1 mm/day every other day for the duration of treatment using an orthodontic light wire clipper. Mice given NL or RL were fed a typical hard diet (PicoLab Rodent Diet 20 5053, LabDiet), and no incisor trimming was performed. To track proliferating cells, 0.1-mg bromodeoxyuridine (BrdU) per gram of body weight was injected intraperitoneally 3 and 19 hours prior to euthanasia, as has been shown previously.[34] Mice were weighed twice per week and sacrificed following 6 weeks of experimentation at 19 weeks old.

Histology and Histomorphometry

Histomorphometry analysis was utilized to determine relative changes to mandibular condylar fibrocartilage morphology in response to DOL and DOL + RL. The mandibular condyle, part of the glenoid fossa of the temporal bone, and intact articulating disc were harvested, fixed in 10% formalin for 2 days, and decalcified in 14% ethylenediaminetetraacetic acid (EDTA) (pH 7.1) for 4 weeks with weekly solution changes. Samples were prepared for paraffin embedding by submerging them in solutions with increasing concentrations of ethanol, followed by xylene. The TMJ was serially sectioned in the anteroposterior direction at 5-mm thickness utilizing a Microm HM 355S microtome (Thermo Fisher Scientific). Three to six sections were obtained based on limitations on the orientation of the TMJ sample to ensure sections were on the same sagittal plane. Sections representing the midsagittal region of the mandibular head were stained with hematoxylin and eosin (H&E) and safranin-O. Histomorphometry measurements, including thickness of the mandibular condylar fibrocartilage, were conducted using the BioQuant computerized image analysis system, and cell counts were done using ImageJ (National Institutes of Health [NIH]). Average

and total cell numbers were determined from an outlined anterior-to-posterior region of the fibrocartilage that included hypertrophic chondrocytes—specifically, a defined frame (width = 3 μm) was drawn to include the region of hypertrophic chondrocytes from anterior to posterior within the sagittal section. Further, the thickness (height) of the fibrocartilage was determined by the upper border at the articular surface and the lower border at the start of subcondylar bone. Image J was utilized to measure the terminal hypertrophic length by calculating the mean diameter of the last hypertrophic cell in the fibrocartilage. Samples from six mice in each group were analyzed, and three to six sections were taken from each sample.

Immunohistochemistry

For immunohistochemistry, sections were deparaffinized with xylene, rehydrated, and digested for 10 minutes with pepsin. Sections were then washed in phosphate-buffered saline (PBS) and treated with a 3% hydrogen peroxide in methanol solution to block endogenous peroxidase activity. All sections were blocked with 10% goat serum to reduce nonspecific binding of the antigen to the primary antibody and then incubated with collagen type II primary antibody (Millipore; MAB8887, 1:100 dilution in 1% bovine serum albumin [BSA]) at room temperature for 60 minutes. Sections were washed twice in PBS. The secondary antibody horseradish peroxidase (HRP) conjugate (SuperPicture, Life Science Technologies) was added and incubated for 10 minutes at room temperature. Following two PBS washes, sections were stained with diaminobenzidene (DAB) chromogen (ImmPACT DAB, Vector Laboratories) for 2 minutes. Hematoxylin treatment for 1 minute was done to counterstain the nuclei.

BrdU immunohistochemical analysis to detect proliferating cells was completed using a BrdU staining kit according to the manufacturer's instructions (Millipore EMD Laboratories, kit#2760). To quantify, the labeling index was calculated as the number of BrdU-positive cells divided by the total number of cells. Three to six sections corresponding to the same anatomical region utilized to determine the total cell numbers were calculated for each sample, and the average of these sections was used as the mean labeling index.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was employed to detect the apoptotic cells according to the manufacturer's protocol (Roche, #11684795910). Quantification of TUNEL-positive cells was performed by averaging five fields of view. Apoptotic cells were quantified by calculating the labeling index as the number of fluorescent cells divided by the total number of cells. Three to six sections corresponding to the same anatomical area used for total cell number were calculated for each animal, and the average of these sections was used as the mean labeling index.

mRNA Extraction and Gene Expression

After 6 weeks of treatment, mRNA from the condylar fibrocartilage of six mice from each group was extracted to determine changes in response to DOL or DOL + RL. Total RNA from the mandibular condylar fibrocartilage was extracted using TRIzol Reagent (Ambion by Life Technologies), purified using the DNase treatment and removal kit (Ambion by Life Technologies), and converted to cDNA utilizing a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Both the left and right mandibular condylar fibrocartilage was pooled together for each sample. For quantification of gene expression, real-time polymerase chain reaction (RT-PCR) was conducted to assess the relative levels of genes of interest using the ViiA 7 Real-Time PCR System (Applied Biosystems) following the protocol detailed in Chen et al.[34] Expression of each gene of interest was determined relative to the Gapdh housekeeping gene (Gapdh – MM99999915_g1) utilizing the ΔΔCT method. Gene expression was performed for the following chondrocyte markers: parathyroid hormone-related protein (Pthrp-Mm00436057_m1); SRY-box containing gene 9 (Sox9-MM00448840_m1); collagen type II (Col2a1-Mm00491889_m1); indian hedgehog (Ihh-Mm00439613_m1); and collagen type X (Col10a1-Mm00487041_m1).

Bite Force

Bite force was assessed utilizing a custom-made force transducer based on previous designs.[26,28] Specifically, the device consisted of two aluminum beams, each affixed with two single-element strain gauges (OMEGA Engineering) and wired in a Wheatstone bridge configuration. Deformation of the parallel beams during biting resulted in a proportional change in output voltage, which was converted to force based on calibrations. The distance between beams was adjusted to 3 mm for maximum bite force. Each day of use, the device was calibrated by suspending a series of weights (0.1 to 0.5 kg) to produce a standard curve of voltage as a function of force. The aluminum bite plate was suspended by a clamp on a ring stand, and mice were introduced a maximum of 10 times to elicit a bite. Baseline values were obtained for 2 weeks prior to testing to acclimate the mice to the testing set-up and to reduce errors in values (three sets of baseline values). Animals were tested four times over the course of the 6-week treatment, at 1 and 2 weeks and at 5 and 6 weeks. On the day of testing, mice were tested five times with intervals of

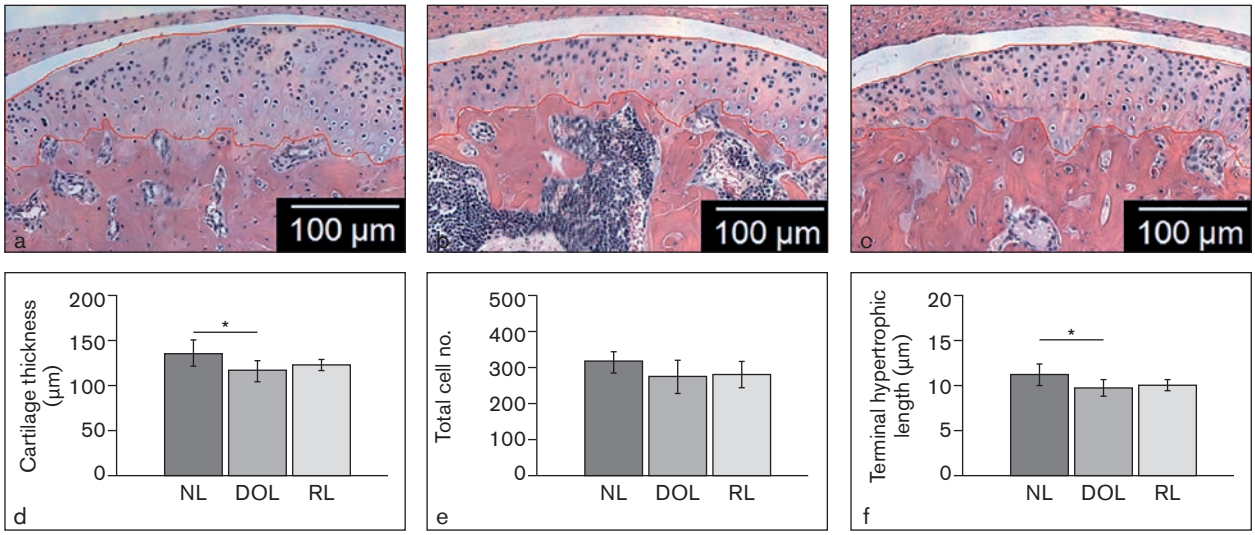


Fig 1 Effect of decreased occlusal loading (DOL) and reloading (RL) on cartilage thickness and cell counts. The data represent male mice under normal loading (NL), decreased occlusal loading (DOL), or reloading (RL) condition. Representative H&E staining of sagittal sections of the TMJ from 13-week-old male mice exposed to **(a)** NL for 6 weeks, **(b)** DOL for 6 weeks, or **(c)** DOL for 4 weeks + RL for 2 weeks. **(d)** Condylar cartilage thickness measurements. **(e)** Total cell number. **(f)** Terminal hypertrophic length. Statistical significance was determined using a one-way ANOVA followed by post hoc analysis with the Bonferonni method. *$P < .05$. Error bars represent standard deviation.

> 1 minute between each trial. The maximum voltage was recorded during each test, converted to force, and averaged for all trials. All data from the last baseline values prior to experimentation were averaged and plotted at Day 0.

Statistical Analyses

Values are presented as the mean ± standard deviation (SD) except for bite force data, which are presented as mean ± standard error of the mean (SEM). For histomorphometry, proliferation, and apoptosis analyses, each data point represents the average value for each mouse sample obtained from three to six histologic sections. Normal distribution of the data was determined using the Shapiro-Wilk test in SPSS. Significant outliers were removed using Tukey outlier method, which was only necessary for the gene expression of Ihh and bite force data; specifically, the interquartile range was multiplied by 2.2, subtracted from the lower quartile, and added to the upper quartile.[35,36] Observations were removed if they fell below or above these determined values. Statistical significance of differences among means was determined using one-way analysis of variance (ANOVA) with post hoc analysis with Bonferroni method using Graphpad Prism 7.0. Differences in means were determined between each group at a time point, and temporal effects within each group for bite force data. Statistical significance was defined as $P < .05$.

Results

All animals were healthy throughout the experiment and were utilized for each detailed analysis unless otherwise stated.

Effect of DOL and RL on Fibrocartilage Thickness and Cell Numbers

Skeletally mature male mice exposed to DOL exhibited a thinner fibrocartilage compared to the NL group (NL vs DOL: $P = .013$; NL vs RL: $P = .147$; DOL vs RL: $P = .766$) (Figs 1a to 1d). No significant differences were measured in total cell numbers between the three groups (NL vs DOL: $P = .307$; NL vs RL: $P = .481$; DOL vs RL: $P > .999$) (Fig 1e). Terminal hypertrophic length was significantly decreased in the DOL group compared to the NL group (NL vs DOL: $P = .049$; NL vs RL: $P = .183$; DOL vs RL: $P > .999$) (Fig 1f). RL did not significantly affect fibrocartilage thickness, total cell numbers, or terminal hypertrophic length compared to NL or DOL (Figs 1d to 1f).

Effect of DOL and RL on Cell Proliferation and Apoptosis

Cell metabolic activity was measured using BrdU and TUNEL assays to determine changes in proliferation and apoptosis. DOL and RL did not result in a significant difference in proliferation compared to NL (NL vs DOL: $P > .999$; NL vs RL: $P > .999$; DOL

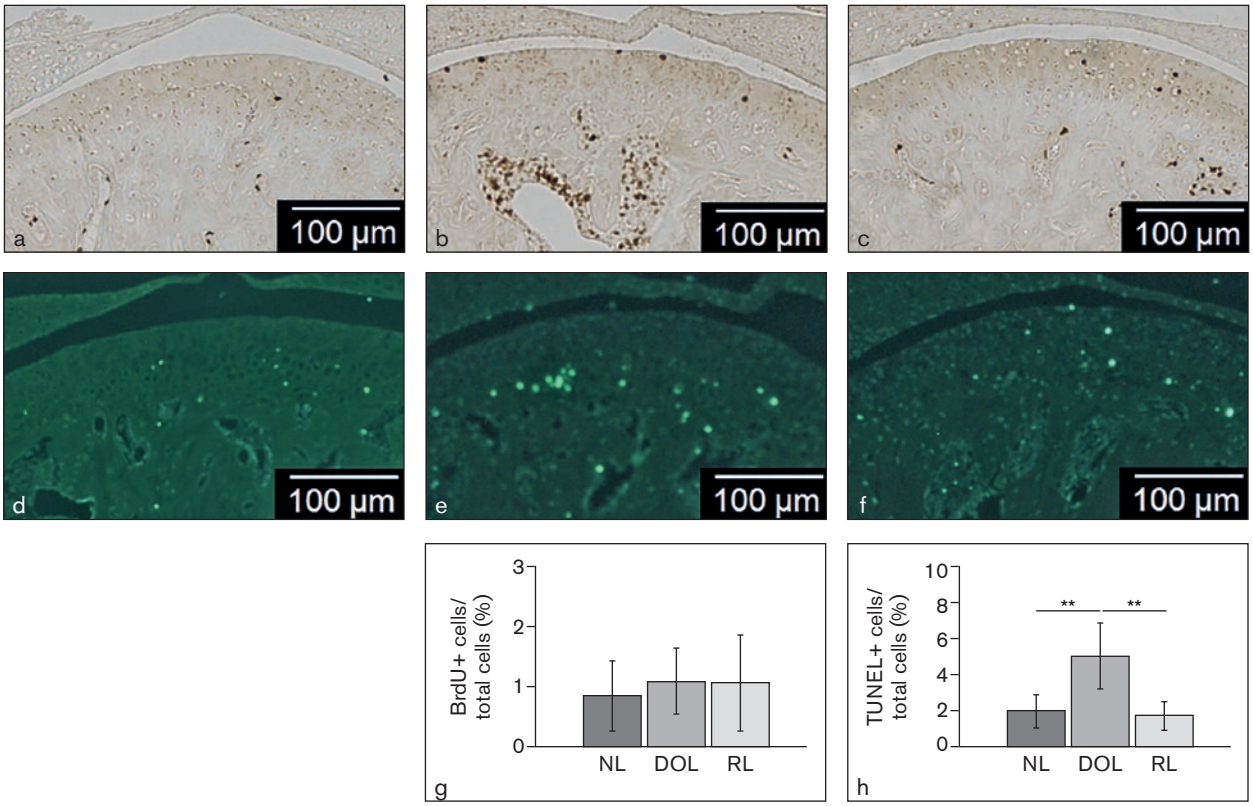


Fig 2 Effect of DOL and RL on cell proliferation and apoptosis. The data represent male mice under **(a, d)** normal loading (NL), **(b, e)** decreased occlusal loading (DOL), or **(c, f)** reloading (RL) condition. **(a–c)** Representative images of BrdU-positive cells and **(d–f)** apoptotic cells as detected by the TUNEL assay. **(g)** Quantified percentage of BrdU-positive cells and **(h)** TUNEL-positive cells. Six mice were utilized for each group, and an average of three to six sections per mouse were analyzed. BrdU- and TUNEL-positive cells were normalized to the total number of cells in the cartilage region as determined from the adjacent H&E section. Statistical significance was determined using a one-way ANOVA followed by post hoc analysis with the Bonferonni method. $^{**}P < .01$. Error bars represent standard deviation.

vs RL: $P > .999$), as seen in Figs 2a to 2f. However, DOL treatment resulted in a significant increase in apoptotic cell numbers compared to the NL and RL groups (NL vs DOL: $P < .0001$; NL vs RL: $P > .999$; DOL vs RL: $P < .0001$) (Figs 2g and 2h).

Effect of DOL and RL on Fibrocartilage Chondrogenesis

The role of DOL and RL on mandibular condylar fibrocartilage chondrogenesis and extracellular matrix composition was assessed via chondrogenic markers at the gene and protein levels. There was a marked decrease of safanin-O staining in the DOL group compared to the NL group, as illustrated in Figs 3a to 3c. There was a partial recovery of safanin-O staining after RL (Fig 3c). A decrease in Col2 staining was observed for the DOL group compared to the NL and RL groups (Figs 3d to 3f). Gene expression analysis revealed a significant reduction in the expressions of Pthrp (NL vs DOL: $P = .003$; NL vs RL: $P = .734$), Ihh (NL vs DOL: $P = .003$; NL vs RL: $P = .112$; DOL vs RL: $P = .469$), and Col10 (NL vs DOL: $P = .029$; NL vs RL: $P = .960$; DOL vs RL: $P = .195$) in the DOL group compared to the NL group (Fig 3g). Also, RL increased Pthrp expression compared to DOL (DOL vs RL: $P = .036$) (Fig 3g).

Effect of DOL and RL on Bite Force

The bite force testing apparatus is shown in Fig 4a, and a representative image of a mouse biting in Fig 4b. Bite force as a function of altered loading is shown in Fig 4c. In investigating temporal effects, bite force was significantly decreased after the initial week of testing for all groups at all time points except DOL week 2 ($P < .05$). In general, bite force values decreased at 5 and 6 weeks of testing compared to 1 and 2 weeks in the DOL group ($P < .05$). In the RL group, bite force 1 week after RL was administered was significantly increased compared to week 2 ($P < .05$). Male mice exposed to DOL for 6 weeks experienced a significant decrease in bite force starting at 1 week that continued throughout testing compared to NL ($P < .01$). Similarly, mice in the RL group that received DOL treatment for 4 weeks experienced a decrease in bite force at 1 and 2 weeks ($P < .05$). However, a recovery in bite force that was statistically similar to NL was measured at both 5 ($P = .95$) and 6 ($P = .91$) weeks after RL was administered.

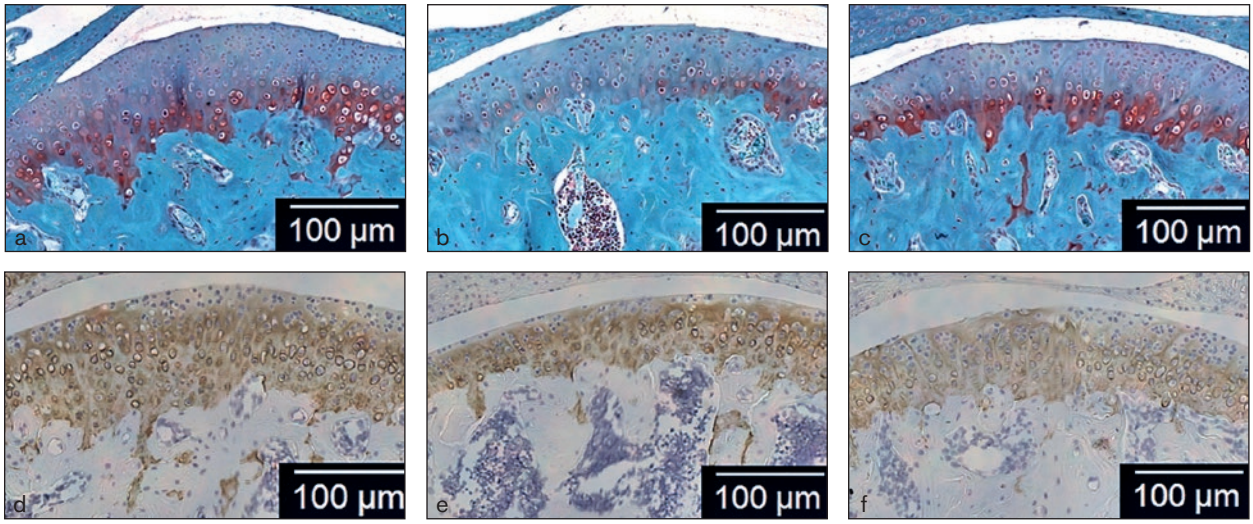
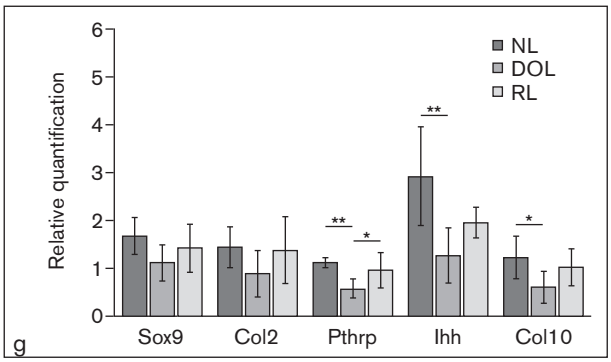
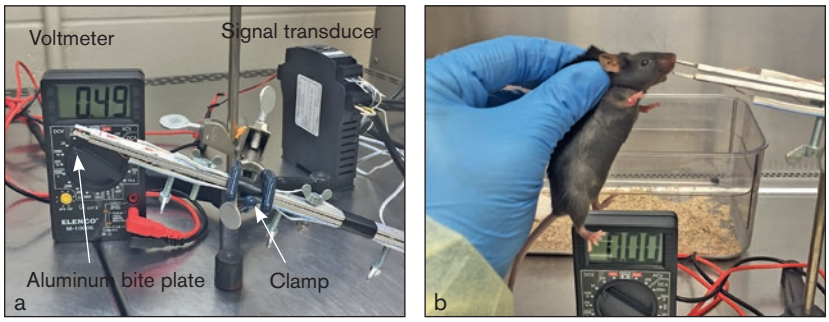


Fig 3 Effect of DOL and RL on chondrogenic markers. The data represent male mice under **(a, d)** normal loading (NL), **(b, e)** decreased occlusal loading (DOL), or **(c, f)** reloading (RL) condition. **(a to c)** Representative safranin-O images and **(d to f)** Col2 immunohistochemical images. Real-time PCR analysis was performed for Sox9, Col2, Pthrp, Ihh, and Col10 gene expression from the mandibular condylar head. For gene expression, six mice were utilized for each group, and **(g)** mandibular condylar fibrocartilage from the left and right sides were pooled together. Statistical significance was determined using one-way ANOVA followed by post hoc analysis with Bonferonni method. **P* < .05, ***P* < .01. Error bars represent standard deviation.





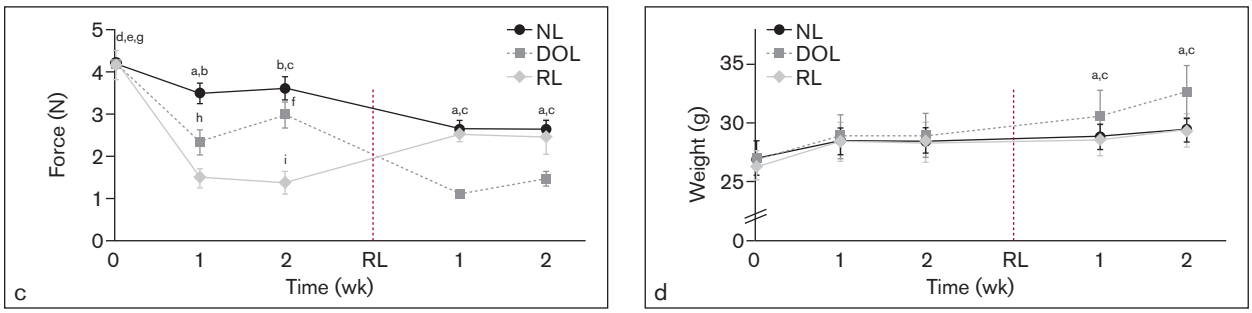


Fig 4 Bite force as a function of altered loading. The data represent male mice under normal loading (NL), decreased occlusal loading (DOL), or reloading (RL) condition. **(a)** Set-up for bite force testing, including the aluminum bite plate with strain gauges, signal transducer, and voltmeter. **(b)** Representative image of mouse preparing to bite the plates. **(c)** Bite force as a function of altered loading measured every 2 weeks for 6 weeks. **(d)** Mice weights over the course of treatment time. n = 12 for NL and n = 14 for DOL and RL. Differences between groups: *P* < .05 for [a]NL vs DOL; [b]NL vs RL; [c]DOL vs RL. Temporal effects: *P* < .01 for [d]NL wk 0 vs NL all other time points; [e]DOL wk 0 vs wks 1, RL1, and RL2; [f]DOL wk 2 vs RL1 and RL2; [g]RL wk 0 vs all other time points. *P* < .05 for [h]DOL wk 1 vs RL1; [i]RL wk 2 vs RL1.

Discussion

The overall goal of this study was to observe the structural and functional changes that occurred in skeletally mature male mice in response to DOL and/or RL. DOL significantly decreased fibrocartilage thickness, hypertrophic chondrocyte thickness, and chondrocyte maturation markers and significantly increased apoptosis in skeletally mature male mice, similar to other studies in adult rodents fed a soft diet.[18,20] These results are also similar to findings in young rodents exposed to DOL[15,37] or to soft diet administration alone.[38] However, unlike in young rodents, DOL did not decrease proliferation or fibrocartilage cell numbers in adult male mice.[15,39] This is likely attributed to an innate decrease in metabolic activity of the TMJ in adults compared to young rodents.[40]

Reloading of the TMJ rescued chondrocyte maturation markers and decreased apoptosis in skeletally mature male mice, which is comparable to results in young rodents. However, RL was not able to fully restore fibrocartilage thickness, as has previously been shown in similar adult rat studies.[18] This lack of full recovery may be a function of a reduced regenerative capacity of the fibrocartilage in skeletally mature mice. Also, a longer duration of RL may be necessary to fully restore fibrocartilage thickness, which is a focus of future studies. Nevertheless, the results from this study highlight the ability of the mandibular condylar fibrocartilage to remodel in response to changes in the mechanical loading environment in skeletally mature male mice.

Bite force has been shown to correlate with masticatory performance and dietary selection.[41] Previous investigations utilizing bite force have been conducted in mouse models of TMJ pain[26,42]; however, in this study, the focus was on correlating masticatory function as a result of structural changes. A decrease in bite force was observed in response to DOL. It is possible that the decrease in fibrocartilage thickness—and thus a corresponding decrease in the relative amount of cartilage-specific extracellular matrix macromolecules—results in alterations in the ability to withstand compressive loading that translates to a decrease in bite force. Also, it is possible that the compressive forces detected by the innervated bone are increased due to the reduction in force dissipation through the thinned fibrocartilage, which may result in sensitivity that would not regularly occur in thicker, noninnervated fibrocartilage.[43] Finally, incisor trimming leads to neuroplastic changes within the sensorimotor cortex, which may influence bite force independent of structural changes to the condylar fibrocartilage.[44] Further investigation on the effects of DOL on the subchondral bone and force dissipation throughout the fibrocartilage in these models would be beneficial to developing a better understanding of the structural-functional changes that affect the TMJ.

In this specific underloading study, the incisors were trimmed and the mice were given a soft diet. While the combined treatment resulted in significant changes, the contribution of each component to the structure and function of the fibrocartilage is unclear. Previously, it was illustrated that incisor trimming or soft-dough diet alone did not produce any significant changes in gene expression after 4 weeks.[15] However, in rats, reducing the load on the mandibular condyle by solely cutting the incisors has been shown to lead to a thinner fibrocartilage layer.[14] Understanding the individual contributions of the soft diet and the incisor trimming on structural changes to the fibrocartilage of skeletally mature mice could provide a mechanism for the functional changes. Lastly, while the results from this study illustrate the ability of the fibrocartilage to remodel in response to loading in skeletally mature mice, these results are currently limited to male mice. Future studies will focus on the role of reloading on the structure and bite force of the mandibular condylar fibrocartilage in skeletally mature female mice.

Conclusions

This is the first study, to the authors' knowledge, illustrating the exciting remodeling capability of the skeletally mature mandibular condylar fibrocartilage as confirmed by cellular, structural, and bite force analyses. Knowledge from these studies provides an understanding of both the remodeling capacity of the fibrocartilage in adults and a model to correlate cellular and structural changes with a translatable measure of bite force. Further, this DOL study provides preliminary evidence that may further be developed into a platform model for studying the effects of pharmacologic and mechanical treatment on TMJ regeneration in older patients.

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