Mandibular condylar process remodeling in rats with different bite-altering devices

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Mandibular condylar process remodeling in rats with different bite-altering devices

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Abstract
The objective of this study was to compare different dental splint models and materials for inducing abnormal loading on the gross morphology and histological appearance of the mandibular condylar processes of Sprague Dawley rats. Three different types of dental splints (resin molar, aluminum incisor, stainless-steel incisor) were placed unilaterally to induce occlusal perturbation for 4 weeks. At that time, mandibular condylar processes were assessed by gross appearance and histology. Quantitative measurements were also conducted on the hematoxylin & eosin images for condyle shape. The results showed that although the condylar cartilage was affected by all splint types, the resin molar splint was associated with the most extensive mandibular condylar process remodeling, which was primarily a slant (skewness) of the lateral aspect of the condylar process. Additionally, quantitative measurements on the histological specimens demonstrated that the split and tilt angle of the left (ipsilateral) condylar processes in the resin molar group (124.8 ± 12.7 degrees and 104.1 ± 12.7 degrees, respectively) increased significantly ($p < 0.05$) when compared to right (contralateral) condylar processes (104.7 ± 5.8 degrees and 91.6 ± 4.4 degrees, respectively). However, no changes were noted on the thickness of the fibrocartilage layer at medial, central, and lateral regions of the condylar process. Another major finding is the high variability of morphology of the naïve animals. Future studies will assess the impact of longer durations of splinting, age, and sex on the remodeling of the mandibular condylar process, allowing for the development of diagnostics and therapies.
Keywords: temporomandibular joint; temporomandibular joint disorder; occlusal splint; degenerative joint disease; TMJ degeneration models

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Introduction

As a load bearing structure, the temporomandibular Joint (TMJ) is under repetitive functional loading from jaw movements and mastication (Boyd et al., 1990; Brehnan et al., 1981). Thus, the homeostasis of anabolic and catabolic events involving the fibrocartilage and subchondral bone in the mandibular condyle is crucial to the normal function of the TMJ. A disruption of this balance could result in Degenerative Joint Disease (DJD) of the TMJ, which might be associated with pain. The etiology of painful DJD is still not understood, as it is multi-factorial across a heterogeneous patient population (Dworkin et al., 1990; Smith et al., 2011; Von Korff et al., 1988). Tooth loss, periodontal diseases, traumas, or dental treatments such as orthodontic and prosthodontic treatments can potentially cause malocclusion or occlusion disharmony, which was believed to be the risk factor for painful DJD (Cao, 2022; Michelotti and Iodice, 2010). Pathological changes such as degeneration of the articular mandibular cartilage are seen on 11% of individuals with TMJ disorders (Mejersjo and Hollender, 1984), which can then lead to a cascade of problems resulting from functional and morphological changes in the joint (Zarb and Carlsson, 1999). The pathological process of joint degeneration includes irreparable abrasion of articular cartilage and thickening and remodeling of underlying bone (Zarb and Carlsson, 1999). However, reviews have pointed out that there was still not enough evidence to indicate a clear relationship between occlusal disharmony and masticatory function as orthodontic treatments, when done properly, were reported to reduce the risk of TMD/DJD (Christensen and Rassouli, 1995; Clark et al., 1999; Greene and Laskin, 1988).

This unknown and complex etiology makes it difficult to develop animal models of the DJD (National Academies of Sciences and Medicine, 2020). A good model of DJD should not require surgical access into the joint and cause secondary inflammation that would confound results. Thus, both the use of irritants and abnormal loading models have been pursued to cause non-invasive joint damage. However, irritants cause acute hypersensitivity and rapid joint damage (Clemente et al., 2004; Fischer et al., 2008; Gameiro et al., 2005; Gameiro et al., 2006; Hutchins et al., 2000; Iwata et al., 1999; Ren, 1999; Roveroni et al., 2001; Shiojiri et al., 2002; Zhou et al., 1999), which may not be representative of the presentation of TMJ patients that is usually associated as a chronic pain disease. Thus, abnormal loading models (Almarza et al., 2011; Guo et al., 2020; Henderson et al., 2015a; Henderson et al., 2015b; Honzawa et al., 1988; Li et al., 2010; Wang et al., 2018a; Xu et al., 2019) is a physiologically relevant approach to drive relatively mild degenerative changes in the joint, or osteoarthritis (OA), over a longer time span and potentially with slowly evolving hypersensitivity.
Increasing number of studies have shown that unbalanced loading on TMJs from bite altering splints/blocks can cause the remodeling of the mandibular condylar cartilage (MCC) and the subchondral bone (Abdalla et al., 2019; Almarza et al., 2018; Buchner, 1982; Ghafari and Degroote, 1986; Henderson et al., 2015a; Kuang et al., 2019; Long et al., 2019; Mao et al., 1998; Wang et al., 2019; Zhang et al., 2015; Zhang et al., 2018). However, there is inconsistency in the literature as to the extent and nature of the remodeling observed following abnormal loading of the joint. This is likely due to differences in splinting techniques, as they can be placed on the incisor teeth (Farias-Neto et al., 2012; Ghafari and Degroote, 1986; Guo et al., 2020; Milena Peixoto Nogueira de et al., 2017; Wang et al., 2018b; Watanabe et al., 2008); or the molar teeth, and if on the molar teeth the splints can be unilateral (Abdalla et al., 2019; Gazit et al., 1987; Mao et al., 1998) or bilateral (Buchner, 1982; Li et al., 2010; Long et al., 2019). Furthermore, the splints can be placed on the maxillary (Gazit et al., 1987; Li et al., 2010; Long et al., 2019; Mao et al., 1998) or mandibular molar teeth (Abdalla et al., 2019; El-Bialy et al., 2015). Moreover, the lack of reliable quantitative parameters to describe the remodeling has limited the ability to make direct comparisons between models.

Marked changes to the condylar process were reported in all studies in which unilateral molar splints were used (Abdalla et al., 2019; Gazit et al., 1987; Mao et al., 1998). However, there was no assessment of the joint contralateral to the splint in these studies, despite the possibility that bilateral changes that might be more reflective of the human condition. As for incisor splints, studies also have shown an impact on condylar process remodeling (Farias-Neto et al., 2012; Ghafari and Degroote, 1986; Guo et al., 2020; Milena Peixoto Nogueira de et al., 2017; Wang et al., 2018b; Watanabe et al., 2008), but the effects observed were the same on both condylar processes. Therefore, the objective of this study was to compare the impact between different splint methods (incisor and molar) and materials (resin, aluminum, stainless steel) for altering the bite on the gross and histological appearance of the mandibular condylar processes of Sprague Dawley rats.

**Materials & Methods**

*Animals*

A total of 28 female Sprague Dawley rats were used in this study. The rats were purchased (Envigo, Indianapolis, USA) at the age of around 11 weeks and were housed in micro-isolator boxes in groups of two in an AAALAC approved animal facility with tight control over temperature (~23 °C) and humidity (50-60 %), in a room on a 12:12 light cycle (lights on at 7:00 AM). Standard rat chow and tap water were provided *ad libitum*. The facility is managed by the Division of Laboratory Animal Resources in the University of Pittsburgh, and all procedures involving animals were approved by the Institutional Animal Care and Use Committee and performed in accordance with the policy on
Humane Care and Use of Laboratory Animals and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

After their arrival in the facility, rats were allowed to acclimate for at least 2 d before any procedures were performed. All the rats were randomly assigned to one of four groups using block procedure: resin molar splint group, aluminum incisor splint group, stainless steel incisor splint group, and naïve control group at 12 weeks of age (n = 7 for each group). This was an exploratory study to investigate the outcome of different splints in inducing DJD in rats in which we did not know what variability to expect from both naïve and the different splinting groups. Thus, N=7 gave enough samples for each phenotype of remodeling observed to be confident that changes between splinting and naïve. However, future studies will need to assess at least 5 if not 10 animals to ensure repeatability. The rats in resin molar, aluminum incisor and stainless-steel incisor group received corresponding bite-altering splints (as described below) at the age of around 12 weeks, while no procedures were performed on the naïve control group.

Sprague Dawley were purchased at the age of 11 weeks and the splints were placed at the age of 12 weeks. According to a previous study (Sengupta, 2013), rats enter their adulthood at the 8th week post-natal and a 12-week-old Sprague Dawley rats is considered an adult. Undeniably, using rats (>20 month) that is similar to a middle-aged women would be ideal for studying the development of DJD. In a pilot study using retired female breeders, the rats were about 10-12 months of age, we saw similar outcomes. Thus, we decided to use 12-week-old rats since their growth is already plateauing and there is no increased cost associated with aging the rats. Future studies should look at the impact of age, specifically using rats at least 20-months of age, on the remodeling of the condylar process on abnormal loading models.

The body weight of each rat was monitored and recorded every week. There was no evidence of severe malnutrition (Figure 1), thus, no rats were excluded from the study. At the end of the 4-week splinting period, all the rats were euthanized. As the objective of this project was to develop/adapt different splint methods, we decided not to blind gross morphology and histology to the groups, as we did not know what differences to expect (if any). Confounders, such as animal/cage location or the order of treatments and measurements, were not controlled.

**Splint Placement**

For the resin molar splint, pilot studies showed that 1 mm resin splint was not enough to induce condylar remodeling consistently, and that such a thin layer of resin was easily grounded down. Therefore, in this study, we placed 1 mm resin splints on both the upper and lower molar teeth of the rats unilaterally. Because of the restricted space in the rat mouth, it was not possible to put a matrix band around the molar teeth and build up the splint with resin composite. Therefore, we used flowable resin to build
up the splint layer by layer. The initial layers were critical, because the resin got into the pits, grooves, and undercuts of the molar teeth to provide a mechanical anchorage, resulting in a good splint retention. Prior to splinting, rats were anesthetized with an intraperitoneal injection of a mixture consisting of 0.55 g/mL Ketamine, 0.275 g/mL Xylazine, and 0.11 g/mL Acepromazine, administered at a volume of 1 mL/kg. When rats were areflexive to tail pinch, they were positioned on their back with their mouth held open with a combination of a rubber band and modified mouth prop. All the left upper and lower molar teeth were cleaned with water, etched with 38 % Phosphoric Acid (Etch-Rite etching gel, Pulpdent, USA) for 30 s, rinsed with water, dried with cotton, and primed with Adper Single Bond 2 (3M ESPE, Germany), followed by Ultraviolet (UV) light cure for 10 s. The splint was then built on the prepared teeth using Beautifil Flow Plus F00/F03 resin (SHOFU Dental Corporation, USA). In addition, due to its low strength, the resin splint is reinforced with a round metal pin (~1 mm in diameter, stainless steel) (Figure 2a). The resin was cured with UV light for 20 s. The thickness of each splint was approximately 1 mm. After the splint placement, rats were anesthetized with 4 % Isoflurane delivered via nose, followed by a brief mouth checkup to make sure the resin splints are still in place at least once a week for 4 weeks.

For incisor splints, pilot studies with the molar splint showed that the rats develop slanted incisor teeth, indicating a lateral shift of the mandible. This finding led us to investigate slanted incisor splints, which have the added benefit of loading each condylar process differently. For the aluminum incisor splint group and the stainless-steel incisor splint group, the rats were sedated with 4 % isoflurane delivered via nose. When rats were areflexive to tail pinch, they were positioned on their back with their mouth held open with a combination of rubber band and modified mouth prop. The upper incisor teeth were cleaned with water and dried with cotton. Then a modified slanted rope sleeve made from either aluminum (1DLD7A, Dayton Electric Mfg, USA) or stainless steel (ST24-4, Loos & Co Inc., USA) was attached to the upper incisor teeth with Zinc Oxide Eugneol Cement (Prevest Denpro, India) (Figure 2b&2c). After the splint placement, the rats were visually checked to make sure the splints are still in place at least once a week for 4 weeks.

Sample recovery, processing, and histology

At the end of the four weeks splint period, all the rats were euthanized with CO₂ followed by cervical dislocation. Their left and right mandibular condylar processes and discs were removed. The condylar processes were then fixed with 15 mL/each (approximately 15 times of the sample size) of 4 % paraformaldehyde at 4 °C for 6 h, followed by decalcification with 15 mL/each of Immunocal (StatLab, USA) at 4 °C for 48 h. Gross images were taken from the anterior and lateral side of the condylar processes using a Leica M165FC microscope (Model: MDG41 with DFC 450C. Leica, Germany) under the brightfield light. The condylar processes were then dehydrated with 70 %
ethanol at 4 °C overnight and embedded with paraffin. To minimize the variation caused by the selection of slides at different depth, all embedded specimens were processed and trimmed with the same protocol. Specimens were placed on the microtome anterior-posteriorly with the anterior end facing the blade. They were then trimmed until the condylar process is in sight. Consecutive sections were collected at the thickness of 8 µm and 14 µm on to microscope slides, which were then dried on a hot plate at 42 °C overnight and stored in slide boxes at room temperature afterwards.

For histology, slides from different rats were chosen at similar depth into the condylar process which was at around the anterior one third of the condylar process. After deparaffinization, 8 µm slides were stained with Hematoxylin and Eosin (H&E), while 14 µm slides were stained with Toluidine Blue. Images were taken from Nikon Eclipse microscope (Model: TE2000-E with DS-Fi3 camera. Nikon, Japan) under the brightfield light. Images were obtained of both right and left condylar processes. All images of right condylar processes were flipped horizontally for easier comparison.

**Image processing**

H&E Images were further processed using ImageJ. Split and tilt angles were determined as illustrated in Figure 6 based on the landmarks of split line, angle divider, and base line. In short, firstly, the medial and lateral boarders of the ramus were marked, of which the angle divider was determined (Figure 5a). Then, the base line was drawn by connecting the medial and lateral convex points below the contour of the condylar process. After, a line (x) was drawn parallel to the base line and tangential to the surface of the condylar process. The split line was determined by connecting the tangent point and the midpoint of the base line. Finally, the split and base lines dictated the split angle, while the angle divider and the base line dictated the tilt angle. In order to measure the thickness at different regions of the condylar process (Figure 5b), central line was drawn from the split line. Then the lateral and medial line were determined by dividing the angles between the central line and the base line on the medial and lateral aspect respectively. Cartilage thickness of the medial, central, and lateral regions were measured.

**Statistical Analysis**

All the weight, angle, and thickness data were reported as mean ± standard deviation (n = 7 for each group, passed Kolmogorov-Smirnov test of normality and Levene’s test of equal variance), over which one-way ANOVA tests were performed followed by the post-hoc Tukey’s HSD. All statistical analysis were performed using GraphPad Prism 8. \( p < 0.05 \) was considered significant. This was an exploratory study to investigate the outcome of different splints in inducing DJD in rats in which we did not know what variability to expect from both naïve and the different splinting groups. Thus, N=7 gave enough samples for each phenotype of remodeling observed to be confident.
that changes between splinting and naïve. However, future studies will need to assess at least 5 if not 10 animals to ensure repeatability.

Results

Rats and Splints

In the aluminum group, there seemed to be a decrease in weight from Week 0 to Week 1, but a bounced back from Week 1 to Week 2 ($p < 0.05$). Similar trend was seen in other groups, but the difference was not significant (Figure 1). As for the splints, all splints were retained for 4 weeks. The wear pattern on the splints varied as a function of splint placement and material. Some of the resin molar splints showed minor wear of the resin holding the metal pin, but the metal pins were still intact (Figure 2a). Uneven wear of both upper and lower incisor teeth of rats in the resin molar group were seen (Figure 2b), likely indicating an abnormal trajectory of the mandible when chewing. All the aluminum incisor splints showed signs of wear (Figure 2c), while at the macroscopic level, the stainless-steel incisor splints remained fully intact (Figure 2d). It is worth pointing out that all the rats in the stainless-steel incisor group developed slanted lower incisor teeth and were offset from the midline. Similar changes were observed in the rats in the aluminum incisor group (6 of 7), but in these, the slant of the incisor teeth was less severe, and there was no detectable shift in the jaw. Gross changes in incisor teeth and jaw in the resin molar splint group were detected in 6 of 7 rats, and in these the slant of the incisor teeth was more severe than that of the steel incisor splint group as were the changes in jaw angle.

Gross morphology of condylar processes

Analysis of the TMJ tissue sections started with an assessment of the gross appearance of the mandibular condylar processes using stereomicroscope. The condylar processes of the naïve control rats looked very similar to their opposing counterparts (left versus right) in both lateral and anterior views (Figure 3 a, a’, b, b’). The area of the lateral surface of the left condylar process (Figure 3c) in the resin molar group enlarged noticeably from the lateral view compared to the right condylar process (Figure 3 c’). The same location on the left condylar process is flattened from the anterior view compared to the right condylar process and the left condylar process in naïve control rats, resulting in a slanted condylar process (Figure 3 d). In contrast, the right condylar process in the resin molar group remained structurally intact with no obvious differences from the morphology of the right condylar process in naïve control rats (Figure 3 c’, d’).

In the aluminum incisor group, besides the slightly enlarged lateral surface, the left condylar process inclined more towards the anterior from the lateral view (Figure 3
A similar inclination was observed in the right condylar process of the aluminum incisor group, but had a smaller lateral surface from the lateral view (Figure 3 e'), which was confirmed by the seemingly missing lateral portion and decreased medial-lateral width of the condylar process when viewing from the anterior (Figure 3 f').

As expected, changes in the stainless-steel incisor group were more extensive than with aluminum incisor group. In this group, the lateral surface of the right condylar process seemed to be enlarged in the lateral view (Figure 3 g), but instead of becoming flattened, it seemed bulged in the anterior view (Figure 3 h). The overall shape of the right condylar process was similar to naïve rats from the lateral view (Figure 3 g'), but had an arched top compared to the naïve control when viewing from the anterior (Figure 3 h').

**H&E Staining of condylar processes**

Since most of the changes on the condylar process were observed on the lateral surface, coronal sections were used to capture the changes on the lateral surface of the condylar processes.

Consistent with what we found in the gross appearance of the condylar process, the lateral surface of the left condylar processes of most rats (6 out of 7) in the resin molar group were flattened (Figure 4 b1, Supplement 2 b1-b7) and seemed to be slanting towards the lateral side when compared to naïve controls (Figure 4 a1). The right condylar processes from most rats (6 out of 7) in the resin molar group (Figure 4 b1') were similar to those of the naïve control group (Figure 4 a1', Supplement 2 b1'-b7'), except for one rat (Supplement 1 b2'), whose right condylar process was so severely deformed that the normal layers of the fibrocartilage were completely disrupted as was the articular surface. Both the left and right condylar processes (Supplement 1 b2, b2') from that rat had numerous circular or elliptic voids in the subchondral bone replacing the normal intertrabecular space.

For the aluminum incisor group, main changes associated with the condylar processes was that their lateral portion was missing, resulting in a sharp and arched top (Figure 4 c1, c1'). It is worth mentioning that the impact of the aluminum incisor splint on condylar process morphology and histology varied dramatically between rats, thus the aforementioned changes were seen in 4 out of 7 rats (Supplement 1 c1-c4, c1'-'c4') and might happen to both sides (Supplement 1 c3, c3', c4, c4'), or one side (Supplement 1 c1, c1', c2, c2'), or neither side (Supplement 1 c5-c7, c5'-'c7').

The lateral surface of the left condylar process also appeared to be site of the primary impact of the stainless-steel incisor splint, where a slightly slanted lateral surface can be seen (4 out of 7) (Figure 4 d, Supplement 1 d1-d7), while no significant changes were noted on the right condylar process (6 out of 7) (Figure 4 d', Supplement 1 d1'-d7'). Same as the aluminum incisor group, histology outcome in the stainless steel incisor group varied between rats. For example, in two rats, the fibrocartilage layer in the lateral portion of the left condylar process was decreased and the stratified layers were
disrupted (Supplement 1 d1, d6), while the right condylar process of one rat appeared to be smaller in size than others (Supplement 1 d5').

**Toluidine Blue staining of condylar processes**

To further investigate how different splinting methods and materials affected the distribution of glycosaminoglycans (GAG), we performed Toluidine Blue staining. The GAG distribution followed a similar pattern to the overall shape of the condylar process. In the naïve control group, GAG was evenly distributed in the hypertrophic layer from medial to lateral, and both condylar processes shared similar pattern (Figure 4 a2, a2'). In the resin molar splint group, the distribution of GAG was very different between left and right condylar processes (Figure 4 b2, b2'). In the left condylar process, the GAG was evenly distributed in the hypertrophic layer from medial to lateral (Figure 4 b2), while in the right condylar process, the GAG was disrupted at the lateral portion (Figure 4 b2').

It can be observed in the aluminum incisor group and stainless steel incisor group that the relative thickness of GAG layer was increased (Figure 4 c2, c2', d2, d2'). It is worth pointing out that the GAG layer and distribution in the left condylar process of one splinted rat did not appear to be continuous (Supplement 2 c4, c4'), a pattern not observed in any condylar processes of naïve control rats. In contrast, there was no lateral GAG staining observed in the condylar processes from all but two rats in the stainless-steel splint group (Supplement 2 d1-d7, d1'-d7'). In these two rats there appeared to be more GAG staining in either the left or the right condylar process (Supplement 2 d6-d7, d6'-d7'). Furthermore, the proportional thickness of the GAG layer of some condylar processes (Supplement 2 d1', d2, d4, d4', d5) was decreased.

**Measurement of landmarks**

We performed measurements with landmarks that enabled us to quantify the morphological changes in the H&E images (Figure 5). As mentioned in the Methods, we defined the split angle as the angle between the split line and the base line (Figure 5a). This split angle was significantly higher in the left condylar process in the resin molar splinted rats compared to the right condylar process in this group (124.8 ± 12.7 degrees and 104.7 ± 5.8 degrees respectively, \( p < 0.05 \)) (Figure 6a). No other significant differences were seen in other groups when comparing the left and right side within the same group or when comparing the same side between the naïve control group and other groups (Figure 6a). The changes in tilt angle (defined as the angle between the angle divider and the base line)(Figure 6b), were largely comparable to those of split angle for both splint type and side (left > right), where the only significance observed were between the left and right condylar process of the resin molar group (104.1 ± 12.7 degrees and 91.6 ± 4.4 degrees respectively, \( p < 0.05 \)) (Figure 6b). We also measured the thickness of the cartilage layer at medial, central, and lateral regions of the condylar process. However, no significant difference was detected between the left and right condylar processes within
the same group, nor between the naïve control group and other groups when comparing the same side (Figure 6c-6e).

**Discussion**

In this study, we investigated the impact of different types of bite-altering splints on the temporomandibular condylar process cartilage. Our results suggest that the resin molar splint is the most consistent across all three bite-altering devices in increasing the split angle, which is observed as a slant of the lateral aspect of the condylar process. Furthermore, this slanting was more drastic with the resin molar splits when compared to the other splinting methods, which also had a higher variability of outcomes. However, the incisor splints still showed marked remodeling when compared to naïve. It is important to note that there was minimal evidence of remodeling in the fibrocartilage layers, especially when compared to the change in shape of the bone (condylar process). This suggests that the fibrocartilage layer is less sensitive to a change in abnormal loading than the bone of the condylar process. Most condylar processes from splinted animals had a change in overall shape, but all still had fibrocartilage tissue.

An unexpected finding was that the naïve tissue had a large variability in the distribution of the GAG-rich layer and condylar process shape in these 12 week old Sprague Dawley rats. It has been reported that some species (e.g., domestic dogs and wolves) have multiple variations in the shape of the condylar process even within the same breed (Curth et al., 2017). Although there is no study regarding the shape variation of the condylar process in rats, we speculate the shape difference seen in the naïve group can be attributed to the morphological variation of the condylar process in rats, which makes it extremely difficult to determine early signs of osteoarthritis as there is no robust GAG-rich layer in naïve animals, and the thickness throughout the condylar process is also highly variable. Thus, histological scoring systems for osteoarthritis, like OARSI (Bannuru et al., 2019), would not be able to detect a difference between joints from naïve and splinted rats.

To achieve the jaw shifting condition, we tried different splinting devices, where differences were observed. Due to the low strength of the flowable resin material, a cylindrical metal pin was added to each resin splint to improve wear-resistance. By unilaterally placing the resin splints on the left upper and lower molar teeth, we were able to create an unbalanced occlusion, which resulted in the perturbation of the jaw movement upon mastication and the likely shift of the load on the TMJs. This is further verified by the slanted upper and lower incisor teeth edges in some of the resin molar splinted rats (Figure 2). Previous studies have shown that the left and right condylar processes to be different from each other in the unilateral splint models or functional lateral shift models (Kure-Hattori et al., 2012; Sato et al., 2006; Wattanachai et al., 2009). In this study, based on the results from the gross appearance, most of the changes were
observed in the condylar process ipsilateral to the splint, manifested by the flattening of the lateral region.

In an attempt to generate a similar load-shifting effect on the TMJs with the incisor splints as with the resin molar splint group, the incisor splints were angled in a manner similar to what was observed on the incisal edges in the resin molar group (the left upper incisor tooth was shorter than the right). We first used the aluminum splint, which turned out to be easily ground down by the rats. In this study, the aluminum splints were ground down differently between rats, which might contribute to the high variation in terms of how left and right joints remodeled. Nevertheless, despite this variability, the aluminum splint was still able to create an observable amount of remodeling in the condylar processes of at least half the animals. The joints remodeled in a different manner compared to the resin molar group, probably owing to the fact that the aluminum splints used were thin anterior-posteriorly, so that the lower incisor teeth moved outside the occlusion plane of the splint during mastication and the incisor teeth were pushed protractively or intrusively by the splints, similar to the setup in other studies (Farias-Neto et al., 2012; Milena Peixoto Nogueira de et al., 2017). This could be why there was a minimal amount of remodeling observed in two of the rats with aluminum splints, at least at the site analyzed, if the changes were primarily manifest in the anterior/posterior region. These would have been difficult to detect using coronal sections that show the medial-lateral view. While changes to the condylar process were not consistent, the constant grinding could have produced damage to the muscles of mastication. Thus, this could be a model for myofascial pain and is worth exploring in the future.

To address the problems of the aluminum splints, stainless steel incisor splints were used. They were fabricated with the same incisor teeth edge angulation as the aluminum splints, but with a much thicker anterior-posterior dimension. The stainless-steel incisor splint maintained its integrity throughout the entire study. However, we did not see a major change on the fibrocartilage of the condylar processes. Instead, the stainless-steel splints induced an overall change in the shape of the condylar process, likely due to bone resorption. There did appear to be more GAG staining in some condylar processes from this group compared to naïve rats, perhaps indicating a transition to hypertrophic and mineralized cartilage, but longer time points would need to be studied to assess this possibility.

To note, the splints placed on the molar teeth might have different compensation mechanism compared to the ones place on the incisor teeth. The incisor teeth of rodents grow continuously, while their molar teeth do not. Therefore, when the splints were placed on the upper incisor teeth, the lower incisor teeth were grinding against the metal splint while the upper incisor teeth kept elongating. As a comparison, the incisor teeth on the molar splint rats were out of occlusion at first, but as they continue to grow, the upper and lower incisors will eventually occlude and grind against each other. The variation in the compensation of the occlusion between splint types can partially explain
the outcome seen in the gross appearance and histology, where the molar splint induced a flattened area on the lateral surface of the condylar process, but the condylar process appeared to be arched in the incisor splint group.

Others have also shown that the growth-rate of the cartilage and the number of chondrocytes increased significantly after applying bilateral molar splints on rats (Buchner, 1982). Unilateral molar splints were also shown to increase the expression of aggrecan in the condylar cartilage and versican related proteoglycan in the disc and articular surface of the condylar process (Mao et al., 1998). Discrepancies between the growth or ablation of the GAG layer are likely due to differences in the splinting technique and splinting material used. It is important to note that the timepoint of 4 weeks of splinting is fairly short when compared to knee osteoarthritis rat models where the timepoints are usually 12-16 weeks post-injury (Miller and Malfait, 2017; Miller et al., 2015). These models also cause abnormal loading by transecting the anterior cruciate ligament (ACL)(Brown et al., 2020) or the medial meniscus(Allen et al., 2012). For these models, transection of the medial meniscus allows for faster articulation of incongruent surfaces (femur on condyle), which produces osteoarthritis faster that ACL transection models. Our TMJ splint model still has an intact TMJ disc protecting the condylar process from the fossa, and thus we would not expect our model to develop late stage/chronic arthritis like these knee osteoarthritis models. Thus, it is worth exploring if similarly long timepoints post splinting (12-16 weeks) produces more extensive damage to the fibrocartilage. Nevertheless, we still found profound changes in shape of the process of the mandibular condylar process in just 4 weeks post-splinting. We have not explored in depth the impact of sex on these changes from splinting, but in a few pilot male animals we did not observe any difference in the slanting/skewness of the lateral aspect from splinting when compared to the females at this 4 week timepoint. We will have to further study these sex differences as it is known that female rodents take much longer to develop osteoarthritis than males in traumatic knee rodent models (Ma et al., 2007; von Loga et al., 2020).

To quantify the remodeling of the condylar process, previous studies used a line connecting the lateral and medial most contour points as a reference to divide the condylar process into medial, middle, and lateral regions (Guo et al., 2020; Sato et al., 2006). However, their method only involved measurements of the area of changes in different regions which does not enable comparisons between groups. In this study we developed two parameters, namely the split and tilt angles, using landmarks from the H&E staining images. These two angles describe different aspects of morphological changes of the condylar processes, namely the skewness and rotation. As shown in Figure 7, the x-axis describes changes in the split angle, during which the skewness of the condylar process outline is changed while the base of the condylar process (Base line) does not rotate against the ramus (angle divider line). In contrast, the tilt angle shown in the y-axis indicates changes of the rotation of the process against the ramus, while the condylar
process outline (skewness) remains the same. After performing the measurements on H&E images, our results indicated that for the resin molar group, the remodeling mainly happened on the left condylar processes, where the condylar process not only skewed laterally, but also rotated counterclockwise when comparing the left to the right. This indicate that the abnormal loading induced by the molar splint was applied to the later side of the condylar process, which agrees with the findings from the gross appearance and histology that the lateral aspect of the ipsilateral condylar process was flattened. Contrarily, no significant difference was detected in the aluminum incisor splint and stainless-steel incisor splint group. In preliminary studies, we verified that the variability of the angle of sectioning did not impact the detectable skewness or rotation of the condylar process in the coronal direction. There is the limitation in that this is a description of a 3-dimensional (3D) change in 2 dimensions (2D), and we could be missing a nuanced change that would be seen in computed tomography (CT) imaging. However, this 2D approach still yields meaning and repeatable comparisons for these splinting models in the coronal plane.

To note, the changes in the skewness and rotation of the condylar process will not be possible without the remodeling of the subchondral bone. Therefore, the split and tilt angles can serve as indicators for subchondral bone remodeling. However, micro computed tomography (µ-CT) or other similar systems are still needed for precisely measuring the histomorphometric changes of the bony structure, which is one of the limitations of this study. Using µ-CT will also enable us to compare the changes in the shape of the condylar process before and after the splint placement within the same individual, eliminating the influence of aforementioned morphological variations of the condylar process seen even in the naïve rats. It is important to note that µ-CT will also show subchondral bone changes, which is key feature of DJD, and is a limitation of this study.

Besides the split and tilt angle, we also measured the thickness of the cartilage layer at the medial, central, and lateral regions of the condylar process. Although in some rats, the thickness at certain regions seemed to be thicker, no significant difference was observed neither when comparing the left with the right side within the same group, nor when comparing the same side between the naive control group and other groups. This is probably due to the large variability within each group. Therefore, a larger sample size is indicated to detect a significant difference, if there is any, in terms of the cartilage thickness.

A limitation of this study is that we did not have comparison to a sham placement of the splints. We did perform a few sham splint placements, and we decided not to pursue this a control since no changes in the lateral aspect of the condylar processes were observed, and thus saving the unnecessary use of animals. While more shams could have yielded changes in morphology, we thought it would be unlikely. First, all three splinting methods caused a side-to-side difference in condylar process remodeling, while the
operation, i.e., mouth opening, alone affected the condylar processes bilaterally. Second, studies have reported condylar process remodeling induced by mouth opening only after a prolonged period of time repetitively over a few days (Kawai et al., 2008; Nicoll et al., 2010), one of which even shows a trend of recovery after 28 d (Kawai et al., 2008). In contrast, the procedure in this study is a one-time procedure that lasts for less than 15 min. Therefore, we estimated the impact of the procedure to be minimum after 28 d. And third, histology from naïve rats can give us more information on what a normal condylar process looks like, allowing us to compare various outcomes caused by different splints. Another limitation of this study lies in the lack of the assessment of the discomfort or hypersensitivity caused by the bite-altering splints. Some rats in this study showed signs of discomfort, manifested by the skin rashes on their lower chin, which was also observed in another study using bilateral molar splints (Long et al., 2019). Therefore, we believe that the bite-altering splints can not only cause the remodeling of the condylar process, but also discomfort or even induce hypersensitivity in the TMJ region. It has also been reported that bite-altering splints are responsible for the stress-induced stimulation of Hypothalamic-Pituitary-Adrenal Axis leading toneurological alterations predisposing the animal to hypersensitivity to stressful stimuli (Piancino et al., 2019). Another limitation is that male rats were not assessed, and a comparison to female needs to be explored in future studies.

In this study, the splints were placed for 4 weeks, which is relatively short considering that it will take much longer to remodel the fibrous layer of the condylar process as shown in the histology. Thus, studies are needed to investigate the effect of the splint on the condylar process in a longer term. Moreover, it is still unclear whether the condylar process will recover after the removal of the splint, and if it does not, knowing the timepoint when the damage becomes irreversible is of clinical significance. Therefore, future studies should also be conducted to investigate the recovery of the condylar process after bite-altering splints. In addition to the changes on the condylar process, it has been reported that the glenoid fossa is remodeled in a rat molar splint (resin + metal crown) model (Liu et al., 2007). In the current study, we did not investigate the changes happened on the glenoid fossa, but it is worthwhile to study the remodeling of the glenoid fossa together with the condylar process to gain a better understanding on how the splint will affect the structure and function of the TMJ as a whole. Due to the scope of this study, we did not look into the neuroinflammatory aspect of the painful DJD. Therefore, future studies are needed to investigate the nociceptive changes of the rats and its potential link to the histological changes. Moreover, according to other studies, bite-altering splints can cause other changes in the craniofacial region, such as the morphological changes of the mandible (Mavropoulos et al., 2004; Sugiyama et al., 1999), changes of the periodontal tissue surrounding the splinted teeth (Liu et al., 2015; Mavropoulos et al., 2005), and the inflammation of the masseter (Wu and Liu, 2019), which are worth of exploring, especially the inflammation of the masticating muscles as
the occlusal perturbation can cause its hyperalgesia in rats (Cao et al., 2009) and TMD pain is sometimes attributed to the myofascial pain (Cairns, 2010).

In conclusion, all three splints were associated with remodeling of the condylar process. However, the molar resin and stainless steel incisor splints were associated with the most consistent changes in histology. It would therefore be most useful for future studies designed to assess the impact of variables such as age and sex, as well as therapeutic interventions.
Acknowledgements
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References


Figure Captions

Figure 1. Rat body weights. Each of the color-coded dot represents one rat in each group. Significance (p < 0.05) were seen within the Aluminum group but not in the Resin Molar nor Stainless Steel Incisor group.

Figure 2. Examples of different bite-altering splints. Resin molar splint (a); Uneven wear of the incisor teeth (b); Aluminum incisor splint (c); Stainless steel incisor splint (d).

Figure 3. Gross appearance of the condylar processes in different groups. Lateral view of left (a, c, e, g) and right (a’, c’, e’, g’) condylar processes of the naïve control, resin molar, aluminum incisor, and stainless steel groups respectively. Anterior view of left (b, d, f, h) and right (b’, d’, f’, h’) condylar processes of the naïve control, resin molar, aluminum incisor, and stainless steel groups respectively. All right condylar processes were flipped horizontally.

Figure 4. Histology of coronally sectioned condyles. Representative H&E and Toluidine Blue staining images for Left & right condyles of the naïve control group (a1, a1’, a2, a2’), resin molar group (b1, b1’, b2, b2’), aluminum incisor group (c1, c1’, c2, c2’), and stainless steel incisor group (d1, d1’, d2, d2’). All right condyles were flipped horizontally. Scale bar: 500µm.

Figure 5. Example of landmarks used in quantitative measurements. Certain landmarks were used to determine the split and tilt angles and the thickness at the medial, central, and lateral regions. a: first, the medial and lateral boarders of the ramus were marked, of which the angle divider was determined. Then, the base line was drawn by connecting the medial and lateral convex points below the contour of the condyle. After, a line (x) was drawn parallel to the base line and tangential to the surface of the condyle. The split line was determined by connecting the tangent point and the midpoint of the base line. Finally, the split and base lines dictated the split angle, while the angle divider and the base line dictated the tilt angle. b: in order to measure the thickness at different regions of the condyle, central line was drawn from the split line. Then the lateral and medial line were determined by dividing the angles between the central line and the base line on the medial and lateral aspect respectively. Cartilage thickness of the medial, central, and lateral regions were measured. Scale bar: 500µm.

Figure 6. Quantitative outcomes of the angle and thickness measurements. Split angle (a); Tilt angle (b); Cartilage thickness at medial (c), central (d), and lateral (e) regions. The result was reported as mean ± standard deviation. Comparisons were made between the left and right condyles within the same group, as well as the same side between the naïve control group and other groups. The only significance was seen between left and right condyles.
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**Figure 7. Schematic of the Changes of Split and Tilt Angles.** Changes in the split angle (x-axis) reflect the changes in the outline of the condylar process (skewness). Changes in the tilt angle (y-axis) reflect the changes in the base of the condylar process relative to the ramus of the mandible (rotation).

**Supplement 1. H&E staining of the coronally sectioned condyles.** Left & right condyles of the naïve control group (a1-a7 & a1’-a7’), resin molar group (b1-b7 & b1’-b7’), aluminum incisor group (c1-c7 & c1’-c7’), and stainless steel incisor group (d1-d7 & d1’-d7’). All right condyles were flipped horizontally. Scale bar: 500µm.

**Supplement 2. Toluidine blue staining of the coronally sectioned condyles.** Left & right condyles of the naïve control group (a1-a7 & a1’-a7’), resin molar group (b1-b7 & b1’-b7’), aluminum incisor group (c1-c7 & c1’-c7’), and stainless steel incisor group (d1-d7 & d1’-d7’). All right condyles were flipped horizontally. Scale bar: 500µm.
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169x160mm (400 x 400 DPI)
Figure 8. H&E staining of the coronally sectioned condyles. Left & right condyles of the naïve control group (a1-a7 & a1'-a7'), resin molar group (b1-b7 & b1'-b7'), aluminum incisor group (c1-c7 & c1'-c7'), and stainless steel incisor group (d1-d7 & d1'-d7'). All right condyles were flipped horizontally. Scale bar: 500μm.

149x120mm (400 x 400 DPI)
Figure 9. Toluidine blue staining of the coronally sectioned condyles. Left & right condyles of the naïve control group (a1-a7 & a1'-a7'), resin molar group (b1-b7 & b1'-b7'), aluminum incisor group (c1-c7 & c1'-c7'), and stainless steel incisor group (d1-d7 & d1'-d7'). All right condyles were flipped horizontally. Scale bar: 500μm.

169x120mm (400 x 400 DPI)