

Effects of Occlusal Change on the Histology of the Mandibular Condyle Cartilage of Rats

by

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We have previous data demonstrating that occlusal change in the rabbit results in degeneration of temporomandibular joint (TMJ) condyle, the major moving bone of the TMJ (Henderson, 2015). In the present study, we sought to determine whether comparable changes are observed in a rat model of occlusal change. Composite bite-raising splints (around 1mm) were applied to the left maxillary molars in 14-week-old male Sprague-Dawley rats for 4 weeks in the first batch (n=4) and 14-week-old female Sprague-Dawley rats 6 weeks in the second batch (n=4). Sham splint placement, where the splint was placed and then removed, served as the sham group (n=4) in both batches. At the end of splinting, all the rats were euthanized and histological analysis of the TMJ was carried out on both splinted and contralateral sides of the jaw with Hematoxylin & Eosin (H&E) for cellularity, Safranin-O staining for glycosaminoglycan(GAG) and Collagen II immunohistochemistry (IHC) staining. The presence of fibrous zone, proliferation zone, mature zone, hypertrophic zone served as primary tissue boundaries on the condyles ipsi- and contralateral to the splint placement. There did not seem to be a difference between the ipsi- and the contralateral sides after 4 and 6 weeks in sham group. While in the splinted group, there seem to be an increase in GAG on the ipsi- compared to the contralateral side after 4 weeks of splinting. Interestingly, after 6 weeks, the difference seemed less consistent compared to 4 weeks. Meanwhile, there appeared to be no GAG in sham group after 6 weeks. The Collagen II staining showed no significant difference in both sham and splinted group after 4 and 6 weeks. It will be important to

further characterize the potential changes in other biochemical components of the joint tissue, time course of both the onset and recovery of the changes in the joint, and the extent to which the histological changes correlate with changes in joint sensitivity.

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1.0 INTRODUCTION

“The Temporomandibular joint (TMJ) is a synovial, bilateral, ginglymo-diarthrodial joint, and it is formed by the articulation of the condyle against the glenoid fossa and articular eminence of the temporal bone (Dolwick et al, 1983).” It is not only one of the most complicated, but also the most used joint in a human body, which consists of the joint capsule, articular disc, mandibular condyles, articular surface of the temporal bone as well as the muscles and ligaments around the joint (Alomar et al, 2017). The mandibular condyle cartilage (MCC) is the fibrocartilage covers the head of the condyle, which consists of fibrous, proliferative, mature, and hypertrophic and subchondral zones, providing the toleration to compressive force during joint movement.

Almost 33% of the population has at least one symptom of temporomandibular joint disorders (TMD), but only 3.6-7% of them seek treatment (Wright et al, 2013). Systematic illness, trauma, and mechanical factors can lead to the degeneration of the TMJ. The excessive or unbalanced mechanical loading may result in the onset and process of osteoarthritis and internal derangement of the TMJ (Tanaka et al, 2008). Furthermore, internal derangement of the TMJ may be induced by excessive or unbalanced stress in the TMJ.

According to Wilkes, internal derangement can be separated into five stages. From stage I to stage III, symptoms start from only painless clicking, and headache to frequent joint pain, and mild limited mandibular range motion. These stages show no difference from a healthy joint under X-ray or MRI except for the anterior disc displacement. However, degeneration is evident in the stage VI and V of TMD with deformed or torn disc, and abnormal bone defects of the condyle (Wilkes, 2013). Patients at these stages will suffer from more severe symptoms such as chronic pain and limited mandibular range motion. When the deformation of the disc starts, non-surgical

treatments might not be as effective as surgical treatments, which including disc repositioning, disc removal, and replacement of the joint with a prosthetic device (Hagandora et al, 2012).

To understand the process of TMD or joint degeneration, several animal models have been studied, which can be classified into chemical approaches and surgical/mechanical approaches. Complete Freund's adjuvant (CFA) is one of the chemical approaches used to induce acute inflammation in the TMJ, containing heat-killed *Mycobacterium tuberculosis* suspended in an oil (Almarza et al, 2011). Mustard oil, formalin and carrageenan are also used as an irritant for provoking pain and inflammation. The mechanical and surgical animal models include disc displacement, condylotomy, disc perforation, discectomy and mechanical perturbation (Almarza et al, 2011). Except for mechanical perturbation, the other models require surgical exposing of the joint cavity, which causes inflammation and introduces an extra variable than expected. Moreover, although chemical approaches and the other mechanical surgeries are sufficient enough to induce hypersensitivity, it does not replicate the slow and sequential development of TMD.

For perturbation, one study used the unilateral bite raise by using 1 mm dental composite in six- and nine-week-old Sprague-Dawley rats. After 4 weeks, histology analysis was conducted. The results showed that the expression of proteoglycans increased in ipsilateral side while the treatment-reversal and the sham-operated sham group showed weaker staining intensity, indicating that the increase of the compressive forces in the TMJ (Mao et al, 1998). Another bite raise model was the incisor bite plane in the 7-week-old male Wistar rats. According to the study, the bite raise was prefabricated by the dental composite that covered the upper incisors of the rats with 2 mm interocclusal space between the molars. The biochemical assays, PCR and western blot analysis indicated that protein accumulation and proteoglycan mRNA expression were influenced by the change of mechanical loading in the TMJ disc (Nakao et al, 2014). In another anterior splint study,

6-week-old female Sprague–Dawley rats were splinted for 2, 4, 8,12 and 20 weeks. At each time point, safranin O staining was conducted, which showed decreasing proteoglycan contents in mandibular condyle cartilage as early as 2 weeks after splint placement (Zhang et al, 2016) (Figure 1.1).

As one of the glycosylated proteins, proteoglycans are molecules that consists of a protein core which covalently attached by glycosaminoglycans (GAG) side chains (Yanagishita, 1993). GAGs are linear, and highly anionic negatively charged polysaccharides that interact with water molecules resulted from the negative charge. The major biological function of proteoglycans is to provide hydration and swelling pressure to the tissue, enabling it to withstand dynamic compressive forces (Nakano and Scott, 1989; Yanagishita, 1993; Mao, 1998). Aggrecan is a large aggregating proteoglycan that presents along the hypertrophic layer of the MCC (Teramoto et al, 2003). In the rabbit (Henderson et al, 2015), previous data showed that after being splinted for four weeks, the content of GAG decreased in the contralateral splinted condyles compared to the native sham group (Figure 1.2).

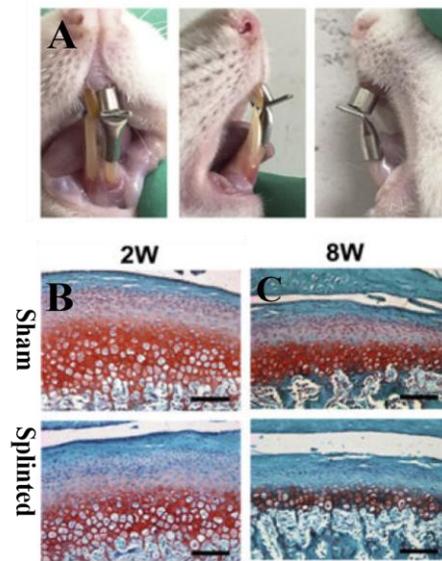


Figure 1 A. Frontal and lateral view of anterior splint B. Histology of rat TMJ showing the condyle cartilage at 10X magnification. (Scale bar=100 μ m) (Modified from Zhang et al, 2016).

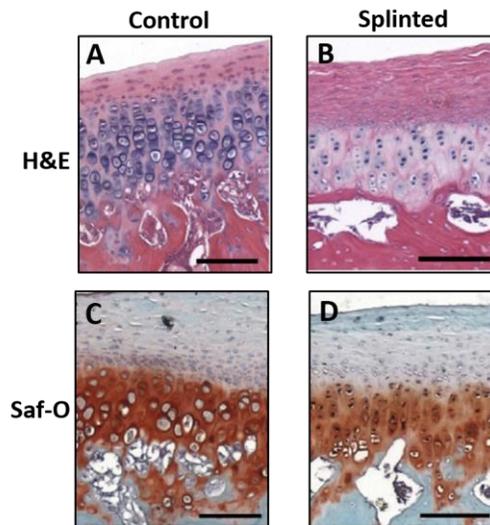


Figure 2. Histology of rabbit TMJ showing the condyle cartilage at 10X magnification. A.C. H&E and Safranin-O staining for the sham group; B.D. H&E staining and Safranin-O staining for the splinted group. (Scale bar=100 μ m) (Modified from Henderson et al, 2015)

In the present study, our objective is to determine whether comparable changes are observed in an older rat model of occlusal change. We applied the composite bite-raising splints (around 1mm) on the left maxillary molars in 14-week-old male and female Sprague-Dawley rats for 4 and 6 weeks in the splinted group (n=4). After 4 and 6 weeks, all the rats were euthanized and histological analysis of the TMJ was carried out on both splinted and contralateral sides of the jaw. Hematoxylin & Eosin (H&E) was conducted to identify the cell arrangement in mandibular condyle cartilage (MCC) and shape of the mandibular condyle. GAG can be identified by Safranin-O staining. The other biochemical content, Collagen II, will be detected by IHC staining.

2.0 HYPOTHESIS

The GAG layer will be larger in splinted rats in the ipsilateral side of the condyle when compare to the sham group because of the increasing mechanical load caused by the change of occlusion.

3.0 MATERIALS AND METHODS

3.1 UNILATERAL BITE RAISE

In the first batch, 14-week-old male Sprague-Dawley rats were anesthetized for rat cocktail consisting of acepromazine (1.1mg/kg), ketamine (5.5mg/kg) and xylazine (5.5mg/kg). Isoflurane (1.5-2%) was delivered via nose before the injection of the cocktail to induce muscle relaxation. The operating table restricted the mobility of the rat with rubber bands (Figure 3.1). The cotton applicator was used to clean and dry the surface of the maxillary molar of the rats before the application of etching agent (Accu-Spense Acid Etch, Phosphoric Acid 37%). 15 seconds later, the target molars were washed and dried. The adhesive agent (3M ESPE) was used later following by the placement of the composite (Beautiful Flow Plus), which was shaped by the dental instrument and cured with a LED curing light (X-lite). All the sham rats have received the same surgeries as the splinted group, except that the composite was taken off in the last step.

Each rat in the splinted group was inspected 7 days after the surgeries to ensure the appliance retention. After 7 days, the splinted rats were inspected once a week. The same surgeries were conducted when the appliance fell off from the maxillary molar in rat 3 and 4 after the splint placement.

In the second batch, 14-week-old female Sprague-Dawley rats were treated 3 months later following the same protocol, except that the splinting period was 6 weeks.

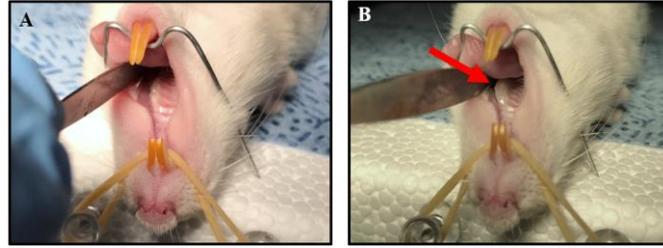


Figure 3. Unilateral bite raise of the rats. A. Splinting operation showing the maxillary molars of the rat. B. Red arrow points to the composite.

3.2 SAMPLE PROCESSING

After 4 and 6 weeks, the rats were euthanized in CO₂ chamber. A 90-degree angle incision was made along the mandibular ramus and the zygomatic bone. The mandibular fossa, TMJ disc and condyle were preserved by careful dissection. Immunocal (Fisher Scientific, 141432) was used to demineralize the tissue for 24 hours. After full decalcification, the samples were embedded in OCT compound (Fisher Scientific, E0114917) and placed in the cold room overnight. Then the samples were frozen in the -80-degree freezer. The sample blocks were sectioned sagittally by Crystat (Microm, HM 505 E) in 10 μ m. The slides were preserved in the fridge for further histology and immunohistochemistry.

3.3 HISTOLOGY

3.3.1 Hematoxylin & Eosin Staining

After fixation in Acetone (VWR Scientific, EM-AX0116) for 10 minutes, the samples were stained by Harris Hematoxylin (Thermo scientific, 6765001) for 30 seconds and Shandon Eosin-Y (Thermo scientific, 6766007) for 30 seconds. Then the samples were dehydrated through 95% alcohol, 2 changes of absolute alcohol, 5 minutes each, cleared in 2 changes of xylene (Azer scientific, ES609), 5 minutes each and mounted with mounting medium (Thermo Scientific, E0153844).

3.3.2 Safranin O staining

Samples were fixed in Acetone (VWR Scientific, EM-AX0116) for 10 minutes before staining. Then 2% fast green (Fisher Scientific, E0071267) and 1% acidic acid were used for 2 minutes and 10 seconds, followed by 0.5% safranin (Fisher Scientific, E0071267) for 5 minutes. Then the samples were dehydrated through 95% alcohol, 2 changes of absolute alcohol, 5 minutes each, cleared in 2 changes of xylene (Azer scientific, ES609), 5 minutes each and mounted with mounting medium (Thermo Scientific, E0153844).

3.4 IMMUNOHISTOCHEMISTRY

Frozen-sectioning samples were fixed in Acetone (VWR Scientific, EM-AX0116), washed by PBS buffer (10mM Na₃PO₄ 0.9% PBS, pH=7.5). Then the slides were incubated with 1% hydrogen peroxide (Fisher Scientific, M15829) in 50 ml methanol (Fisher Scientific, A4114) for 30 minutes. After rinsing, the horse serum (Vector Laboratories, PK-4002) was used to block non-specific binding. Primary antibody (1: 1000, Mouse anti-Collagen II, Fisher Scientific, 0863171) was used for 30 minutes. Then the positive slides were incubated with the secondary Horse anti-Mouse antibody (1:400, Vector Laboratories, PK-4002) for 30 minutes. The slides were incubated with ABC reagent (Vector Laboratories, PK-4002) for 30 minutes, and then the slides were stained with DAB substrate (Vector Laboratories, SK-4100) for 2 minutes and washed with tap water. The samples were dehydrated through 95% alcohol, two changes of absolute alcohol, 5 minutes each, cleared in 2 changes of xylene (Azer scientific, ES609), 5 minutes each and mounted with mounting medium (Thermo Scientific, E0153844). The negative control was exposed to all the steps without the primary antibody.

3.5 IMAGES PROCESSING

Image J (NIH image) was used to analyzed the histology and immunohistochemistry. GAG and collagen type II intensity was qualified by the percentage of positive staining area divided by the MCC area between ipsi- and contralateral sides as well as sham and splinted group. The thickness of the MCC was measured at anterior, median and posterior portion.

3.6 STATISTIC ANALYSIS

15 samples were analyzed (n1=8; n2=7) by student t-test for comparison between ipsi- and contralateral sides in batch 3 and 4 respectively. The differences were identified by the same test between sham and splinted groups. Statistically significant is $p < 0.05$.

4.0 RESULTS

4.1 14-WEEK-OLD MALE RATS SPLINTED FOR 4 WEEKS

4.1.1 H&E Staining

In Hematoxylin & Eosin staining, the connective tissue was stained in pink, and the nucleus was in purple (Figure 4.1). The MCC consists of fibrous, proliferative, mature, and hypertrophic and subchondral zones from superior to inferior. It can be divided into anterior, median and posterior portion. In H&E staining the five layers were observed on both ipsi- and contralateral side of the MCC with a continuous and complete fibrous layer. In image analysis, there were no statistically differences in the thickness of the MCC between the anterior, middle and posterior portions between sham and splinted group (Figure 4.2) ($p>0.05$).

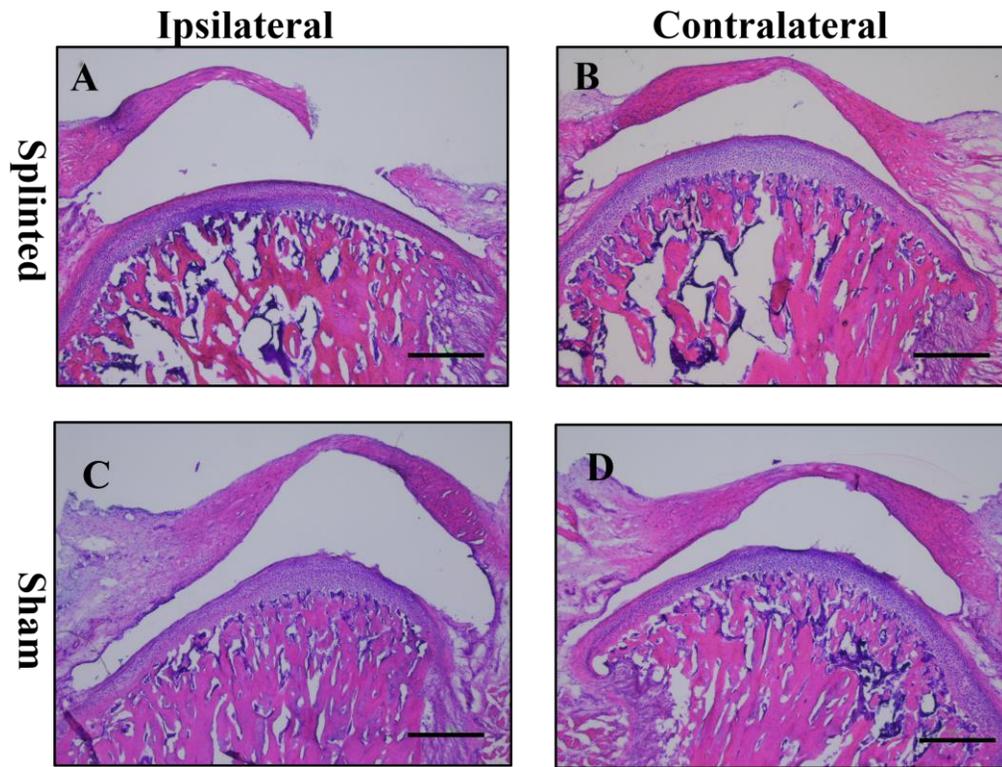


Figure 4. Histology showing the ipsi- and contralateral of TMJ discs and condyles in H&E staining of the splinted group at 4X magnification (scale bars=500 μm). No histological difference in the cell arrangement of MCC was observed between ipsi- and contralateral side (A-D).

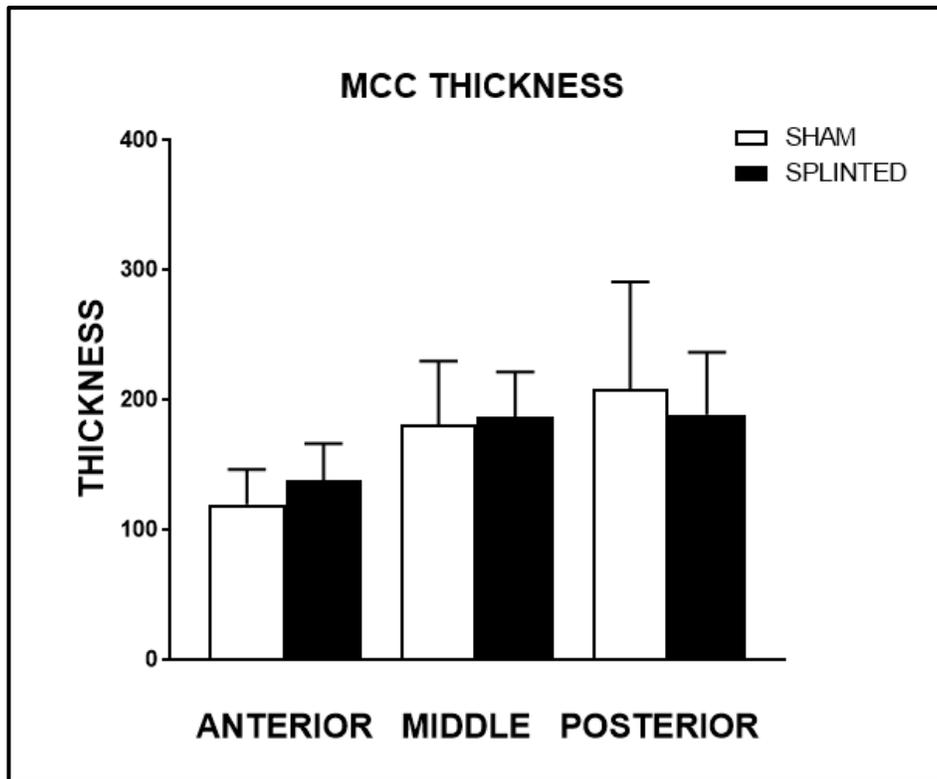


Figure 5. Comparison between MCC in sham and splinted group. No significant differences between the anterior, middle and posterior portions of the MCC between sham and spl

4.1.2 Safranin O Staining

GAG, the highly anionic side chains of proteoglycans that present on cell surfaces in the extracellular matrix, was stained in red to pink in Safranin O staining while the connective tissue was stained in green (Gandhi, 2008). In the splinted group, there was an increase in safranin-O staining along the mature and hypertrophic layer on the ipsi- compared to the contralateral side, especially on the posterior band of the MCC for most rats (Figure 4.3, A, B). Although GAG was stained faintly in the two rats in the sham group (Figure 4.3, C, D), these did not appear to have a difference in GAG distribution between sides. The other two rats did not stain for GAG (Figure 4.3, E, F). Part of the samples did not have TMJ disc because histology artifacts. In image analysis, splinted group had more GAG than sham group in ipsilateral side (Figure 4.4. A) ($p < 0.05$). The ipsilateral side had more GAG compared to contralateral side in splinted group ($p < 0.05$). In the sham group, there was no difference between the two sides (Figure 4.4. B) ($p > 0.05$).

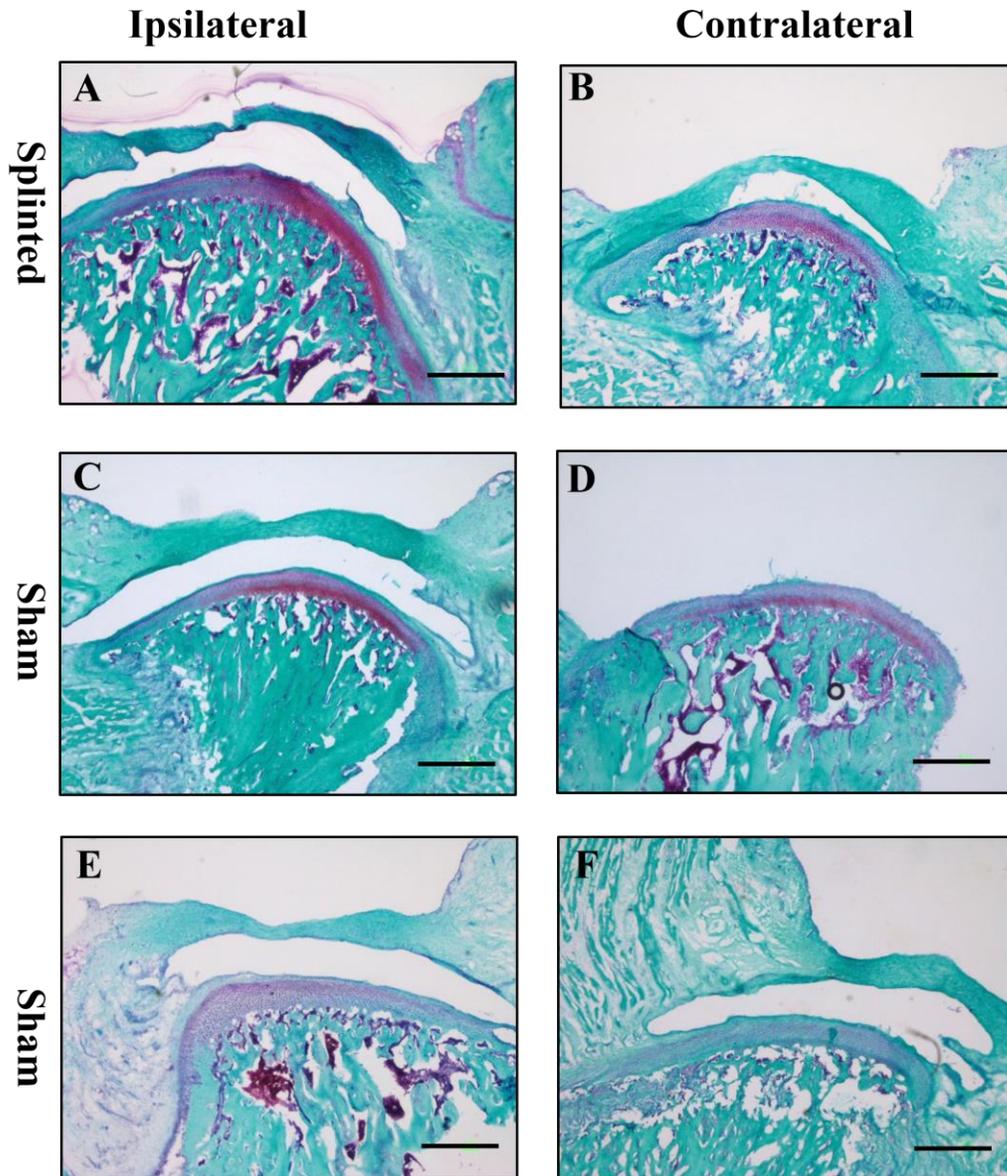


Figure 6. Histology showing the ipsi- and contralateral of TMJ discs and condyles in Safranin O staining of the splinted group at 4X magnification (scale bars=500 μ m). In the splinted group, there was an increase in safranin-O staining along the mature and hypertrophic layer on the ipsilateral side (A, C, E, G) compared to the contralateral side (B, D, F, H).

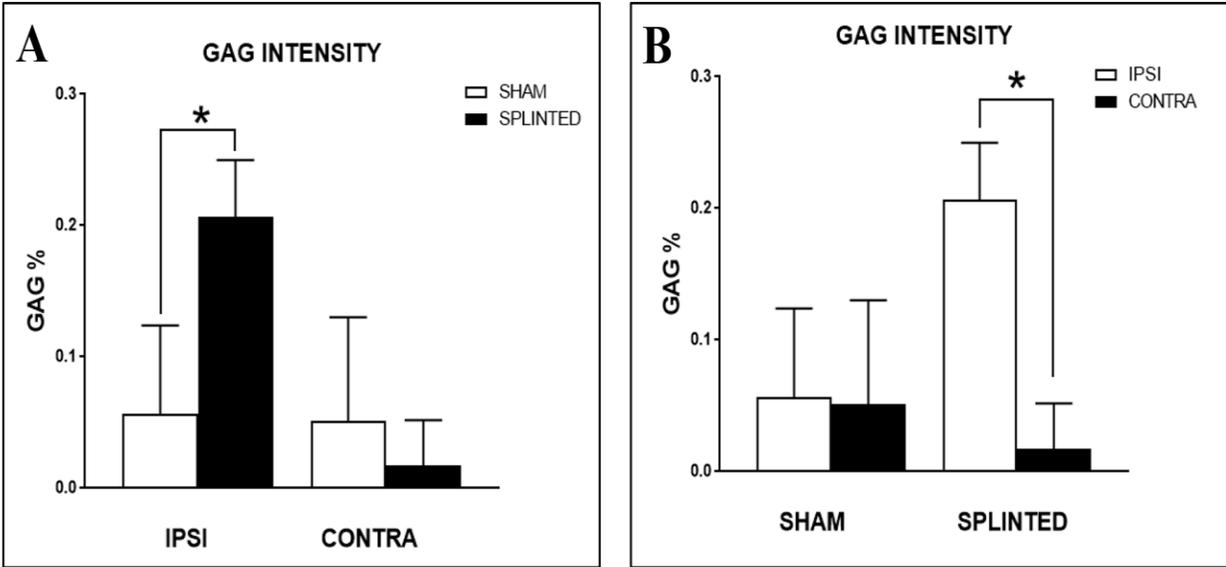


Figure 7. Comparison of GAG staining between sham and splinted, ipsi and contralateral sides. A. Splinted group had more GAG than sham group in ipsilateral side. B. The ipsilateral side had more GAG compared to contralateral in splinted group. In the sham group, there was no difference between the two sides. ($p > 0.05$)

4.1.3 Collagen II Staining

Collagen I and Collagen II are the primary biochemical contents of the articulate cartilage (Figure 4.5). While Collagen I is predominant in the fibrous zone, Collagen II is mainly expressed in the mature and hypertrophic zone (Mizoguchi, 1996). According to the IHC staining, Collagen II was stained in brown along the mature and hypertrophic layer of the MCC in the ipsi- and contra-lateral side of the sham and splinted group (Figure 4.6. A-D). The image analysis indicated that there was no difference between the ipsi- and contralateral sides, and sham and splinted groups (Figure 4.7) ($p>0.05$). There was no non-specific staining according to the histology of the negative sham ((Figure 4.6. E, F).

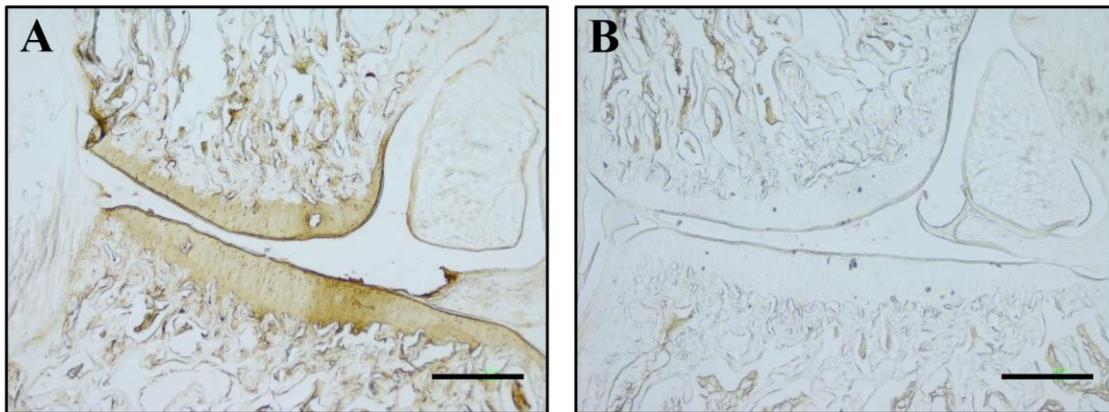


Figure 8. Histology showing Collagen type II IHC staining of rat's knee at 4X magnification (scale bars=500 μm). A. Collagen II was stained in brown along the articulate cartilage of femur and tibia. B. There was no non-specific staining in negative sham.

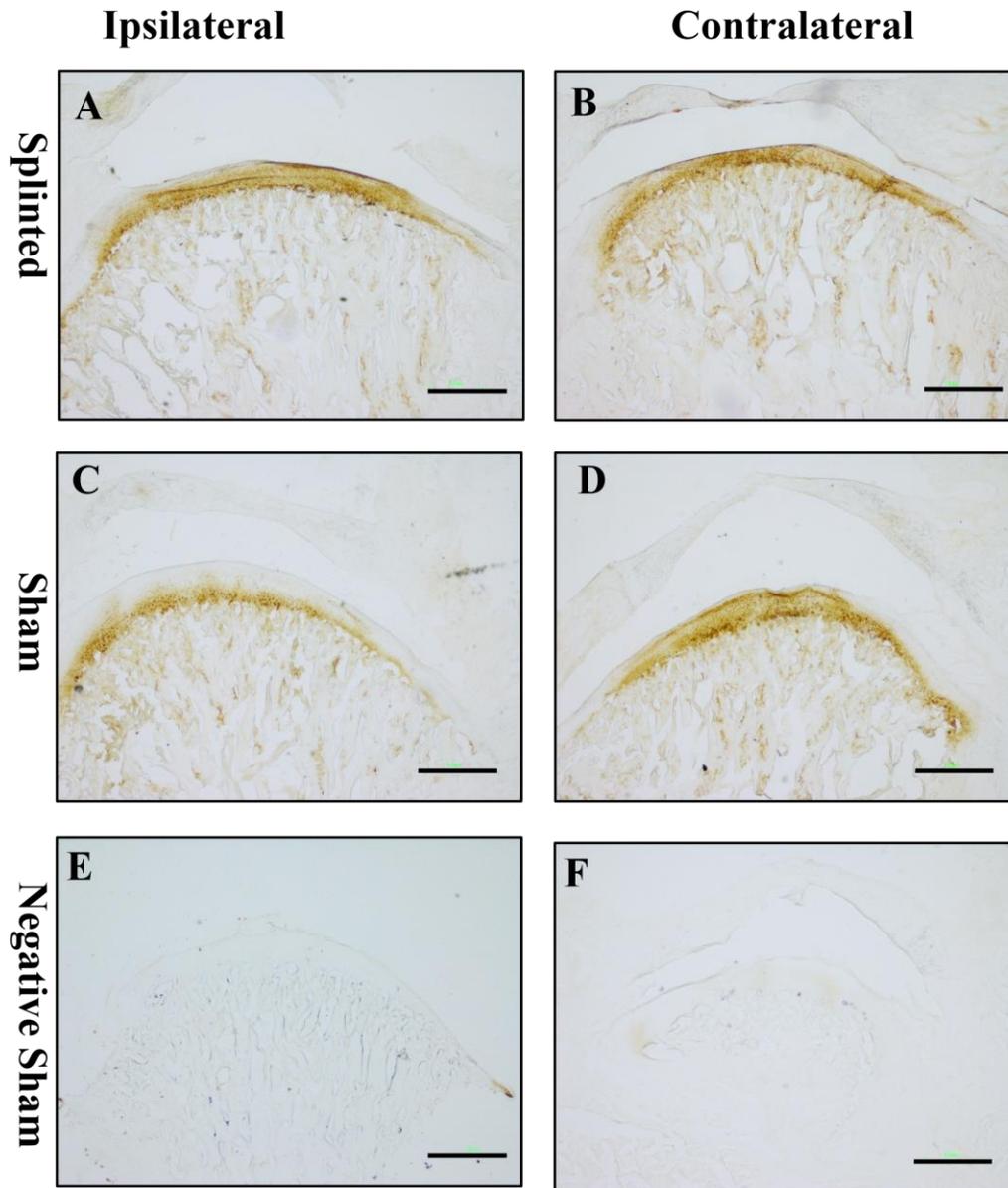


Figure 9. Histology showing the ipsi- and contralateral of TMJ discs and condyles in Collagen type II IHC staining of the splinted group at 4X magnification (scale bars=500 μ m). Collagen II was stained in brown along the mature and hypertrophic layer of the MCC in the ipsi- and contra-lateral side of the splinted and control group (A-D). No difference was observed between the two sides. No non-specific staining can be observed (E, F).

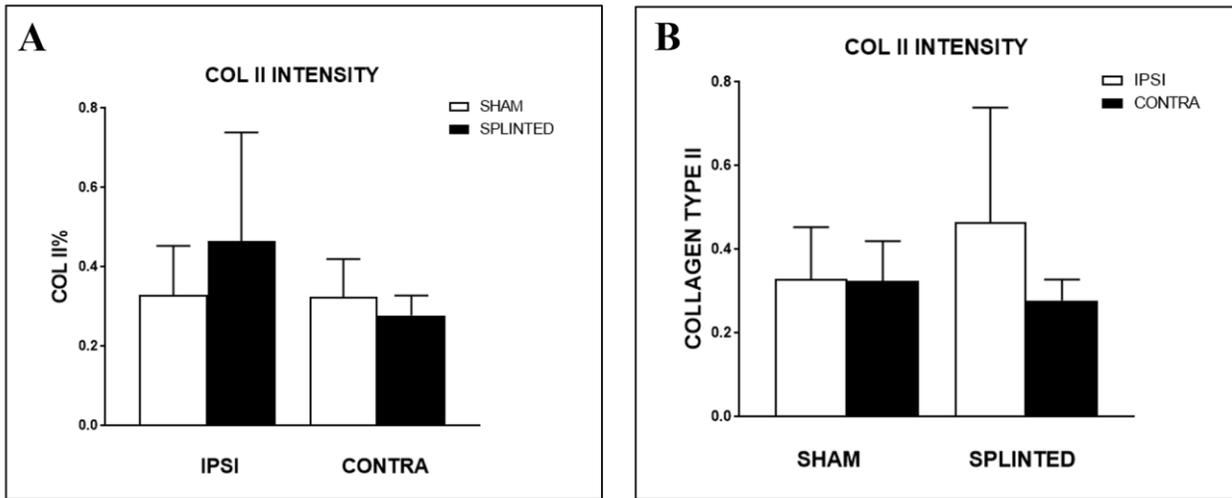


Figure 10. Comparison between ipsi- and contralateral side, and sham and splinted group in Collagen type II staining. A. No difference in collagen type II staining between ipsi- and contralateral sides; B. No difference in collagen type II staining between sham and splinted groups. ($p > 0.05$)

4.2 14-WEEKS-OLD FEMALE RATS SPLINTED FOR 6 WEEKS

4.2.1 H&E Staining

There seemed to be no histological difference in the cell arrangement, and the thickness of MCC between the two sides in both groups (Figure 4.8). One rat in the splinted group died after anesthesia was injected. The image analysis showed that there were no significant differences in MCC thickness between the anterior, middle and posterior portions of the MCC between sham and splinted group (Figure. 4.9) ($p>0.05$).

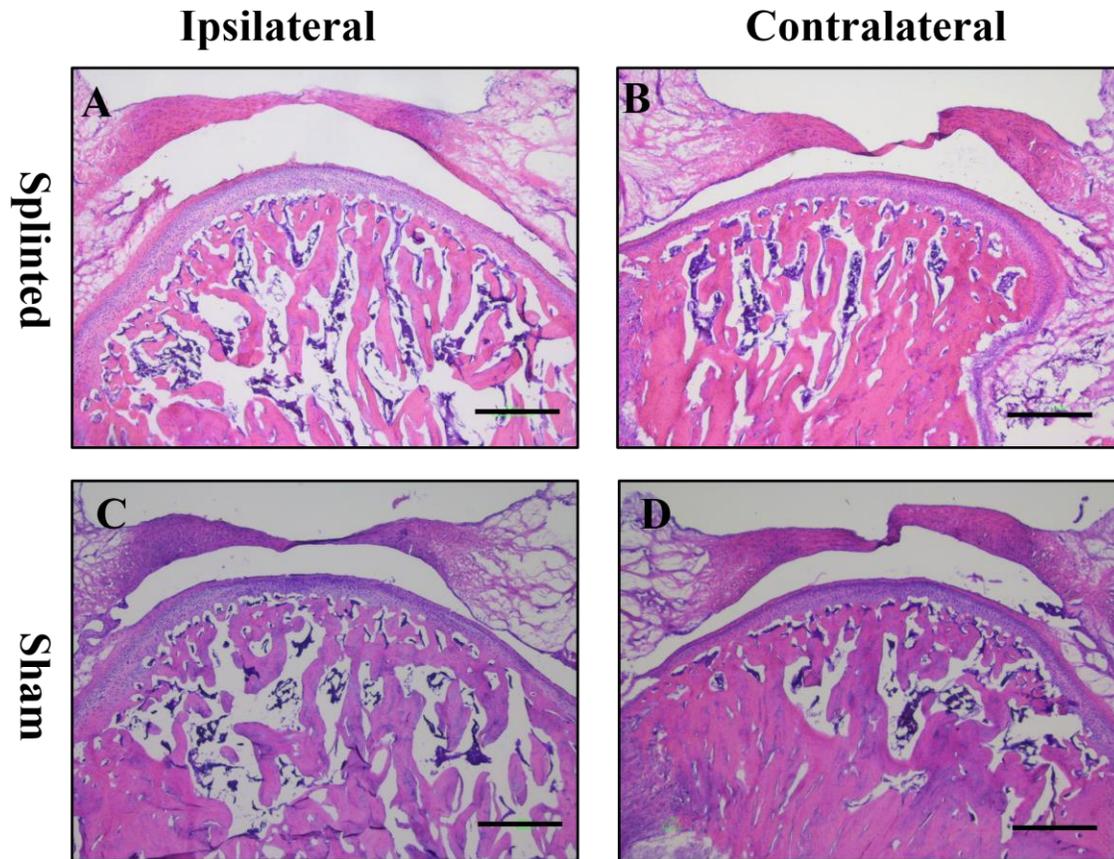


Figure 11. Histology showing the ipsi- and contralateral of TMJ discs and condyles in H&E staining of the splinted group at 4X magnification (scale bars=500 μ m). No histological difference in the cell arrangement of MCC was observed between ipsi- and contralateral side, sham and splinted group (A-D).

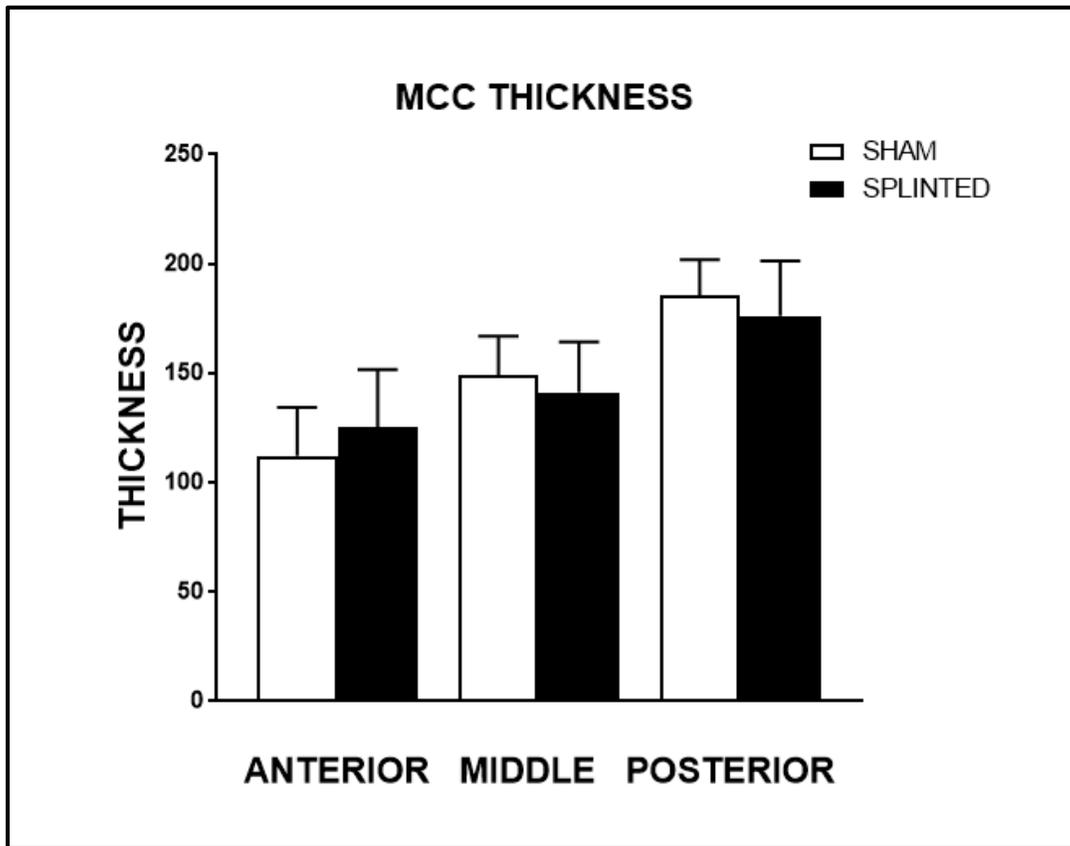


Figure 12. Comparison between MCC thickness in sham and splinted group. No significant differences between the anterior, middle and posterior portions of the MCC between sham and splinted group.

4.2.2 Safranin O Staining

In the splinted group, GAG was stained along the mature and hypertrophic layer of the MCC in the ipsi- and contralateral side of the first rat (Figure 4.10. A, B). Staining was only observed in ipsilateral side of the second rat (Figure 4.10. C, D). In the third rat, the staining appeared on the opposite side of the condyle (Figure 4.10. E, F). In the sham group, no staining was detected on both the ipsi- and contralateral side of the condyle (Figure 4.10. G-H). The image analysis indicated that there was no difference of GAG was found between ipsi- and contralateral side. Overall, splinted group had more GAG than sham group in both ipsi and contralateral side (Figure 4.11) ($p>0.05$).

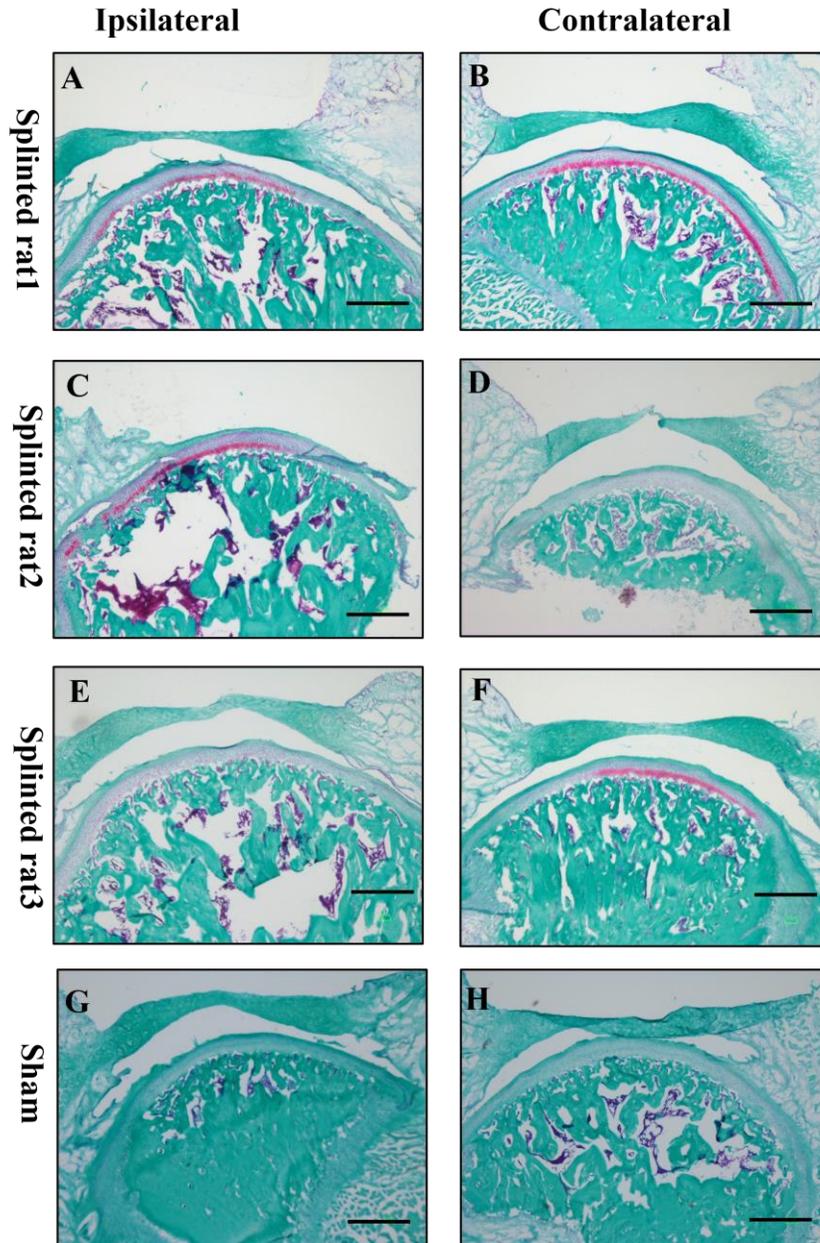


Figure 13. Histology showing the ipsi- and contralateral of TMJ discs and condyles in H&E staining of the sham and splinted group at 4X magnification (scale bars=500 μm). GAG was stained on both sides of the first rat (A, B). GAG was detected only in ipsilateral side of the second rat (C, D). The staining appeared on the opposite side of the condyle (E, F). No GAG was stained on Sham group.

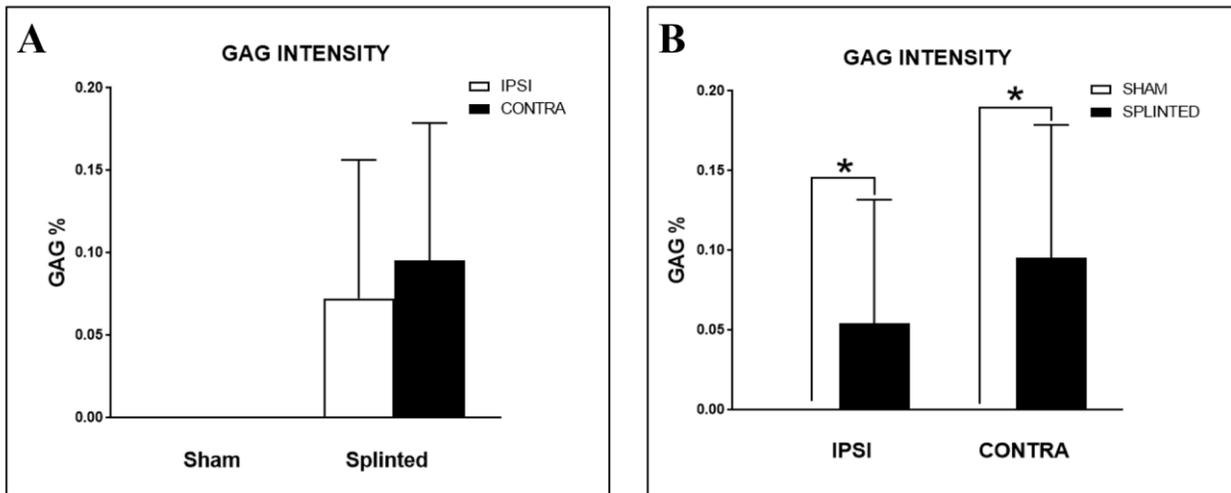


Figure 14. Comparison between ipsi- and contralateral side, and sham and splinted group in GAG staining. A. No difference of GAG was found between ipsi- and contralateral side ($p > 0.05$); **B.** Splinted group had more GAG than sham group in both ipsi and contralateral side ($p < 0.05$).

4.2.3 Collagen II Staining

Both the splinted and sham group showed Collagen II stained along the hypertrophic layer of the MCC (Figure 4.12. A-D). No histological difference was identified between the ipsi- and contralateral sides of the condyle in both groups. There was no non-specific staining according to the histology of the negative sham group (Figure 4.12. E, F). In image analysis, there was no difference between ipsi- and contralateral side as well as sham and splinted groups (Figure 4.13) ($p>0.05$).

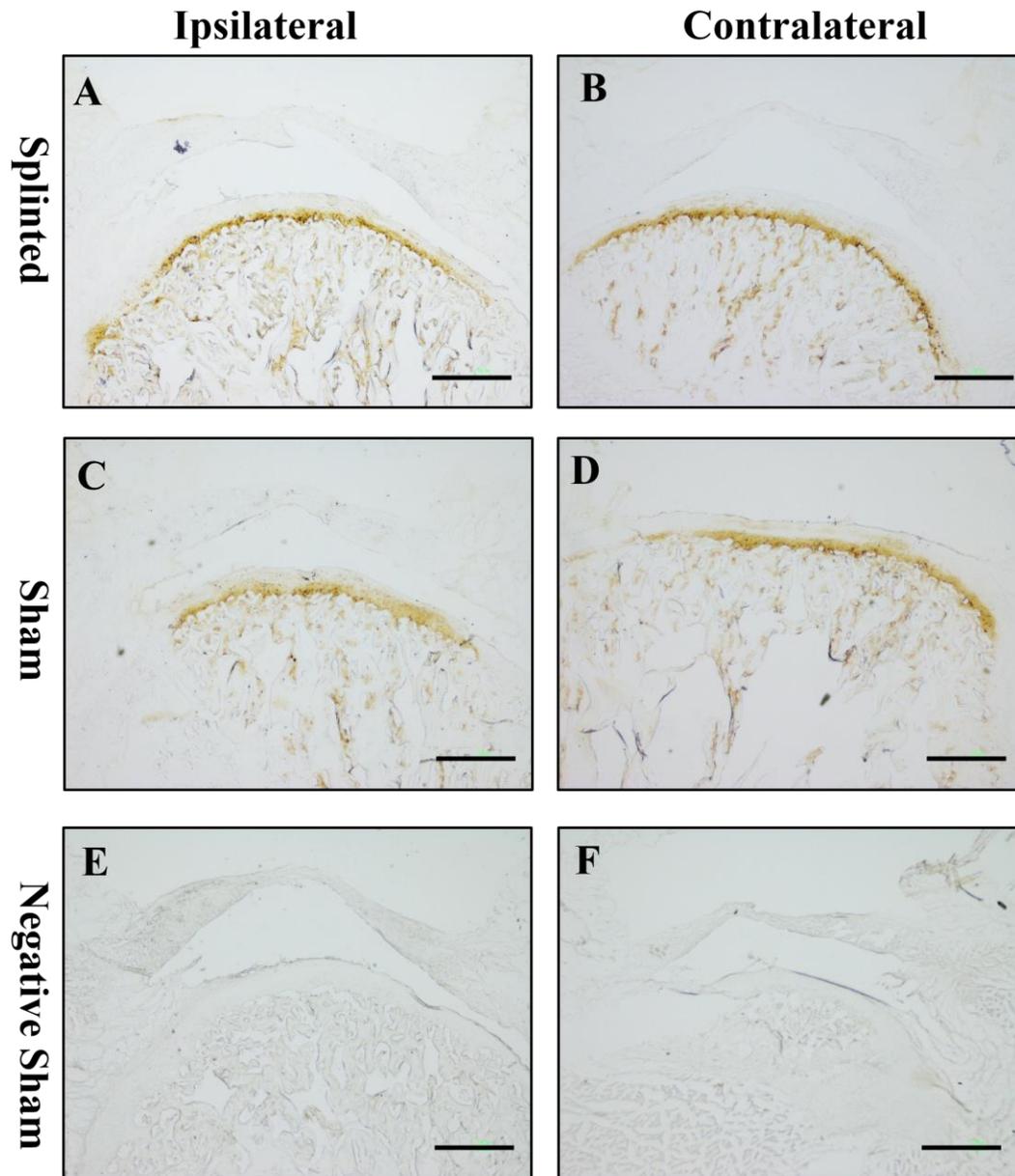


Figure 15. Histology showing the ipsi- and contralateral of TMJ discs and condyles in Collagen type II IHC staining of the splinted group at 4X magnification (scale bars=500 μ m). Collagen II stained along the hypertrophic layer of the MCC in splinted and sham group (A-D). No non-specific staining in negative sham group (E, F)

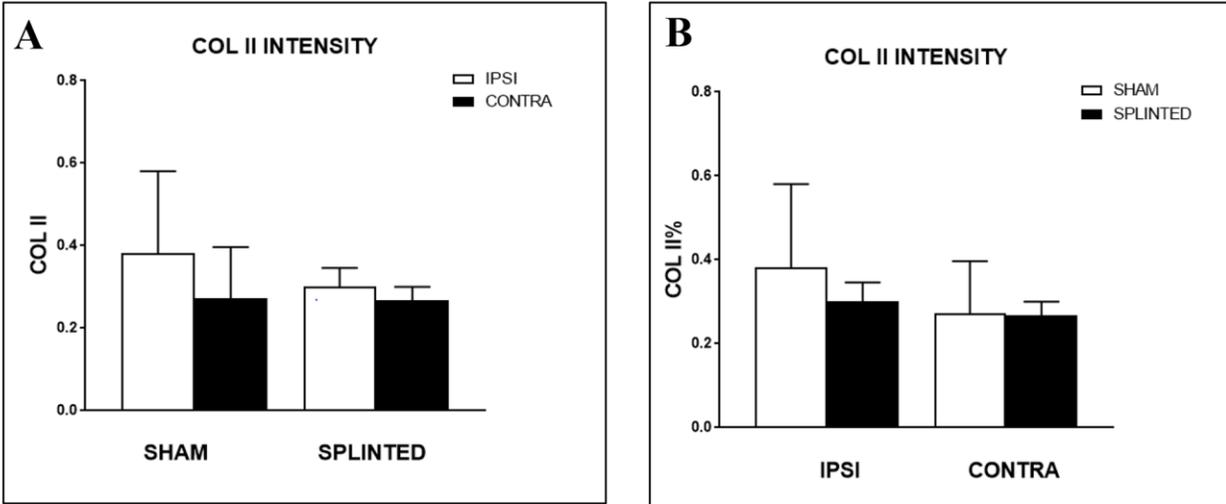


Figure 16. Comparison between ipsi- and contralateral side, and sham and splinted group in Collagen type II staining. A. No difference in collagen type II staining between ipsi- and contralateral side; B. No difference in collagen type II staining between sham and splinted group. ($p > 0.05$)

5.0 DISCUSSION

5.1 ANIMAL MODEL

Compared to other animal models of degenerative joint disease, splint models had the advantage over the others for the fact that the joint capsule was not penetrated (Henderson, 2015). Incision and mastication are the two main jaw movement of rodents (Nakao, 2015). When the rats obtained food, the splinted rats could only masticate through the ipsilateral side while the maxillary teeth of the contra-lateral side could not form the normal occlusion with the mandibular teeth. Under this circumstance, the mechanical load that MCC received in mastication increased in the ipsilateral side and decreased in the contra-lateral side. Age could be another factor that influenced the distribution of GAG. In Mao's study, the unilateral bite raise was manifested used in in six- and nine-week-old Sprague-Dawley rats (Mao et al, 1998). After 4 weeks of splinting, GAG distribution was different between the two age groups. It may indicate that the adaptability to mechanical loading decreased with age. In the present study, older Sprague-Dawley rats were used, which may eliminate the variable that results from exponential growth in the early 10 weeks.

Occlusal splint is designed through detailed study model using articulator for people with TMJ pain and malocclusion (Crout, 2016). Different from unilateral molar splint, it is usually therapeutic, and bilateral, and has a coverage of all the teeth to adjust the occlusion. In comparison, unilateral molar splint changes the occlusion extremely, which can induce dysfunction of the jaw in short period.

5.2 HISTOLOGY AND IMMUNOHISTOCHEMISTRY

The MCC consists of fibrous, proliferative, mature, and hypertrophic and subchondral zones from superior to inferior. According to Henderson et al., after 6 weeks of splinting in the rabbit, the subchondral layer of the fibrocartilage showed loss of GAG (Henderson, 2015). However, Mao et al. found that the unilateral bite in rats increased the thickness of the MCC and GAG after 4 weeks (Mao et al, 1998). In this study, the result of H&E staining in the present study revealed that both 4 and 6 weeks of splinting did not induce cell disarrangement, loss of fibrous layers, and increase thickness in ipsi- and contralateral side of the condyle. Interestingly, there appeared to be an increase in Glycosaminoglycan (GAG) content with splinting that was greater ipsilateral to the splint placement after 4 weeks in the male rats. The distribution of GAG was mostly in the posterior band which is regarded as the major pressure-bearing band, revealing the biochemical content change as well as the beginning of MCC degeneration (Mizoguchi, 1996). In comparison, the safranin O staining after 6 weeks in male rats did not show the same trend between ipsi- and contra-lateral sides. It might indicate that the degeneration is dependent on gender. Meanwhile, it is possible that the distribution of the mechanical force changed resulting from the adaption. In the rabbit, the GAG only appeared on the anterior band of the MCC instead of the posterior band in the rabbit, which was different from GAG staining in the splinted rat, which might indicate that the rabbit adapted the bite raise differently (Henderson et al, 2015).

In the sham group, GAG was expected to express along mature and hypertrophic layer. One of the reasons for the absence of GAG is that it might decrease with the growth of the rats, which is consistent with the results from Mao and his colleagues (Mao et al, 1998). It is also possible that the sham surgeries conducted in the sham group caused the decrease of GAG. It could also dissolve during processing and frozen sectioning.

Collagen II is predominate in the mature and hypertrophic layer of the MCC. According to IHC staining, after 4 weeks and 6 weeks of splinting, no histological difference can be recognized, which indicates that the occlusal change did not influence the concentration and location of collagen II. According to Henderson et al, collagen type II decreased in rabbit with the unilateral splint. The difference suggested that changing occlusion influenced collagen type II between those two species in various ways.

5.3 LIMITATIONS AND FUTURE DIRECTIONS

Although there was an increase in Glycosaminoglycan (GAG) content with splinting that was greater ipsilateral to the splint placement in male rats after 4 weeks of splinting, only 4 rats were splinted. In the second batch, the safranin O staining was less consistent and predictable in only 3 female rats. More experimental animals in the future study will increase the statistical power.

We are in the process of establishing the splint model in the rats. As such, we are trying to determine an effective splint duration to observe degeneration changes in the condyle, and also determine if gender has an impact. In the first batch, the splinting surgery was conducted in female rats for 4 weeks while the second batch was performed in male for six weeks. Therefore, conclusions were made within the same batch. It will be inconclusive to compare batch 1 and batch 2 for the reason that there were 2 variables, which were gender and splinting time.

In spite of the fact that there was no histological difference in collagen type II, it is not accurate to conclude that the collagen type II was expressed equally on the MCC based on

observation. Therefore, a quantitative method should be considered to compare the biochemical contents between groups.

Considering the fact that GAG might dissolve during processing, it is necessary to test other biochemical contents such as Aggrecan to confirm the potential changes. Versican is one of the proteoglycans that mainly located on the fibrous layer and the TMJ disc (Roth, 1997). In our study, we only investigated the biochemical content that expressed in the mature and hypertrophic layer but not in the other layers, where degenerative change could happen.

In the first batch, there was no GAG staining in the 14-week-old female rats sham group which only received the surgery but not splinted on the molars. It is possible that the absence of GAG was caused by the splinting surgery. Another sham group without receiving any surgeries should be added to exclude the possible damage of the surgery. A different age group will also be considered because the age of the rats could be a factor in GAG content.

Some gaps and tears were seen in the narrow space of the condyles, which was clearly histological artifact. In the future, the tissue could be infiltrated with sucrose first, to then allow full penetration of OCT compound so that smooth tissue will be expected in frozen sectioning.

Last but not least, it will be important to further characterize the time course of both the onset and recovery of the changes in the joint, and the extent to which the histological changes correlate with changes in joint sensitivity.

6.0 CONCLUSION

There was an increase in Glycosaminoglycan (GAG) content with splinting that was greater ipsilateral to the splint placement after 4 weeks in the male rats. In female rats which were 14-week-old and splinted for 6 weeks, the result was not consistent with the first batch. More GAG was detected in the splinted group compared to the sham in both batches. Overall, frozen sectioning was successful in obtaining high quality sections without wrinkles or rips. Furthermore, both histology and IHC staining were validated and worked well on the rat condyles. It will be important to further characterize the potential changes in other biochemical components of the joint tissue, for example, Aggrecan, and time course of both the onset and recovery of the changes in the joint, and the extent to which the histological changes correlate with changes in joint sensitivity.

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