Anti-fibrotic Effect of Exosomes on Human Temporomandibular Joint Tissues: A Pilot Study

2021 Grants

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FollowUp Form

Award Information

In an attempt to make things a little easier for the reviewer who will read this report, please consider these two questions before this is sent for review:

- *Is this an example of your very best work, in that it provides sufficient explanation and justification, and is something otherwise worthy of publication? (We do publish the Final Report on our website, so this does need to be complete and polished.)*
- *Does this Final Report provide the level of detail, etc. that you would expect, if you were the reviewer?*

Title of Project*

Anti-fibrotic Effect of Exosomes on Human Temporomandibular Joint Tissues: A Pilot Study

Award Type

Biomedical Research Award (BRA)

Period of AAOF Support

July 1, 2021 through June 30, 2023

Institution

University of Iowa

Names of principal advisor(s) / mentor(s), co-investigator(s) and consultant(s)

Dong Rim Seol, PhD Aaron Figueroa, DDS Xian Jin Xie, PhD

Amount of Funding

$29,998.00

Abstract

(add specific directions for each type here)

Respond to the following questions:

Detailed results and inferences:*

If the work has been published, please attach a pdf of manuscript below by clicking "Upload a file".
OR
Use the text box below to describe in detail the results of your study. The intent is to share the knowledge you have generated with the AAOF and orthodontic community specifically and other who may benefit from your study. Table, Figures, Statistical Analysis, and interpretation of results should also be attached by clicking "Upload a file".

Final Report _Kyungsup Shin.pdf

Were the original, specific aims of the proposal realized?*

Yes.

Were the results published?*

No

Have the results of this proposal been presented?*

Yes

To what extent have you used, or how do you intend to use, AAOF funding to further your career?*

1) My former AAOF funding (PFA and BRA) supported my preliminary research, which worked as the step stones for me to receive NIH/NIDCR grants (R03 and R01). The AAOF funding support was publicly appreciated on the website (https://dentistry.uiowa.edu/news/2022/08/nih-r01-grant-awarded-orthodontics-faculty).

2) With the support from my former AAOF BRA and current AAOF BRA (Anti-fibrotic Effect of Exosomes on Human Temporomandibular Joint Tissues: A Pilot Study), I, as the leading inventor, registered our discovery entitled "Preventative Therapy for Post-Traumatic Osteoarthritis" to the University of Iowa Research Foundation (UIRF) (https://uiowa.flintbox.com/technologies/a3f92e08-8d9f-4458-9153-070274015ab3, published on March 1, 2023). I, as the leading inventor, submitted this novel delivery system for treating TMJ disorders to a US patent (US 2022/0233598), which is currently pending. I attribute this opportunity of US patent submission to the AAOF funding opportunity and I truly appreciate that.

Accounting: Were there any leftover funds?

$0.00

Not Published

Are there plans to publish? If not, why not?*

We are currently working on a manuscript to publish the outcomes of this study in a peer-reviewed journal.

Presented

Please list titles, author or co-authors of these presentation/s, year and locations:*

1. Ino Song, Dong Rim Seol, Martin A James, Kyungsup Shin, "Therapeutic potentials of mesenchymal stem cell-derived exosomes for fibrocartilage repair," Poster presentation, Iowa Section of the AADOCR Research Day, February 15, 2022

2. Kyungsup Shin, Ino Song, Dong Rim Seol, Martin A James, "Therapeutic potentials of mesenchymal stem cell-derived exosomes for fibrocartilage repair," Interactive talk, 2022 AADOCR/CADR 51st Annual Meeting, Atlanta, March 21-26, 2022

3. Ino Song, James Martin, Kyungsup Shin, "Therapeutic effects of the highly purified extracellular vesicles from stem cells for temporomandibular joint repair" Poster Presentation, 2022 ISEVxTech EV Technology and Methods Summit, Honolulu, Hawaii, November 16-18, 2022

Was AAOF support acknowledged?

If so, please describe:

Yes, all presentations included the AAOF support acknowledgement in the proceeding abstract, poster, and/or slides). Also, we will continue acknowledging the AAOF support in all upcoming publications in the future.

Internal Review

Reviewer Comments

Reviewer Status*

File Attachment Summary

Applicant File Uploads

- Final Report _Kyungsup Shin.pdf

1. Specific Aims (List original specific aims)

Aim 1. Isolate and characterize exosomes derived from bone marrow stromal cells (BMSC-Exos). Working hypothesis: Highly-purified exosomes derived from MSCs will have exosome characterization with cellular uptake in temporomandibular joint (TMJ) cells. Highly-purified human BMSC-Exos will be characterized for size distribution, zeta potential, morphology, and protein markers. Exosome uptake will be evaluated on human fibrochondrocytes, synoviocytes, and disc cells.

Aim 2. Characterize human fibrous ankylosis tissues and evaluate anti-fibrotic effects of exosomes. Working hypothesis: Myofibroblasts will show dominant expression of α-SMA in human fibrous ankylosis and exosomes will reduce myofibroblast activities. Fibrosis will be characterized at the tissue level by analyzing human TMJ tissues (fibrocartilage, disc, and synovium) obtained as surgical discards. Tissue architecture will be assessed using α-SMA and type I collagen immunohistochemical staining to identify myofibroblasts. Myofibroblasts isolated from the human ankylosis TMJ tissues will be treated with BMSC-Exos to evaluate the suppression of α-SMA, type I collagen expression, and contractile activities.

2. Results

Aim 1-1: Isolation of human BMSC-Exos

Brief description of isolation methods: Three batches of human BMSCs were purchased from PromoCell (Heidelberg, Germany, Catalog #: C-12974) (Table 1). When the cells were grown to 70-80% confluency, the culture medium was replaced with a fresh cell culture medium containing MEM-alpha with 10% exosome-depleted fetal bovine serum (FBS), 1% amphotericin B, and 1% penicillin/streptomycin (all from Gibco, NY). The conditioned medium (CM) was collected after 48 hours from 3rd-5th passaged cells, and BMSC-Exo in CM was isolated and purified by size-exclusion chromatography (SEC) and ultrafiltration (UF).

Isolation outcomes: Three of human BMSC which were identified by putative stem cell markers (positive for CD73, CD90, and CD105 / negative for CD14, CD34, CD45, CD19, and HLA-DR) and multi-differentiation potency (adipogenesis, osteogenesis, and chondrogenesis) showed a homogeneous fibroblastic morphology. BMSC-Exo was successfully purified by SEC with qEV10 column (IZON Science, Medford, MA), and according to the manufacturer's instruction and the results of bicinchoninic acid (BCA) assay, 1st – 4th fractions were collected as exosomal fractions, which contain less than 5 μg of total proteins per 1 ml of input CM into the SEC column (Figure 1A). SEC with ultrafiltration provided over 76 percent of recovery rate with desired concentration of the exosomes compared to SEC only (Figure 1B).

Table 1. The information of human BMSC donors.

	Age	Sex	Race	Tissue origin
H-BMSC #1	63	Male	Caucasian	Femoral head
H-BMSC #2	44	Female	Caucasian	Femoral head
H-BMSC #3	68	Male	Caucasian	Femoral head

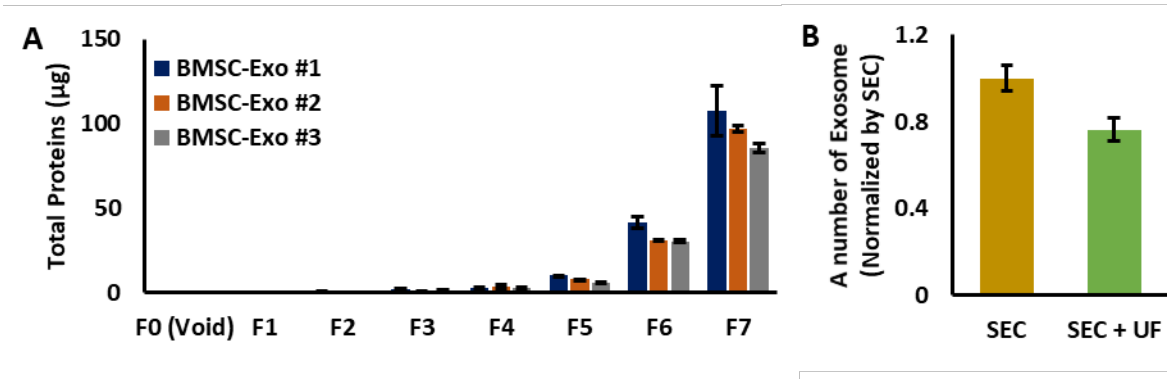

Figure 1. Isolation and Purification of BMSC-Exo. (A) Total amount of protein in each fraction per 1 ml of CM input (n = 3). (B) Exosome yield of size exclusion chromatography (SEC) with or without concentration step by ultrafiltration (UF). The result was normalized by SEC only group (n = 3).

Aim 1-2: Characterization of human bone marrow stem cell derived exosomes (BMSC-Exos)

Brief description of characterization methods: Purified exosomes were characterized in terms of size (nCS1, Spectradyne, Torrance, CA), surface charge (zetasizer Nano ZS), morphology (transmission electron microscope: TEM), and exosome-specific protein markers (Exo-Check Exosome Antibody Array, System Biosciences, CA) as previously described by the International Society of Extracellular Vesicles (ISEV).

Characterization outcomes: The average size and zeta potential on the membrane of BMSC-Exo were 95.8 nm and -16.5 mV, respectively (Figure 2A and 2B). TEM image showed the cup-shape morphology of BMSC-Exo, and contaminant protein aggregates were barely observed indicating that SEC provided highly purified exosomes (Figure 2C). As shown in Figure 2D, BMSC-Exo showed a high signal of the putative exosome positive markers including CD63, ANXA5, TSG101, FLOT1, ICAM, and CD81, whereas no cellular contamination (GM130) was observed.

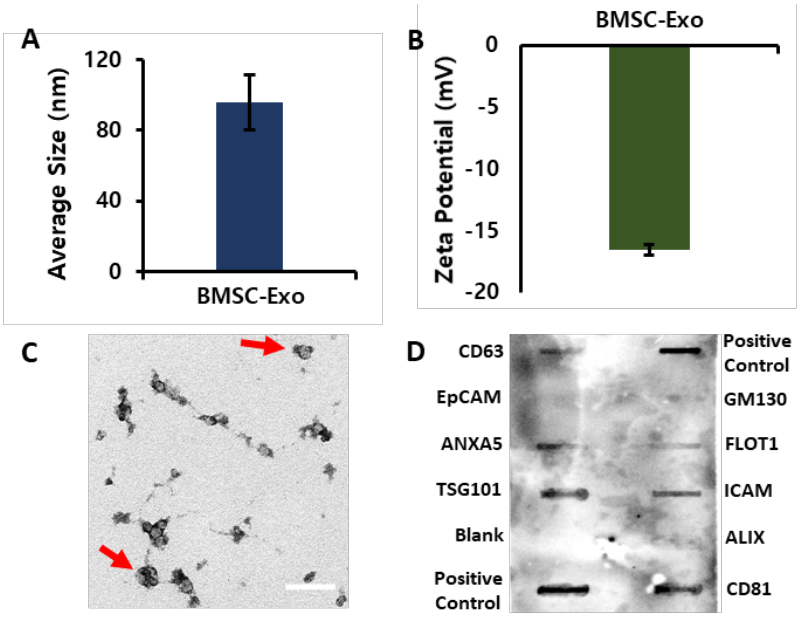

Figure 2. Characterization of BMSC-Exo. (A) Average size of BMSC-Exo measured by nCS1 (n = 3), (B) Average membrane potential of BMSC-Exo measured by Zetasizer (n = 3), (C) Morphology of BMSC-Exo (red arrows) in TEM (scale bar = 100 nm). (D) Immunoblotting of BMSC-Exo proteins for putative exosome positive markers (CD63, EpCAM, ANXA5, TSG101, FLOT-1, ICAM, ALIX, and CD81) and one negative marker (GM130).

Aim 1-3: Exosome uptake in cells in the TMJ

Brief description of exosome uptake assay methods: Due to the difficulties we experienced in purchasing freshly retrieved normal human TMJ tissues for cell isolation (from Anatomy Gift Registry: www.anatomygifts.org), we selected a human chondrocyte cell line (TC28a2, Millipore Sigma) and rabbit synoviocytes. The synoviocytes were harvested from the synovial membrane of the rabbit TMJ by the explant method. Cellular internalization of BMSC-Exo into the target cells was evaluated using a PKH67 Green Fluorescent Cell Linker Mini Kit (Sigma-Aldrich, MO). In brief, BMSC-Exo was mixed with PKH-67 staining reagent and diluent C. PBS was used as a negative control. Excessive PKH-67 staining reagent was removed by Exosome Spin Columns (MW 3000; Thermo Fisher Scientific). The cells were seeded in 6 well plates and incubated with PKH-67-labeled BMSC-Exo at 37 °C for 48 hours. For the counterstaining, Hoechst 33342 (Thermo Fisher Scientific) was used with 15 min incubation. Confocal images were taken using an Olympus FV1000 confocal microscope with a 20x water immersion objective.

Exosome uptake outcomes: To investigate the interplay between BMSC-Exo and their target cells, exosome uptake experiment was performed. After 48 hours of co-culture, PKH-67-labeled BMSC-Exo was internalized into both rabbit TMJ synoviocytes and human articular chondrocytes (Figure 3). In both cell batches, exosomes were observed in the cytoplasm surrounding the nuclei, while it was more widely spread in the synoviocytes due to the fibroblastic morphology compared to that of chondrocyte. No PKH-67 signal was observed in PBS

groups indicating all excess dye was clearly removed by Exosome Spin Columns.

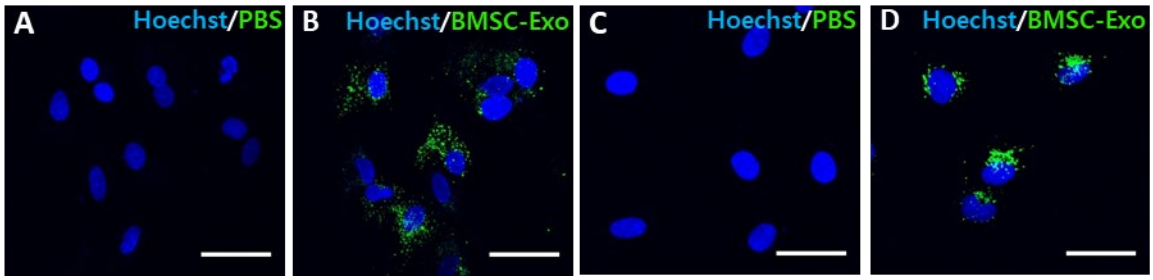

Figure 3. Internalization of BMSC-Exo into the target cells. (A and B) Rabbit TMJ synoviocytes and (C and D) Human articular chondrocytes cultured with PKH-67-labeled BMSC-Exo or PBS for 48 hours (blue: Hoechst 33342, green: PKH-67-labeled BMSC-Exo or PBS, scale bar = 50 nm).

Aim 2-1: Characterize human fibrous ankylosis tissues

Brief description of human sample characterization methods:

Following the IRB approved for human subject research (ID: #201906771: Kyungsup Shin), three de-identified patients (batch #1, #2, and #3), who underwent total TMJ replacement surgeries (#1 and 2) or fibrous ankylosis removal surgery (#3), were recruited through written informed consent (Table 2). Surgically discarded TMJ tissues (fibrocartilage, synovium, retrodiscal tissue, and disc) were tested (Figure 4). Prepared tissues were stained with Weigert's iron hematoxylin and eosin (H&E: Electron Microscopy Sciences, Hatfield, PA). Ankylosed joints were categorized into four groups. As a marker for myofibroblasts, α-SMA immunohistochemical (IHC) staining was performed using an automated staining instrument, Discovery ULTRA system (Roche Diagnostics, Indianapolis, IN). To quantify the amount of IHC positive expression, we followed a semi-quantification procedure using ImageJ Fiji software (Version 1.53c; NIH, Bethesda, MD) (Figure 5).

Table 2. List of human TMJ tissues

Batch	Date	Surgery	ID	Tissue	Histology	Cell isolation
#1	8/20/2020	TMJ total replacement	hTMJ-C-#1	Fibrocartilage	Available	N.A
			hTMJ-D-#1	Disc	Available	Available
			hTMJ-R-#1	Retrodiscal tissue	Available	Available
			hTMJ-S-#1	Synovium	N.A	N.A
#2	4/29/2021	TMJ total replacement	hTMJ-C-#2	Fibrocartilage	Available	N.A
			hTMJ-D-#2	Disc	Available	Available
			hTMJ-R-#2	Retrodiscal tissue	Available	N.A
			hTMJ-S-#2	Synovium	Available	N.A
#3	3/30/2023	Fibrous ankylosis removal	hTMJ-DR-#3	Disc + retrodiscal tissue	N.A	Available

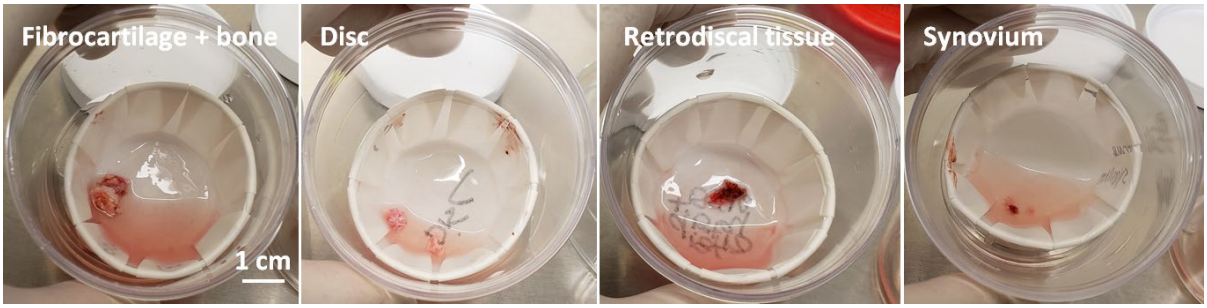

Figure 4. Collection of human TMJ tissues (batch #2).

Table 3. Ankylosis classification

Type I: non-bony ankylosis of the joint with almost-normal joint space	**Type II**: lateral bony ankylosis marked by a normal joint space that coexists with a radiolucent line
Type III: complete bony ankylosis of the joint characterized by only a radiolucent line	**Type IV**: extensive bony ankylosis without any radiolucent line

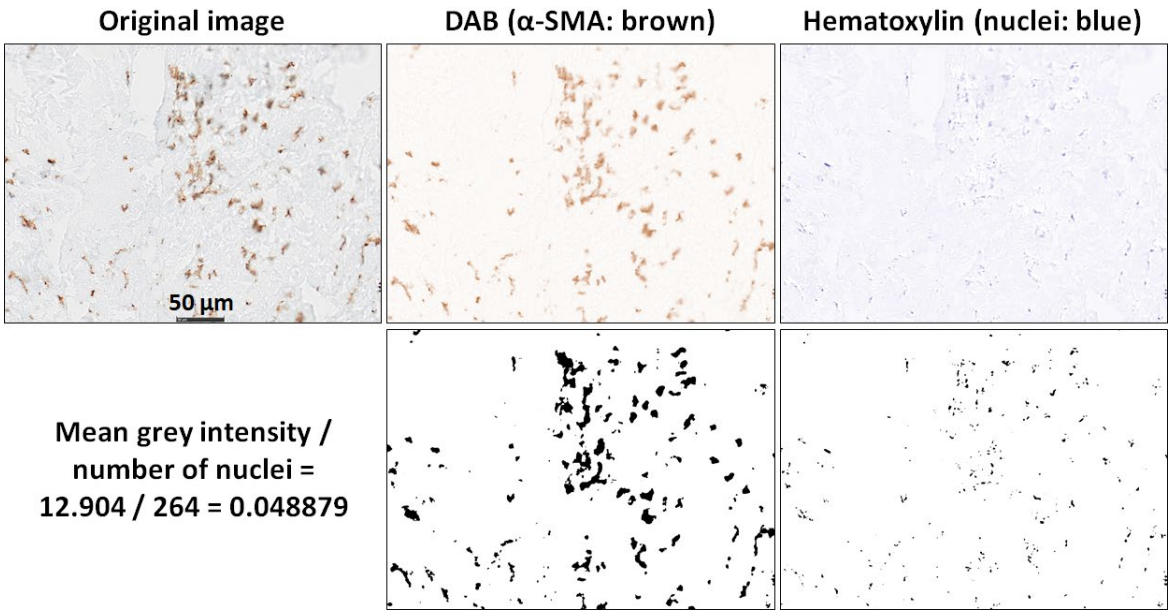

Figure 5. Semi-quantification procedure of α-SMA IHC staining. Maximal threshold: 200, minimal nuclei size: 10 pixels.

Characterization of human TMJ samples: Subintimal fibrosis was observed with heavy diffuse collagenous staining in loose connective tissue from the human TMJ synovium stained in H&E (batch #2) (Figure 6A and B). Inflammatory cells were highly infiltrated at 2 focal spots (Figure 6C).

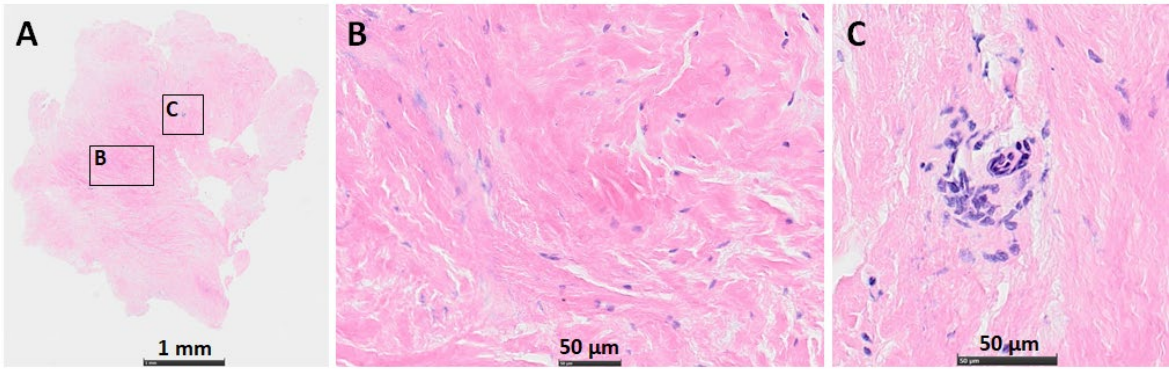

Figure 6. H&E staining of human TMJ synovium (batch #2). (A) Whole image of synovium, (B) Interstitial fibrosis, (C) Infiltrated inflammatory cells.

The retrodiscal tissue is vascular and highly innervated connective tissue, which is attached to the posterior aspect of the disc. Therefore, it is known as a major contributor to the pain of temporomandibular disorder. As this tissue functions to restrict disc displacement, patients with TMJ ankylosis may have a strong deposition of fibrous tissues. In particular, batch #2 showed a strong distribution of blood vessels and inflammatory cell density (Figure 7).

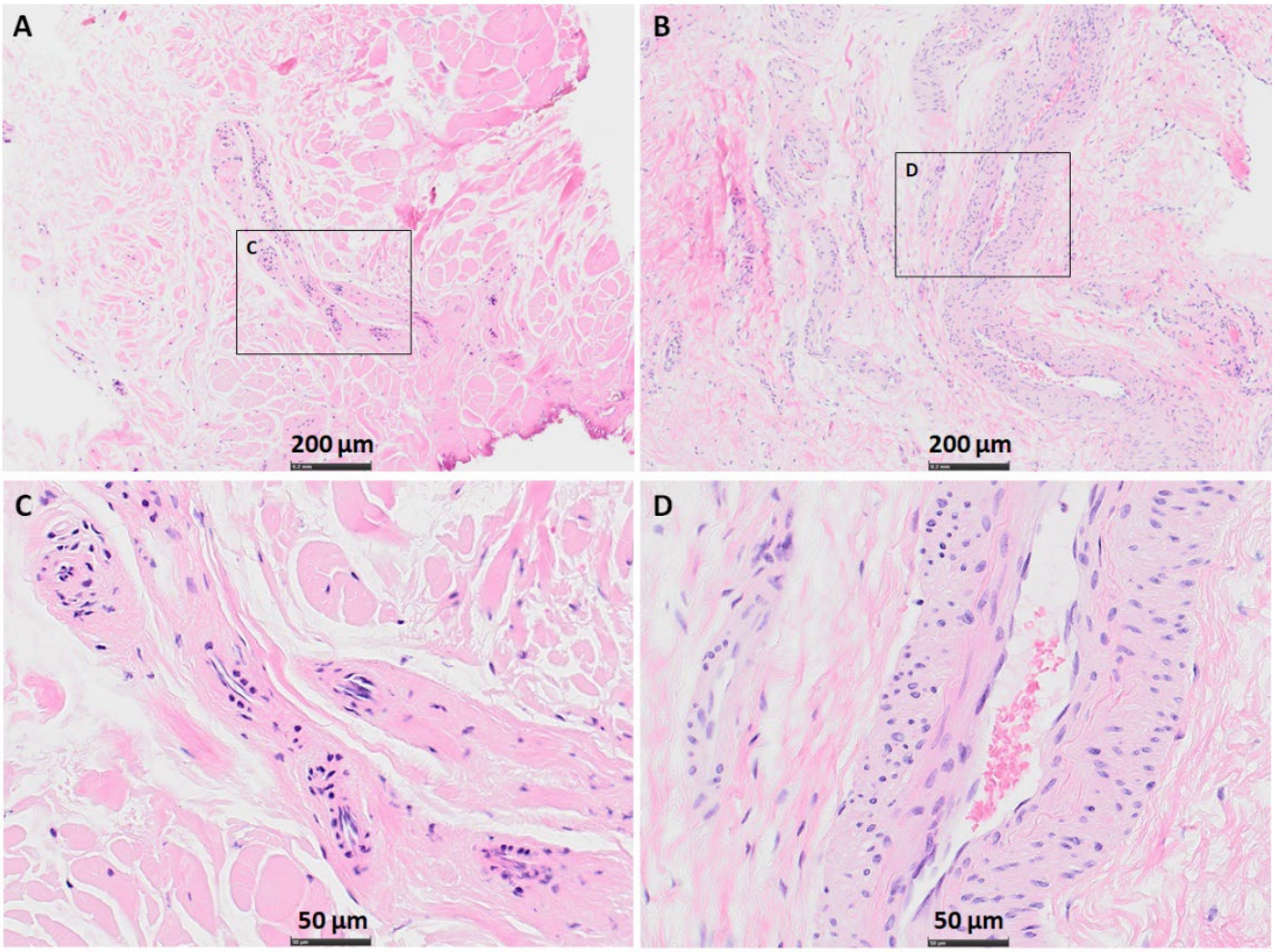

Figure 7. H&E staining of the human TMJ retrodiscal tissue. (A and C) Batch #1, (B and D) Batch #2.

The fibrocartilage was totally eroded in batch #1 (Figure 8A). Batch #2 also showed severe clefts to the calcified zone with hypercellularity. Disorganized collagen and fibrochondrocyte alignment were observed in the whole area (Figure 8B and C).

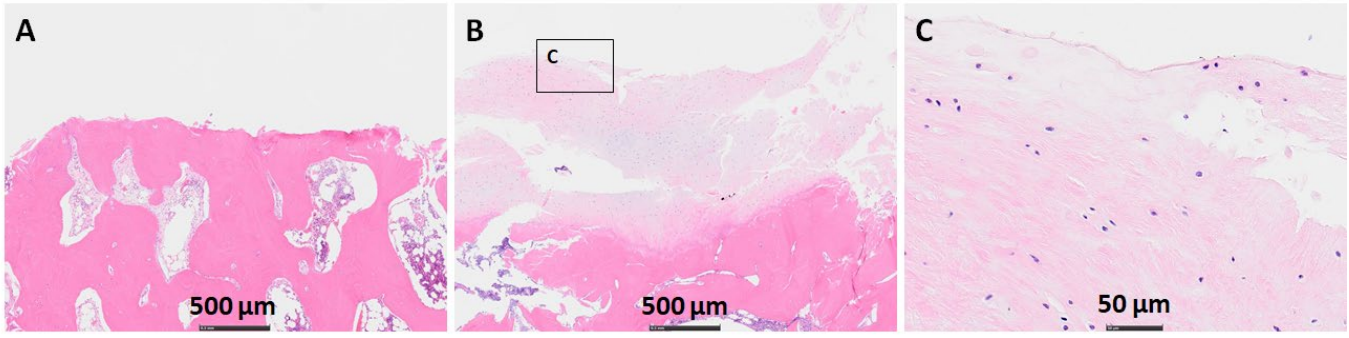

Figure 8. H&E staining of human TMJ fibrocartilage. (A) Batch #1, (B and C) Batch #2.

In the disc, vascular formation and inflammatory cell infiltration were observed in the peripheral region of batch #1 (Figure 9A and C). Batch #2 presented hypercellularity and apparent interstitial fibrosis in the whole region (Figure 9B and D).

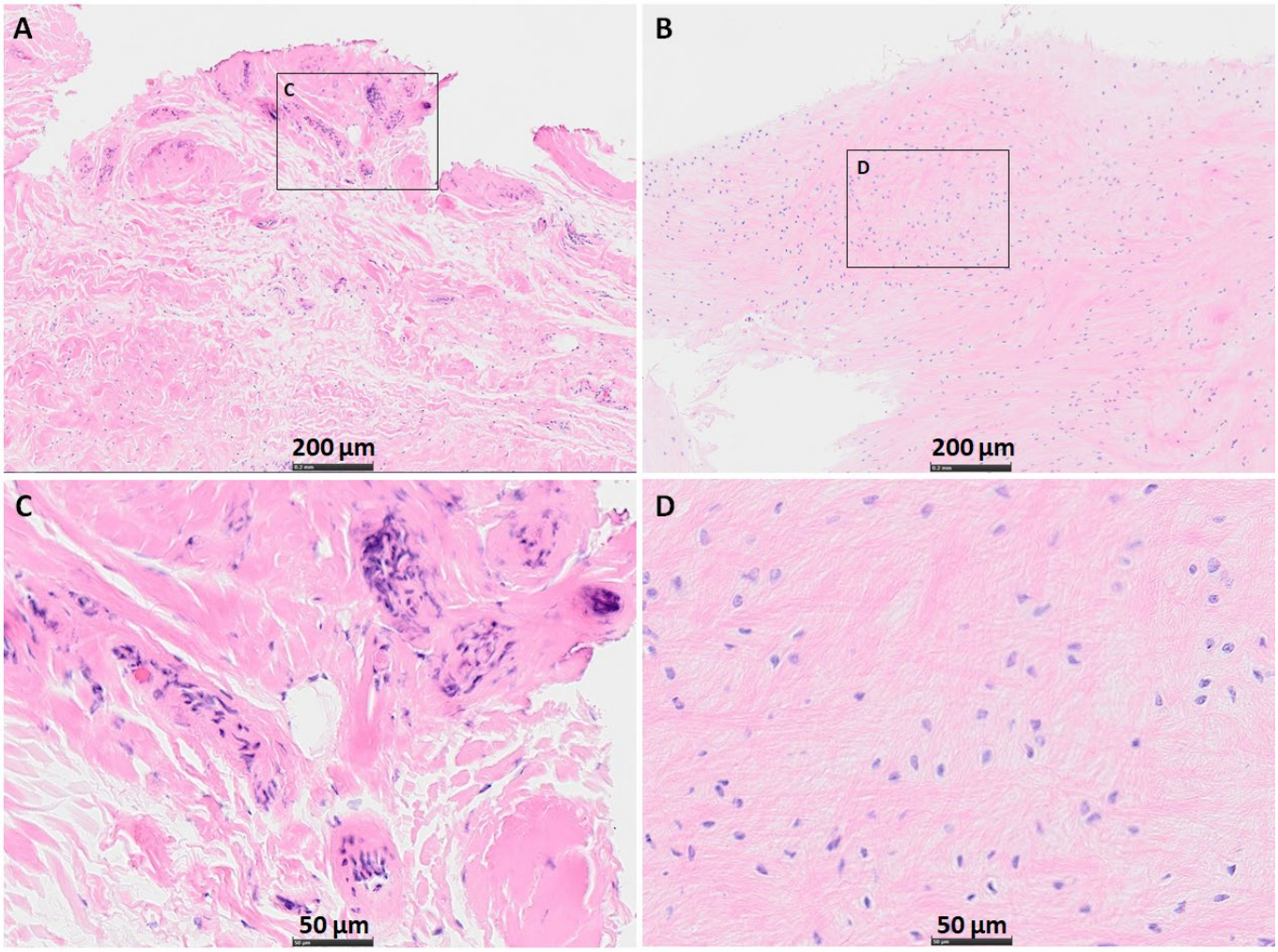

Figure 9. H&E staining of human TMJ disc. (A and C) Batch #1, (B and D) Batch #2.

Overall, human fibrocartilage was severely damaged, and other tissues had moderate infiltration of fibrous tissue and inflammatory cells. We categorized batches #1 and #2 for type II-III and type I ankylosis, respectively, according to the grades of severity (Table 3). Batch #3 was unable to be categorized due to eroded tissues on retrieval.

α-SMA IHC staining as a fibrosis marker was performed to evaluate the degree of fibrosis ankylosis. The synovium (batch #2; Figure 10A and B) and the retrodiscal tissue (batch #2; Figure 7E and F) showed strong deposition of α-SMA expression. In contrast, the retrodiscal tissue (batch #1; Figure 10C and D) and disc (batch #1; Figure 10G and H) were expressed relatively less positive staining. In order to quantify the α-SMA expression, the mean grey intensity was calculated (Table 4). Similar to H&E images, α-SMA expression in the retrodiscal tissue was higher in batch 2 compared with batch 1 ($158.3x10^{-3}$ batch #2 versus $15.0x10^{-3}$ batch #1). The α-SMA expression in the synovium (batch #2) was evenly and strongly distributed in the whole area with $44.7x10^{-3}$ mean grey intensity. Although these data will be compared with normal TMJ tissues to confirm fibrous deposition in ankylosis tissues, it is known that scattered α-SMA positive cells were observed in normal TMJ discs and tissues [3].

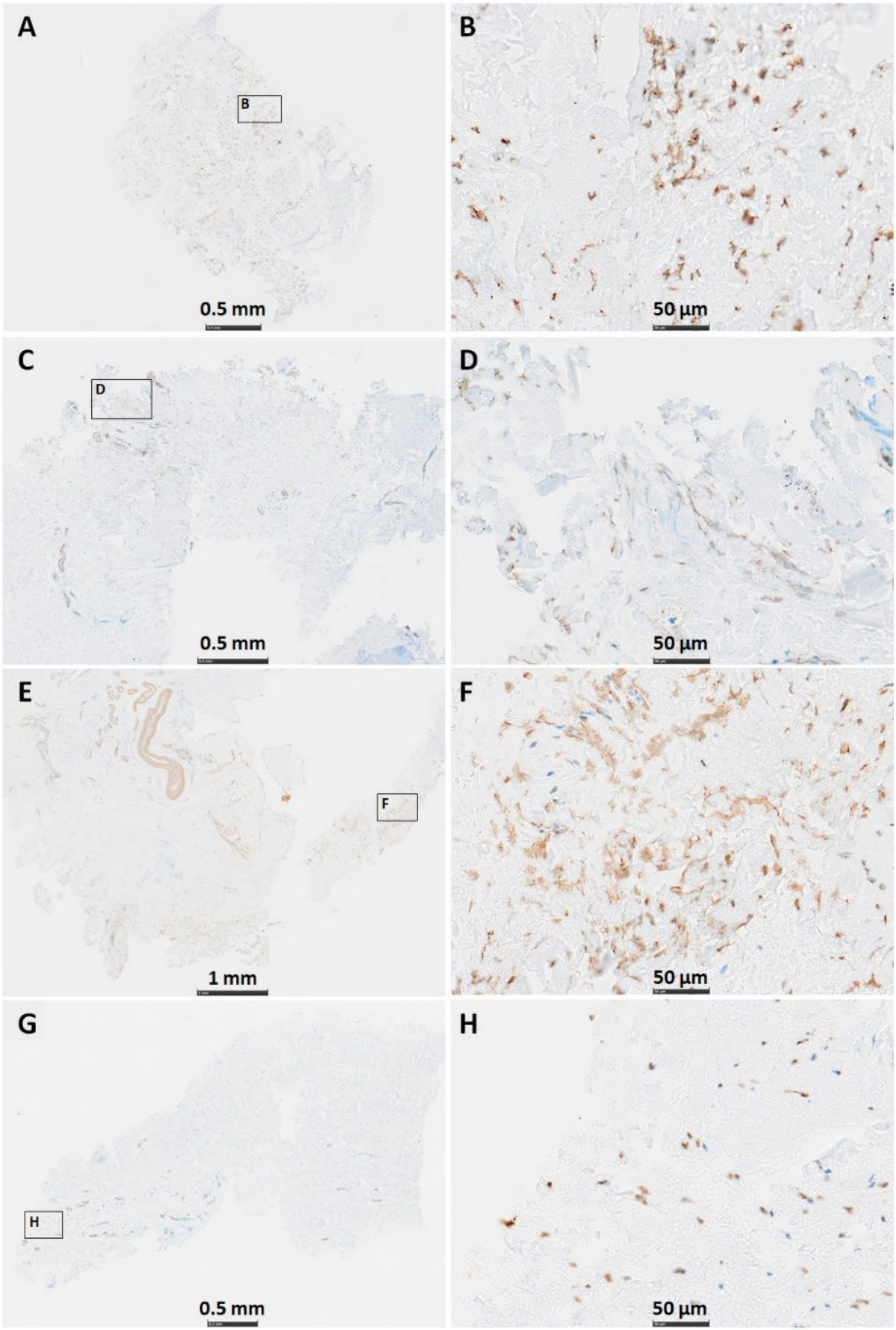

Figure 10. α-SMA IHC staining of human TMJ disc. (A and B) Synovium from batch #1, (C and D) Retrodiscal tissue from batch #1. (E and F) Retrodiscal tissue from batch #2. (G and F) Disc from batch #1. Brown: α-SMA, blue: nuclei.

Table 4. Semi-quantification of α-SMA expression (mean ± standard deviation)

	Synovium (batch #2)	Retrodiscal tissue (batch #1)	Retrodiscal tissue (batch #2)	Disc (batch #1)
Mean grey intensity / number of nuclei ($1x10^{-3}$)	**44.7 ± 8.8**	**15.0 ± 3.7**	**158.3 ± 44.5**	**20.6 ± 14.8**

***Aim2-2: In vitro* anti-fibrotic effect of BMSC-Exos**

Brief description of in vitro anti-fibrosis test:

We originally proposed to evaluate the *in vitro* anti-fibrotic effect of exosomes using human myofibroblasts isolated from fibrous pathological TMJ tissues (fibrocartilage, synovium, disc, and retrodiscal tissue). Although we isolated putative myofibroblasts from disc and retrodiscal tissue, we identified the characteristics of these cells without naïve cells from healthy tissues. Therefore, as an alternative approach, we exogenously induced myofibroblast using transforming growth factor beta 1 (TGF-β1). For inducing myofibroblast differentiation, human fibroblasts were treated with 1 or 10 ng/ml TGF-β1 including a non-treated control. The cells were stained with vimentin as a fibrosis marker and 4′,6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain, and the images were obtained using a confocal microscope.

Anti-fibortic effect of BMSC-Exos outcomes: After 24-hours treatment of TGF-β1, elongated fibroblasts (Figure 11A) morphologically differentiated into widespread-shaped myofibroblasts with intense expression of vimentin (Figure 11B and 11C). The vimentin expression in myofibroblasts was dramatically reduced when treated with BMSC-Exos, especially in the concentration of 1 x 10^6 exosomes/ml/cell ($p < 0.001$ versus TGF-β1 with no BMSC-Exos) (Figure 12).

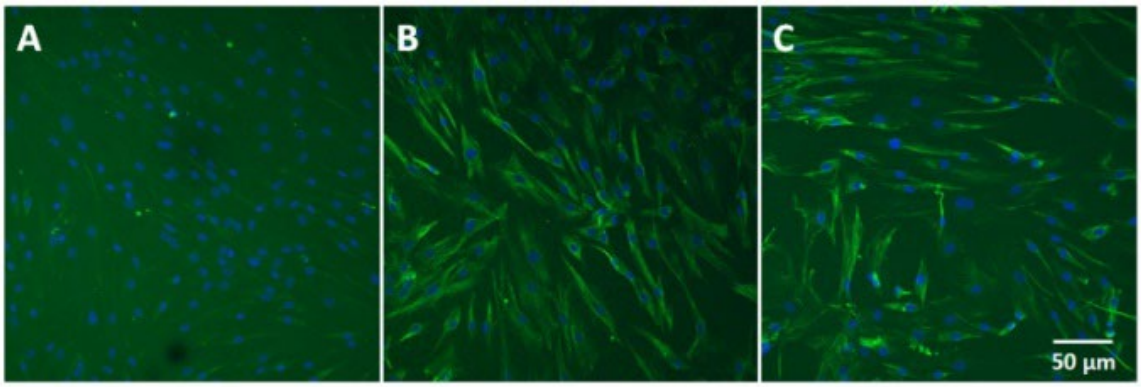

Figure 11. Determination of optimal concentration of transforming growth factor beta 1 (TGF-β1) to induce myofibroblast differentiation using vimentin immunofluorescence staining. (A) Control (no TGF-β1). (B) 1 ng/ml TGF-β1. (C) 10 ng/ml TGF-β1. Green: vimentin. Blue: DAPI.

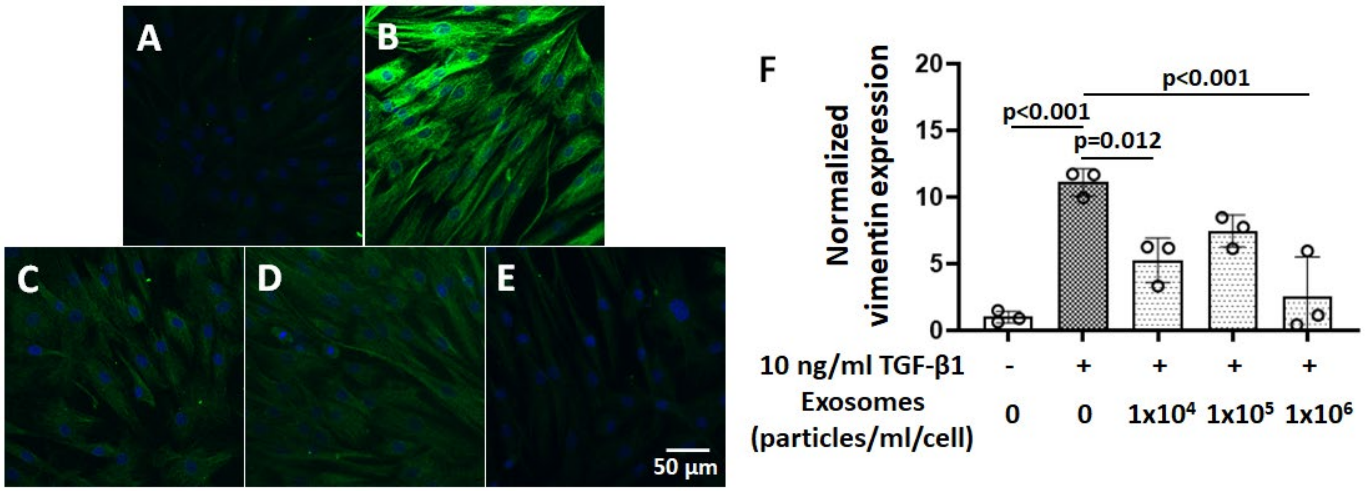

Figure 12. Anti-fibrotic effect of BMSC-Exos on vimentin expression. (A-E) Confocal images for negative

control (no TGF-β1 and BMSC-Exos) (A), positive control (TGF-β1) (B), and BMSC-Exos treatment with 1 x 10^4/ml/cell (C), 1 x 10^5/ml/cell (D), and 1 x 10^6/ml/cell (E). Green: vimentin. Blue: DAPI. (F) Quantified vimentin expression by area measurement.

3. Conclusions

Aim1:

- Highly-purified exosomes were successfully isolated from human bone marrow stromal cells (BMSC-Exos) using a combinational method of size-exclusion chromatography (SEC) and ultrafiltration (UF).
- Exosomes derived from the human BMSCs were successfully characterized in terms of size, zeta potential, morphology, and exosome-specific protein markers.
- Human BMSC-Exos were successfully uptaken by the target cells and internalized into the cytoplasm.

Aim2:

- Pathological TMJ tissues were retrieved from TMD patients who underwent TMJ tissue removal surgeries.
- Fibrous tissues of the patients' TMJ tissues were characterized for pathological findings: subintimal fibrosis, inflammatory cell infiltration, eroded fibrocartilage, disorganized collagen, interstitial fibrosis with hypercellularity.
- Patients' pathological TMJ tissues were categorized for Type II-III and Type I ankylosis classification.
- H&E and α-SMA IHC staining consistently indicated pathological findings, which contrast with normal references.
- BMSC-Exo successfully suppressed vimentin (fibrosis marker) expression of myofibroblasts, indicating promising potential of BMSC-Exo as an anti-fibrosis therapy.