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Effects of Mechanical Stretch on Stem Cells from Human Exfoliated Deciduous Teeth (SHED)

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ABSTRACT

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Objective: To define the effect of mechanical stretch on SHED *in vitro*.

Design: Cryopreserved SHED were expanded and seeded onto collagen type-I BioFlex culture plate. Cells were subjected to cyclic stretching for 2 hours every 48 hours for 7 days on the Flexcell-FX5000 tension system. Cells are then grown in osteogenic and adipogenic medium for 4 weeks. Control groups grown under the same protocol without stretching. After 4 weeks, both groups are stained with 1% Alizarin Red S stain and Oil Red O stain accordingly.

Results: Results showed significant increase in osteogenic gene expression and calcium deposition and decrease in adipogenic gene expression and lipid deposition post-stretching.

Conclusion: *In vitro* cyclic tensile strain significantly enhances SHED's osteogenic properties and minimizes SHED's adipogenic properties.

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PREFACE

Craniofacial problems such as periodontal disease, cleft lip and palate, craniofacial microsomia, and head and neck cancers are common and burdensome surgical problems (Sanchez-Lara PA et al. 2012). Regenerative therapy research for the treatment of congenital or acquired craniofacial deformities is a rapidly growing field.

Regenerative medicine requires three important components: scaffold, stem cells, and bioactive markers. Although various sources of stem cells are available, Stem Cells from Human Exfoliated Deciduous Teeth (SHED) have gained popularity in the last decade. They are multipotent stem cells that can be readily acquired with minimal legal and ethical concerns (Kashyap R 2015). SHED possess the ability to differentiate into functional odontoblast-like cells *in vitro* and odontoblasts *in vivo*. Additionally, SHED also have osteoinductive capacities *in vivo*. (Muira M et al. 2003).

Stem cell differentiation could be affected by the matrix stiffness, external mechanical signals, and mechanotransduction (Steward AJ et al. 2014). Four types of external mechanical signals have been studied on mesenchymal stem cells: fluid flow, pressure, tension, and compression. It is interesting to note that cyclic tensile strain seems to induce osteogenesis and helps to maintain bone formation (Steward AJ et al. 2014).

What would happen if we apply cyclic tensile strain on SHED? Could we use SHED in regenerative therapy to repair bony defects caused by periodontal diseases, cancer or cleft palate? How can we enhance the osteoinductive properties of SHED? This project will attempt to answer some of these questions.

Steward et al. (2014) reviewed twelve articles published between 2006 to 2011 involving tension

and MSC differentiation. They found that several articles report that applying cyclic tensile strain has been shown to promote osteogenic gene expression and calcium deposition. In addition, they also reported that cyclic tensile strain has also been shown to induce the expression of proinflammatory cytokines known to inhibit bone resorption.

Seo BM et al. (2008) used SHED to repair critical-size calvarial defects in mice. Their findings include SHED were able to repair the defects with substantial bone formation and SHED were found to co-express mesenchymal stem cell marker, with an array of growth factor receptors implying their comprehensive differentiation potential. They concluded that SHED may select unique mechanisms to exert osteogenesis and might be a suitable resource for orofacial bone regeneration. No literature has been found on the effect of cyclic tensile strain on SHED differentiation. Therefore, this study will try to define the effect of tension on SHED.

CHAPTER 1

Study Design

1.1. Objective: To define the effect of mechanical stretch on SHED *in vitro*.

1.2. Hypothesis: *In vitro* cyclic mechanical stretch of SHED will promote osteogenic gene expression and induce their osteoinductive properties.

1.3. Null Hypothesis: *In vitro* cyclic mechanical stretch of SHED will not affect SHED's differentiation, and will not promote osteogenic gene expression and induce their osteoinductive properties.

1.4. Study Significance: SHED is a readily available source of stem cell and enhancement of SHED's osteoinductive properties can help the management of bony defects due to periodontal diseases, congenital or acquired defects.

CHAPTER 2

Material and Methods

2.1. Isolation and Culture of SHED

SHED used for this study were expanded from cryopreserved cells collected previously. Cells were between passage 2 and 10. The following paragraph will briefly describe isolation and culture of the SHED used.

To isolate SHED, pulp tissue was gently separated from the crown and resorbed root of healthy human exfoliated deciduous mandibular incisors collected from 6-year-old girl in the private clinic with parental consent. The pulp tissue was then digested in a solution of 3 mg/mL collagenase type I and 4 mg/mL dispase for 1 h at 37°C. A single-cell suspension of BM-derived all- nuclear cells (ANCs; 15 x 10^6) was seeded into 100mm culture dishes (Corning) and cultured at 37°C with 5% CO_2. 48 h later, non-adherent cells were removed, and attached cells were cultured for 16 days in α-MEM (Invitrogen) supplemented with 20% FBS, 2 mM L-glutamine (Invitrogen), 55 mM 2-mercaptoethanol (Invitrogen), 100 U/mL penicillin and 100 mg/ml streptomycin (Invitrogen). The medium of the attached single colonies was changed frequently.

2.2. Mechanical Stimulation

The Flexcell FX-5000 Tension System (FX5K; Flexcell International Corp, Hillsborough, NC) was used to apply mechanical cyclic tensile stretch to the SHED. The Flexcell FX-5000 is a computer-based system that uses a vacuum to strain cells adhered to the flexible silicone membranes (BioFlex plates; Flexcell International Corp) arranged in a format of six wells per plate with a total growth area of 9.62 cm^2/well and a membrane thickness of 0.05 mm. The deformation of

the flexible membrane of the plates also causes the attached cells to deform. Programming the magnitude, duration, and frequency of the negative pressure in the Flexcell apparatus creates desired strain profiles. SHEDs were seeded onto the collagen type-I-coated BioFlex plates at a density of 4×10^4 cells/well. When cultures reached approximately 70% to 80% confluence, undifferentiated SHED were subjected to continuous mechanical stimulation with equibiaxial waveform with 15% elongation and a frequency of 0.5 Hz for 2 hours every 48 hours for 7 days. Each cycle consisted of 10 s strain and 20 s relaxation. Control cultures were grown under the same conditions but without the strain protocol. All cells were kept in an incubator during active stretching at 37˚C in a humidified atmosphere containing 5% $C0_2$.

2.3. Osteogenic and adipogenic differentiation assays of SHED

For differentiation induction in vitro, SHED were seeded into 6-well plates (Corning) and cultured in growth medium until the cells reached confluence. To induce osteogenic differentiation, the SHED were cultured under osteogenic culture conditions after stretching completed, containing 2 mM b-glycerophosphate (Sigma–Aldrich), 100 mM L- ascorbic acid phosphate (Wako), and 10nM dexamethasone (Sigma–Aldrich) in the growth medium. After 4 weeks induction, 1% Alizarin Red S (Sigma–Aldrich) staining was performed to detect matrix mineralization (Chen et al., 2015). To retain Alizarin Red S, images were analyzed and quantified using NIH ImageJ software with a 50% threshold by determining the area positive for dye staining expressed as a fraction of the total area. For adipogenic induction, 500nM isobutylmethylxanthine (Sigma–Aldrich), 60 mM indomethacin (Sigma–Aldrich), 500 nM hydrocortisone (Sigma–Aldrich), 10 mg/ mL insulin (Sigma–Aldrich), and 100 nM L-ascorbic acid phosphate were added to the growth medium. After 4 weeks, the induced cells were stained

with Oil Red O (Sigma–Aldrich). The positive cells were quantified under microscopy and are shown relative to the total cell count.

2.4. Western blot analysis

Cells were lysed in M-PER mammalian protein extraction reagent (Thermo Fisher Scientific) with protease and phosphatase inhibitors (Roche), and then protein content was quantified using the Pierce^{TM} BCA Protein Assay Kit (Thermo scientific). 30mg of protein was separated on an SDS-PAGE gel and transferred to nitrocellulose membranes (Millipore). The membranes were blocked with 1% non-fat milk (Santa Cruz), 4% BSA, and 0.05% Tween-20 for 1h and then incubated overnight with primary antibodies diluted in incubation buffer, according to the manufacturer's instructions. Antibodies to human alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX2), osteocalcin (OCN), Peroxisome proliferator-activated receptor gamma (PPAR-γ), and lipoprotein lipase (LPL) were purchased from Santa Cruz Biotechnology, Inc. Antibody to human ß-actin was purchased from Sigma–Aldrich. The membranes were incubated for 1h in HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) diluted at 1:100,000 in incubation buffer. Immunoreactive proteins were then detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and BioMax film (Kodak).

CHAPTER 3

Results

3.1. Osteogenesis

Western blot analysis showed noticeable increase in expression of OCN and RUNX2 in the stretch group compared to control. See Figure 1.

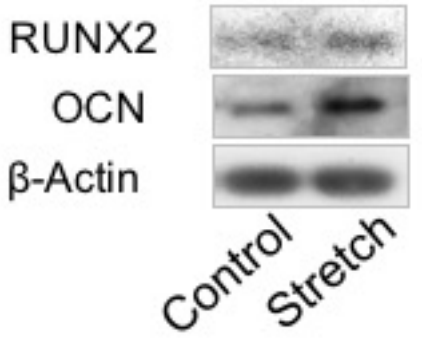

Figure 1. Noticeable increase in RUNX2 and OCN expression in the stretch group.

3.2. Adipogenesis

Results showed decreased expression of LPL and PPAR-γ in the stretch group compared to control. See Figure 2.

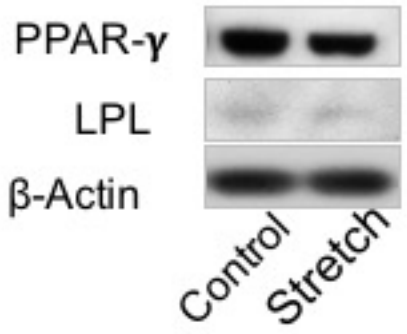

Figure 2. Decreased expression of LPL and PPAR-γ expression noted in stretch group compared to control.

3.3. Alizarin Red S staining

24 hours post stretching, SHED was induced with osteogenic medium previously described. Medium was changed regularly for a period of 4 weeks. After 4 weeks, SHED was stained with 1% Alizarin Red S for calcium deposition. Results showed significant increase in calcium deposition in stretch group compared to control group. See Figure 3.

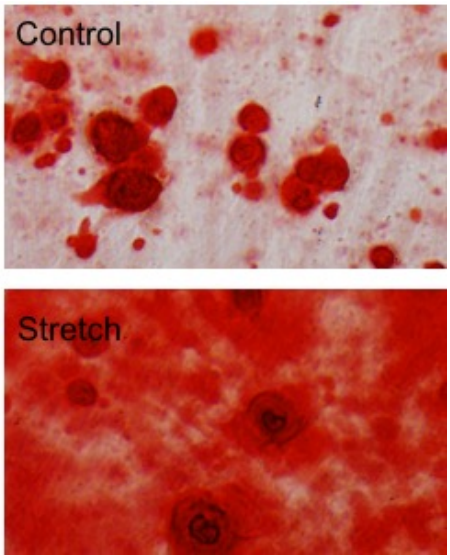

Figure 3. Alizarin Red S staining 4 weeks post-stretching. SHED was cultured in an osteogenic medium changed regularly. Stretch group show significantly increased calcium deposition.

3.4. Oil Red O staining

24 hours post stretching, SHED was induced with adipogenic medium previously described. Medium was changed regularly for a period of 4 weeks. After 4 weeks, SHED was stained with Oil Red O for lipid presence. Results showed minimal adipogenesis in control group and no lipid deposition in stretch group. See Figure 4.

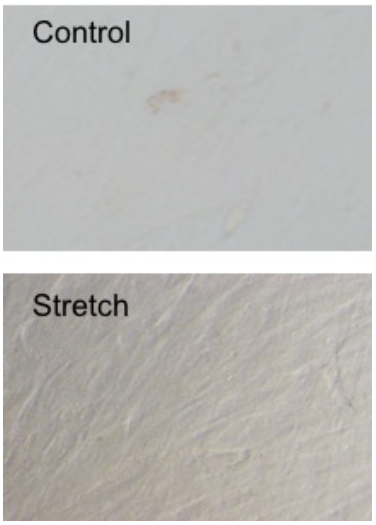

Figure 4. Oil Red O staining 4 weeks post-stretching on the Custom Stretch Apparatus. SHED was cultured in an adipogenic medium changed regularly. Minimal lipid deposition in control group and no lipid deposition in stretch group.

CHAPTER 4

Discussion and Conclusion

Results from the present study showed that *in vitro* mechanical strain can potentially increase SHED's osteogenic properties. *In vitro* cyclic stretching of SHED showed significant increase in calcium deposition. This finding confirmed that SHED, similar to MSCs show enhanced osteogenic properties under cyclic tensile strain. Although the results obtained from the analysis of Western blot from Flexcell-FX5000 tension system are inconclusive, results from the Custom Stretch Apparatus showed promising increase in calcium deposition after stretching.

The current study also confirmed that *in vitro* cyclic tensile strain decreases SHED's adipogenic abilities. Overall, SHED showed poor adipogenic properties which are further affected after cyclic tensile strain.

SHED are a readily available source of stem cells that can be used in regenerative medicine (Arora et al. 2009). Their therapeutic values are being studied and pursued by many researchers at institutions around the world (Arora et al. 2009). Scientists are increasingly proposing banking of SHED for future therapeutic purposes because the multipotent properties of SHED are unaffected by cryopreservation (2008).

For dental professionals, congenital and acquired bone defects in the oral facial region are cumbersome issues. In current practice, the gold standard bone graft material is of autogenous origin. Although it shows the best results in terms of healing, harvesting autogenous bone requires a second surgical site which could be traumatic to the patient. Hence, using stem cells derived from human exfoliated teeth could eliminate the painful secondary surgery. SHED are readily available and harvesting them generate a minimal amount of ethical and legal debates. They could be banked and preserved for later usage without their properties being altered. Studies using SHED have shown promising results in regenerative surgery and treatment of

autoimmune diseases. In fact, a recent animal study by Zheng et al has shown that stem cells from exfoliated deciduous teeth can successfully repair a critical-size orofacial defects in swine (2009). Therefore, by enhancing the osteogenic properties of SHED prior to grafting using mechanical tensile strain, reparative and regenerative surgery could yield improved outcome. Although current study pf cyclic stretching decreases SHED's adipogenic properties, this is concurrent with SHED's poor ability to differentiate into adipocyte. Further study is required to understand the signaling pathway that how mechanical cyclic strain is triggering in SHED. Future studies should look into cell proliferation after mechanical stretch and also the effect of loss of mechanical loading on the behavior of SHED.

In conclusion, *in vitro* cyclic tensile strain has the ability to enhance SHED's osteogenic properties and decreases SHED's adipogenic properties. Further study is required to elucidate and understand the signaling pathway.

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