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Research Article A Novel Tissue Engineering Technique of Mesenchymal Stem Cells

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Abstract

Background and Objective: Mesenchymal stem cells isolated from different types of tissues have the ability to self-renew and have the potential of differentiation into countable and uncountable progenitor. The objective of the study was to develop tissue engineering technique with maximum yield and measurement the power of synovium-derived mesenchymal stem cells. **Materials and Methods:** The 1.8 g patient⁻¹ human synovial membrane were collected from each patient during the time of the arthroscopic surgical procedure. Each aliquot was divided into three aliquots each containing 0.6 g of the synovial membrane and minced. The first, second and the third aliquot were treated as digestion in 0.1% collagenase IV solution followed by filtration, only filtration and no treatment. Each aliquot of all groups was further subdivided into subgroups of 5 mL each for further process. The subgroups were subjected to *in vitro* quantitative measures of cells using the automatic cell counter plate, osteogenic, adipogenic, chondrogenic differentiation and cell proliferation assay. The one-way ANOVA/the Turkey's *post hoc* test was performed to show statistical significance between results at 95% of confidence level. **Results:** The density was measured as group 1< group 2< group 3. The cell viability was found more than 85% in all groups. There were significant differences for yields, DNA content, osteogenic, adipogenic, chondrogenic differentiation and cell proliferation between subgroups 1 and 2 (p<0.05 for all), between subgroups 1 and 3 (p<0.05 for all). There were insignificant differences for yields, DNA content, osteogenic, adipogenic, chondrogenic differentiation and cell proliferences for these parameters between subgroups 2 and 3 (p<0.05 for all). **Conclusion:** The study provided simple and effective tissue engineering technique for isolation of stem cells from the synovial membrane with the 8-fold higher percentage yields than the technique of isolation of cells by filtering.

Key words: Admeasurement power, cell proliferation, chondrification promoter, mesenchymal stem cells, synovial membrane, cartilage repair

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The chondral injuries usually do not heal spontaneously due to its avascular surroundings and unique matrix organization¹. Therefore, several methods are adopted for the same. Among these, chondrocyte-based therapies among these are studied frequently. However, it has limitations of the sacrifice of undamaged cartilage within the same joint and² alterations of the chondrogenic phenotype associated with the *in vitro* expansion of the cells¹. To overcome such limitation, stem cells therapy is adopted. Synovium-derived Mesenchymal Stem cells (SMSs) exhibit the most enhanced chondrogenic potentia³ and are the most appropriate cells for cartilage repair till date used by orthopedics in tissue engineering techniques (Fig. 1). Mesenchymal stem cells (MSs) were isolated from different types of tissues. The origin, the characteristics of the cells and the local environment and age of donor of MSs in the tissue engineering technique, have a countable effect on their ability to self-renewability, ossification promoter, chondrification promoter, adipogenesis promoter, cardiac muscle cells regenerator⁴ and the nerves regenerator³. The SMSs have the highest chondrification promoter effect and admeasurement power compared to MSs obtained from the other tissues of the body. The preclinical and clinical studies have been also reported the same⁵. However, the tissue engineering technique adopted also have a booming effect on chondrification promoting property of SMSs⁶. There was need of $1.31 \times 10^8 \pm 1.1 \times 10^3$ SMSs for chondrification promoter effect. The most of the tissue engineering techniques were failed to achieve such concentration in the final yield⁷. Till date availed study for the isolation of MSs/SMSs are for digestion of standard collagen followed by filtering to remove debris and then carried out primary culture⁸. However, digestion process leads to decrease quantity as well as admeasurement power of MSs⁹. Thus there is need of novel tissue engineering technique which gives high yield and admeasurement power of synovium-derived mesenchymal stem cells.

The objective of the present study was to develop novel tissue engineering technique with a maximum yield from the cultured human SMSs and to evaluate their osteogenic, adipogenic, chondrogenic differentiation and cell proliferation activities.

MATERIALS AND METHODS

Materials: Hanks' balanced salt solution, fetal bovine serum, high-glucose Dulbecco's modified eagle medium, Alcian Blue, Kernechtrot and collagenase IV were purchased from Sigma-Aldrich, Hong Kong, China. The ampicillin, streptomycin and fluconazole were purchased from Poultry antibiotics, China. Collagenase A, paraformaldehyde and Alizarin Red S (ARS), Oil Red O (ORO), Safranin O (SO) dyes were purchased from Royal Pharma, China. OsteoLife[™]-An osteogenesis medium was purchased from Lifeline[®] cell technology US. StemPro[®]-An adipogenesis differentiation kit and CellTrace[™]



Fig. 1: Anatomy of bone a: Yellow marrow, b: Spongy bone with red marrow, c: Compact bone, d: Blood vessels in bone marrow, e: White blood cells, f: Blood stem cells, g: Platelets and h: Red blood cells

				Sex	
	Quantity of		Age		
No. of samples	sample (No.)	Diagnostic condition	(Mean±SD)	Male	Female
1-121	121	Anterior cruciate ligament injury	23.00±1.2	75	46
122-197	75	After anterior cruciate ligament reconstruction synovitis	25.00±1.3	57	18
198-267	71	Osteochondromatosis	27.00±1.4	32	39
268-323	56	Meniscal injury	30.32±1.4	29	27

Table 1: Synovial samples used in the study

Table 2: Groups for tissue engineering technique					
Groups	Components used for cell culture	Composition			
1	Digested tissue fragments removed by filtration	Cs only			
2	No filtration; only separation	Cs+UTFs			
3	No filtration and no separation	Cell+UTFs+PTFs			

Cs: Cells, UTFs: Undigested tissue fragment, PTF: Precipitated tissue fragments

CFSE cell proliferation Kit for flow cytometry were purchased from Thermo Fisher Scientific, MA, US. MesenCult[™]-ACF chondrogenic differentiation medium was purchased from STEMCELL Technologies China Co., Ltd., Shanghai, People's Republic of China. CytoTrack cell proliferation dyes were purchased from NeoBioscience Technology Company, Shenzhen, People's Republic of China. The other chemicals used in this study are analytical grade.

Ethical statement: The Ethics Committee for Human Experiments of the Guangdong Second Provincial General Hospital, Guangzhou, P.R. China approved the experimental protocol under the reference number of ChiCTR-TRC-14004311 and the Ethical Guidelines for biomedical research on human participants in accordance with Chinese law was followed¹⁰. There were 323 patients of Guangdong second provincial general hospital, Guangzhou, China, the first affiliated hospital of Guangzhou University of Traditional Chinese Medicine, Guangzhou China and Deping hospital, Chongqing, China during the period of April, 2014 to February, 2016 enrolled in the study (Table 1).

Aliquots preparation: The 1.8 g patient⁻¹ human synovial membrane were collected from each patient via either postmortem within a day of death or during the time of the arthroscopic surgical procedure. The maximum and minimum ages of donors were 49 to 17 years. The 54 samples were collected from postmortem and the 269 samples were collected during the time of the arthroscopic surgical procedure. The samples rinsed thrice with Hanks' balanced salt solution and antibiotic-antimycotic solution (80 mg mL⁻¹ ampicillin, 100 mg mL⁻¹ streptomycin and 0.20 mg mL⁻¹ fluconazole) was added. Each sample was divided into three aliquots each containing 0.6 g of the synovial membrane and minced with stainless steel angle rongeur surgical scissors

(Shanghai Medical Instruments Group Ltd., Corp.) and add 1% antibiotic-antimycotic solution, allowed to put in an incubator (Yorko, USA) at 37°C for 15 h, allow to grow was centrifuged by clinical centrifuged (Remi equipment, Mumbai, India) at 2000 rpm for 5 min¹. Three different groups were divided as per the Table 2. The final volume of each aliquot was maintained 10 mL with high-glucose Dulbecco's modified eagle growth medium supplemented with 10% fetal bovine serum. After 4 h of the minced method, in group 1 the synovial membrane was digested in 0.1% collagenase IV solution and the undigested tissues were removed by filtration using 22 µm Nylon filter (High Media, USA). In group 2 the floating undigested tissue fragments were removed by centrifugation. However, in group 3 synovial membrane was not digested and not filtered. The study flow chart is given in Fig. 2.

Outcome measures

Primary end aim: The primary aim of the study was to achieve maximum percentage yield.

Secondary endpoint: The secondary endpoint of the study was to get SMSs with high osteogenic, adipogenic, chondrogenic differentiation and cell proliferation activities.

Microscopic observation: About 10 μ L cell culture were applied to the automatic cell counter plated (BD Biosciences, CA, US) for analysis¹¹.

Preparation of primary cell culture: There was the final volume of 10 mL in each aliquot of all groups. These aliquots were further subdivided into subgroups of 5 mL each. The aliquots of subgroups were plated at 4500 cell cm⁻² and were cultured in growth medium containing 10% of BS at 37°C in an incubator. The medium was replaced every 5 days¹².



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Fig. 2: Flow diagram of the study

In vitro quantitative measures of cells: The aliquots were further plated at 4500 cell cm⁻². After 21 days of culture for each subgroup, the final cell number/0.3 g tissue was counted using the automatic cell counter plated¹³.

Osteogenic, adipogenic and chondrogenic differentiation: There were 2.01×10⁴ cells in each subgroup plated in 12-well plates and cultured in complete medium for 49 h. The medium was converted to OsteoLife[™], StemPro[®] and MesenCult[™]-ACF cultured for another three weeks, one week and two days, respectively. The medium was replaced every fourth day and walls were washed two times with phosphate buffer saline, fixed in 4% of paraformaldehyde and then stained with 0.5% of ARS dye, ORO and SO, respectively².

Cell proliferation assay: There were 5×10^3 cells in each subgroup plated in 96-well plates and cultured in

complete medium for 49 h. The medium was converted to CellTrace[™] CFSE cultured for another 2 weeks. The medium was replaced every 4th day and walls were washed two times with phosphate buffer saline. CytoTrack cell proliferation dyes were used for evaluation method¹⁴.

Statistical analysis: All results were represented as Mean \pm SD of five independent results. The one-way ANOVA (Microsoft excel[®] 2013, Raymond, US) was used to show statistical significance between results. The SPSS 24.0 (SPSS Inc., USA) for Windows was used¹⁵. The results were considered significant at 95% of confidence level. The significant difference (p<0.05) between two subgroups was further checked by the Turkey's *post hoc* test¹⁶. For statistical analysis of osteogenic and mineralization, adipogenic induction and lipid, vacuoles, Chondrogenic induction and cell proliferation efficiency were graded as in the range of 0-4 from absent, mild, medium, moderate to strong and strong.



Fig. 3(a-c): Density of cells in culture (a) Group 1, (b) Group 2 and (c) Group 3

Table 3: Comparison of number of samples, DNA content and stem cells between groups

Groups	No. of samples (n)	DNA content	Stem cells $\times 10^7$ (subgroup/0.3 g tissue)
1	306	4.11±0.12	0.712±0.0001
2	318	45.20±1.13	5.460±0.0090
3	320	51.20±1.15	4.470±0.0080
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Data are expressed in Mean \pm SD, n = 5

RESULTS

The density of groups was measured as group 1<group 2<group 3 (Fig. 3). The p-value of the one-way ANOVA for the DNA content between groups 1 and 2 was <0.05, between groups 1 and 3 was p<0.05 and between groups 2 and 3 were >0.05. There were significant higher DNA content in groups 2 than group 1 (p = 0.018) and in group 3 than group 1 (p = 0.015). There was insignificant higher DNA content for groups 3 than group 2 (p = 0.21). There was a significant high yields of SMSs in subgroup 2 than subgroup 1 (p = 0.0165) and subgroup 3 than subgroup 1 (p = 0.0191). There were insignificant high yields of SMSs in subgroup 2 than subgroup 2 than subgroup 3 than subgroup 3 (p = 0.291, Table 3). The cell viability was reported more than 85% in all groups.

The collagen digestion time was increased from 4 h to one day, there was no any significant increase in the cell number in group 1 (p>0.05). However, at the 4 h, there was a significant increase in the cell number and more cells with aggregates and undifferentiated tissues in group 2 (p<0.05) and group 3 (p<0.05). The cell population within the undigested tissue was not more existing even more exposed to collagenase IV in all groups (p>0.05 for all).

The one-way ANOVA following the Turkey's *post hoc* test showed that there were significant incensement of osteogenic/mineralization with calcium deposition (Fig. 4), adipogenic induction/lipid vacuoles (Fig. 5), chondrogenic induction (Fig. 6) and cell proliferation activity (Fig. 7) in subgroups 3 and subgroup 2 as compared to subgroup 1.

However, there was the insignificant incensement of osteogenic and mineralization with calcium deposition, adipogenic induction/lipid vacuoles, chondrogenic induction and cell proliferation activity in subgroups 2 as compared to subgroup 3.

DISCUSSION

The present tissue engineering technique was performed without any sophisticated equipment and biological reagent in the preparation of SMSs. Till date, available studies are in the preparation of SMSs by filtering process followed by collagenase digestion and required sophisticated equipment like bioreactor and biological reagents, growth factors^{17,18}. With respect to the process adopted in the present study, it was simple and effective.

The present study revealed that there was a significant loss of DNA and cell as compared to the unfiltered method. There is a significant loss of DNA and cell in the filtration process^{17,18}. As majorities of SMSs are present in undigested tissues of the synovial membrane. Therefore filtration process was significantly decreased (p<0.05) proliferation.

The study first time provided superior osteogenic induction activity for undigested medium. The previous study is failed to evaluate superior osteogenic induction activity of developed SMSs without filtering to that with filtering¹⁹. With respect to the secondary endpoints of the study, it was novel in character.

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Fig. 4(a-d): Osteogenic and mineralization with calcium deposition (a) Sample collected from subgroup 1, (b) Sample collected from subgroup 2, (c) Sample collected from subgroup 3, (d) Control Magnification view 10× and magnification bar 90 μm for all photographs



Fig. 5(a-d): Adipogenic induction and lipid vacuoles (a) Sample collected from subgroup 1, (b) Sample collected from subgroup 2, (c) Sample collected from subgroup 3, (d) Control
Magnification view 10× and magnification bar 90 μm for all photographs

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Fig. 6(a-d): Chondrogenic induction (a) Sample collected from subgroup 1, (b) Sample collected from subgroup 2, (c) Sample collected from subgroup 3, (d) Control Magnification view 45× and magnification bar 25 µm for all photographs



Fig. 7(a-c): Cell proliferation assay (a) Sample collected from subgroup 1, (b) Sample collected from subgroup 2 and (c) Sample collected from subgroup 3

 $Magnification \ view 45 \times and \ magnification \ bar 25 \ \mu m \ for \ all \ photographs. Figure \ 5c \ was \ somewhat \ yellow \ in \ color \ due \ to \ undigested \ and \ precipitated \ tissue \ fragments$

The study showed that filtration process had an adverse effect on cell proliferation efficiency. The previous studies demonstrated that SMSs derived by filtration has high cell proliferation property^{17,20}. There is the logic behind the results of the present study that filtration process was

removed SMSs and low percentage yield was not enough for high proliferation efficiency.

The study reported high SMSs yield and the cell proliferation without filtering method than the enzymatic method and with filtering method. However, till date, the studies for adipogenic induction and lipid vacuoles by the enzymatic method are reported the same yield by filtering and non-filtering methods^{21,22}. With respect to the primary aim of the study, it was more effective.

However, the study reported that the proliferation potential of SMSs was affected by digestion of synovial membrane. The present studies stated that growth medium affected the proliferation potential of SMSs²³. With agreements of the data reported in the study²³, the current tissue engineering technique was quite logical and authentic.

The current tissue engineering technique first time provided superior chondrogenic induction activity. Till date, the studies are failed to evaluate superior chondrogenic induction activity without filtering to that with filtering²⁴. The previous studies were authentic but were not effective in cartilage repair because of failed to provide superior chondrogenic induction properties.

The study used the cultured human SMSs for tissue engineering technique. The previous studies were suggested the feasibility of tissue engineering technique in animal studies². The study was more acceptable because of clinical and data are of a human.

The present study did not use any synthetic polymers or biological materials. Most of the tissue engineering techniques of SMSs utilized Scaffolds¹. With respect to materials used in the study, scaffold-free tissue engineering technique could be an excellent alternative.

The study showed large population growth without filtering. The filtration process had led to decreasing population growth²⁴. The study was more effective than previous studies.

CONCLUSION

The study demonstrated the unique tissue engineering technique for the isolation of mesenchymal stem cells from synovial membrane. The current study was reported the 8-fold higher percentage yield of cells than the technique of isolation of cells by filtering. Further characterization of the stem cells for osteogenic, adipogenic, chondrogenic differentiation and cell proliferation assay was found that the stem cells obtained from the current technique were more effective than the present tissue engineering technique. The study was failed to evaluate the effect of duration of digestion on the yield of SMSs. The study also limited for effects of physiological and anatomical characteristics of human on quality and quantity of SMSs.

SIGNIFICANCE STATEMENTS

This study discovered the novel tissue engineering technique with high quality and quantity of synovium-derived mesenchymal stem cells that can be beneficial for cartilage repair. This study will help the researcher to uncover the effect of filtration technique on the potential of cells in tissue engineering technique.

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