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ORIGINAL ARTICLE

Obtaining Stem Cell Spheroids from Foreskin Tissue and the Effect of *Corchorus olitorius* L. on Spheroid Proliferation

Sünnet Derisinden Kök Hücre Sferoidlerinin Elde Edilmesi ve *Corchorus olitorius* L.'nin Sferoid Proliferasyonuna Etkisi

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ABSTRACT

Objectives: Mesenchymal stem cells are self-renewing stem cells. The human foreskin has potential to be used as a source of stem cells. The aim of the study was to obtain spheroid formation of human foreskin cells (hnFSSCs) isolated from newborn human foreskin tissue. In addition, the apoptotic and proliferative effects of a traditional plant, *Corchorus olitorius* L. (*C. olitorius*), on hnFSSC spheroids were investigated.

Materials and Methods: After a routine circumcision procedure the cells were isolated and cultured in suitable medium. The plant leaves was extracted with ethanol and their composition was analyzed by liquid chromatography coupled with mass spectrometry (LC-MS/MS). The foreskin stem cells were characterized immunocytochemically by CD45, CD34, and CD90 antibodies. hnFSSC spheroids were formed using the hanging drop technique. Immunofluorescence staining was used on the obtained spheroids to determine the distribution of caspase-3 and Ki-67 after being treated with *C. olitorius* extract for 48 h.

Results: Immunostaining analysis showed that hnFSSCs were positive for CD45 and CD34 and negative for CD90. According to LC-MS/MS *C. olitorius* was rich in flavanols and hydrocinnamic acid derivatives. Although the spheroids obtained were loose and floating, the cells interacted with each other. Caspase-3 activity was higher in the control group than in the extract-treated group and Ki-67 was higher in the extract-treated group than in the control group, suggesting that the plant might have the capacity to increase stem cell proliferation due to its rich polyphenolic content.

Conclusion: The results suggest that hnFSSCs and spheroids might be used in stem cell generation, tissue repair and renewal as human foreskin tissue has potential to be used as a stem cell source. *C. olitorius* also increased proliferation of hnFSSCs, showing that polyphenols might increase proliferation of stem cells.

Key words: *Corchorus olitorius*, spheroid, human foreskin, stem cell

ÖZ

Amaç: Mezenkimal kök hücreler kendi kendini yenileyebilme özelliğine sahiptir. Sünnet derisinin kök hücre kaynağı olarak kullanılma potansiyeli vardır. Çalışmanın amacı, yeni doğmuş insan sünnet derisi izole hücrelerinden (hnFSSCs) sferoid oluşumunu elde etmektir. Buna ek olarak, Kıbrıs'a özgü geleneksel bir bitki olan *Corchorus olitorius* L. (*C. olitorius*) bitkisinin hnFSSCs sferoidleri üzerindeki apoptotik ve proliferatif etkileri de araştırılmıştır.

Gereç ve Yöntemler: Rutin sünnet prosedüründen sonra hücreler izole edildi ve uygun besi yeri ortamında kültüre edildi. Bitki yaprakları etanol ile ekstre edildi ve içerik analizi sıvı kromatografi-kütle spektrometresi (LC-MS/MS) yöntemi ile yapıldı. Sünnet derisi kök hücreleri CD45, CD34, CD90 antikorları kullanılarak immünositokimyasal olarak karakterize edilmiştir. hnFSSC sferoidleri asılı damla tekniği kullanılarak oluşturuldu. Elde edilen sferoidler daha sonra *C. olitorius* ekstraktı ile 48 saat süre muamele edildikten sonra kaspaz-3 ve Ki-67'nin dağıtımı için immünofloresan boyama yöntemiyle boyandı.

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Bulgular: İmmünoboyama analiz sonuçları, hnFSSC'lerin CD45, CD34 için pozitif fakat CD90 için negatif olduğunu gösterdi. LC-MS/MS sonuçlarına göre *C. olitorius* bitkisinin, flavanoller ve hidro-sinnamik asit türevleri içerdiği saptanmıştır. Elde edilen sferoidler gevşek ve yüzer vaziyette olmalarına rağmen hücreler birbirleri ile etkileşim halindeydi. Kaspaz-3 aktivitesi kontrol grubunda ekstrakt grubuna göre daha yüksekti ve ekstraksiyon uygulanan grupta Ki-67 aktivitesi kontrol grubuna göre daha yüksek bulundu. Bu sonuçlar, bitkinin polifenol içeriğinden dolayı kök hücre proliferasyonunu artırma kapasitesine sahip olabileceğini göstermektedir.

Sonuç: hnFSSC'lerin ve sferoidlerin kök hücre üretimi ve doku onarımı ve yenilenmesinin bir parçası olarak kullanılabilme potansiyeli, sünnet derisinin kök hücre kaynağı olarak kullanılması durumunda mevcuttur. *C. olitorius* içerdiği polifenollerinden dolayı kök hücrelerin proliferasyonunu artıran etki göstermeyi başarmıştır.

Anahtar kelimeler: *Corchorus olitorius*, sferoid, sünnet derisi, kök hücre

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent, self-renewing adult stem cells that are isolated from multiple tissues such as adipose tissue, bone, umbilical cords, dental pulp, and skin. MSCs are fibroblast-like cells and *in vitro* studies have shown that they have the potential to differentiate into adipocytes, osteoblasts, and chondrocytes.[1] According to the International Society of Cellular Therapy criteria, human MSCs are defined by positive expression for cell surface markers including CD29, CD44, CD90, CD49a-f, CD51, CD73 (SH3), CD105 (SH2), CD106, CD166, and Stro-1 and lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR surface molecules.[2] Because of their easy isolation and lack of ethical issues, MSCs are among the first stem cell types to be used in the treatment of various conditions, including autoimmune diseases, orthopedic injuries, and liver and cardiovascular diseases.[3]

Skin is the largest organ of the human body and a source of multipotent mesenchymal cells with the capacity for multipotential differentiation. Human newborn foreskin tissue is part of the skin that is obtained by noninvasive techniques and can proliferate without cell differentiation over a long period.[4] Recent studies reported that human foreskin isolated cells (hnFSSCs) have stem cell properties and multipotent and pluripotent abilities. Skrzypczyk et al.[5] showed that storage of hnFSSCs and newborn foreskin tissue might be very beneficial in terms of disease development potentials and treatment actions.

Spheroids are 3D cell culture models to be used as *in vitro* models for screening new anticancer therapeutics. There are multiple methods for spheroid creation, namely hanging drop, spinner culture, nonadhesive hydrogel micromolds, pellet culture, liquid overlay, rotating wall vessel, external force, cell sheets, and microfluidics.[6] 3D spheroids models have been shown to be advantageous compared to traditional two dimensional (2D) cell culture. 2D monolayer culture mostly focuses on cell growth conditions, cell proliferation, and gene and protein expression profiles. However, 3D spheroids are able to accurately mimic some properties of normal or tumor tissue structure, such as their micro-environments, spatial architecture, physiological responses, signaling cascades, gene expression patterns, and drug resistance mechanisms. Thus, the behavior of 3D cultured cells is more reflective of *in vivo* cellular responses.[7]

Corchorus olitorius L. (*C. olitorius*) is a plant that is commonly consumed in Eastern Mediterranean and Middle Eastern countries. The plant is known to have medicinal properties, showing anti-inflammatory, anticancer, antibacterial, and antioxidant effects.[8-11] It is also known that the plant content is rich in polyphenols, antioxidant vitamins, and minerals that are part of endogenous antioxidant systems.[8,9,12] *C. olitorius* contains quercetin and its derivatives and chlorogenic acid derivatives, which are thought to provide the plant with its medicinal properties.[9,12,13] Polyphenols also tend to improve proliferation and have the potential to increase stem cell viability due to differentiation in stem cells.[14,15]

The aim of the present study was to obtain spheroid formation of hnFSSCs isolated from newborn human foreskin tissue. Furthermore, the proliferative and apoptotic effects of *C. olitorius* on hnFSSC spheroids were assessed.

MATERIALS AND METHODS

Isolation and culture of human foreskin stem cells

Human newborn foreskin tissue was obtained following routine circumcisions. Foreskin samples were obtained from donors 4 to 40 weeks of age at Near East University Hospital after informed consent was obtained from their parents. The study was approved by Near East University Health Sciences Ethics Committee (YDU/2018/62-658). The mucosa part of the foreskin was collected. The mucosa was digested enzymatically with 1 mg/mL collagenase type 1 (Sigma, C0130) for 1 h at 37 °C and 5% CO_2. Cells were collected and centrifuged to remove collagenase. The hnFSSCs were cultured in DMEM-F12 medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 25 µg/mL amphotericin B in a humidified atmosphere at 37 °C and 5% CO_2. When the cultured cells reached 80% confluence state, they were subcultured using 0.25% trypsin-EDTA solution (Biochrom, L 2143) for further studies.

Characterization of human foreskin stem cells

hnFSSCs were characterized immunocytochemically for distribution of CD45, CD34, and CD90 (Thy-1 glycoprotein). The hnFSSCs were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) at 4 °C for 30 min. For permeabilization, 0.1% Tween 20 (Sigma-Aldrich) was added for 15 min on ice. The cells were washed with PBS and endogenous peroxidase activity was quenched by incubation with 3% H_2O_2 for 5 min at room temperature. After the cells were washed with PBS three times for 5 min, primary antibodies anti-CD45 (sc-1178), anti-CD34 (sc-74499), and anti-CD90 (Thy-1 glycoprotein) (sc-19614) were added, followed by incubation overnight at 4 °C.

Biotinylated secondary antibody and streptavidin-peroxidase (Histostain-Plus, IHC Kit, HRP, 859043, Thermo Fischer) were added and each secondary antibody was incubated for 30 min followed by PBS wash (×3) for 5 min. Cells were then stained with diaminobenzidine for 5 min for enhancement of immunolabeling. After being washed with distilled water, they were counterstained with Mayer's hematoxylin for 5 min and mounted with mounting medium (Merck Millipore, 107961, Germany). All specimens were examined under a light microscope (Olympus BX40, Tokyo, Japan).

Plant material and extraction

Mature *C. olitorius* leaves were collected from Kyrenia, Cyprus. The collected plant sample was registered with the Near East Herbarium at Near East University under the Herbarium number 6904. The dry leaves of *C. olitorius* (100 g) were powdered (Waring Commercial Blender, USA) and extracted with 80% ethanol during incubation overnight at room temperature with occasional stirring. The extract was vacuum filtered and concentrated to 200 mL by rotary evaporator (BUCHI Rotavapor R-210). The extract was evaporated and lyophilized (Christ Alpha 1-4 LD Plus, Germany) to yield 14.8 g of crude extract.

Liquid chromatography-mass spectrometry (LC-MS/MS) analysis of C. olitorius leaf extract

The extract composition of *C. olitorius* leaves was investigated by LC/MS-MS analysis. LC separation was performed using an Agilent 1200 high performance LC (HPLC) system (Agilent, USA) equipped with an automatic degasser, a quaternary pump, and an autosampler. Chromatographic separation was carried out on a Waters SunFire™ C18 column (150 mm×4.6 mm, 5 m) at 40 ˚C. The flow rate of the mobile phase was maintained at 0.5 mL/min. The mobile phases were (A) acetonitrile:water:formic acid (10:89:1, v/v/v) and (B) acetonitrile:water:formic acid (89:10:1, v/v/v). The HPLC system was connected to a 3200 Q TRAP LC/MS/MS system with a hybrid triple quadrupole/LIT (linear ion trap) mass spectrometer equipped with an ESI ion source (Applied Biosystems/MDS Sciex, USA). The instrument control and data acquisition were carried out by the software Analyst 1.6.

Cell viability and growth assay

The extract was dissolved with dimethyl sulfoxide [(DMSO), Sigma-Aldrich)] to 100 mg/mL. It was further diluted in culture medium (5 µg/mL, 10 µg/mL, 20 µg/mL, 50 µg/mL, and 100 µg/mL). The final concentration of DMSO in cell lines was less than 0.05%. hnFSSCs were collected, suspended in medium, and seeded in 96 well culture dishes at a density of 5×10^4/mL cells in each well with 100 µL of medium. The hnFSSCs were incubated for 24 h and 48 h.

Cell viability was estimated by MTT assay. MTT solution (Biotium, #30006) was heated to 37 ˚C and then 10 µL was added to each well. After 4 h incubation at 37 ˚C in 5% CO_2, 200 µL of DMSO was added to dissolve the formazan salts. The absorbance was measured at 570 nm with a spectrophotometer (Versa Max, Molecular Device, Sunnyvale, CA, USA).

Preparation of the 3D spheroid model and determination of the effects of C. olitorius leaf extract

hnFSSC spheroids were formed using the hanging drop technique with 600 cells per 20 µL droplet (Y5, Fermenne 2013). The cells were incubated in a humidified atmosphere at 37 ˚C in 5% CO_2 for 36 h and 72 h. Two different culture time spheroids were collected and transferred in two different 24 well plates and all of them were incubated with 50 µg/mL *C. olitorius* extract for 48 h.

Immunofluorescence of 3D spheroids

The spheroids were fixed with 4% paraformaldehyde at room temperature for 30 min and then washed three times with PBS. The spheroids were then embedded in OCT compound (Jung, 0201-08926) and cross-sectioned with a cryostat at 8 µm thickness. Sections were kept at -20 ˚C until the staining procedure. The sections were warmed at room temperature overnight and washed with PBS for 2×30 min at 37 ˚C. The sections were traced around with a PAP pen (Diagnostic BioSystems, K039). The blocking solution [10% sheep serum (sc-2488) in PBS with 0.05% Triton X-100)] was added, followed by incubation for 1 h. The blocking solution was aspirated and primary antibodies [rabbit polyclonal anti-caspase-3 (sc-98785) and mouse monoclonal anti-Ki-67 (BioGenex mv370-uc)] diluted in 2% sheep serum in PBS with 0.05% Triton X-100 were added overnight in a humidified chamber at 4 ˚C. The cells were then incubated with secondary antibodies (goat anti-rabbit TRITC sc-2091, goat anti-mouse FITC Millipore AP308F) for 2 h after washing with PBS with 0.05% Triton X-100. They were then washed and stained with DAPI (Applichem A1001-0025) for 2 min. All sections were covered with mounting media (JA1750) and then evaluated under a fluorescence microscope (Olympus IX71, Tokyo, Japan).

Staining of Ki-67 and caspase-3 was also graded semiquantitatively using the intensity of staining with a value of 1, 2, or 3 (mild, moderate, or strong, respectively).

Statistical analysis

The results were analyzed using GraphPad Prism 7 software. The results were expressed as mean ± standard deviation and standard error where appropriate.

RESULTS

Cell morphology

Fibroblast-like and spindle-formed cells were isolated from the mucosal part of human newborn foreskin tissue. After 4 days, fibroblast-homologous, spindle-formed morphology cells were visible and it had been observed that the cells covered the surface after 7 days. After subculture of the cells, the proliferation rate was triggered and accelerated (Figure 1).

Immunocytochemical characterization of human foreskin stem cells

Immunostaining analysis showed that the hnFSSCs were positive for CD45 and CD34 and negative for CD90. Mucosa-derived foreskin fibroblast-like stromal cells expressed MSC surface markers at passage 1 (Figure 2).

LC-MS/MS Analysis of C. olitorius extract

The extract of *C. olitorius* was analyzed by LC-MS/MS. Caffeoyl glucose, 3-caffeoylquinic acid, quercetin glucoside, quercetin acetylglucoside, 3,5-dicaffeoylquinic acid, 1,3-dicaffeoylquinic acid, luteolin, and/or kaempferol acetylglucoside were identified in *C. olitorius* extract (Table 1).

Cell viability and cytotoxicity

The hnFSSCs were treated with different concentrations (5-100 µg/mL) of *C. olitorius* extract for 24 and 48 h. None of the dilutions showed any cytotoxic effects on the hnFSSCs and 50 µg/mL concentration at 48 h incubation period was optimal as cell viability was 100% (Figure 3).

Effects of C. olitorius extract on the 3D culture hnFSSC spheroids model

The cells started to aggregate after 24 h (Figure 4). After 36 and 72 h, two different groups of spheroids were collected and placed in 6 well plates. The clumps increased in size with time until day 7 and started to disintegrate after 7 days of incubation. Day 7 was therefore chosen as the time for collecting the spheroids for assays.

Immunoreactivity of caspase-3 was detected in both the control and extract-treated groups. However, the intensity of caspase-3 was less in the extract-treated group than in the control group (Table 2). Immunoreactivity of caspase-3 was higher in the 36-h-incubated control group than in the extract-treated spheroid group (Figure 5). Immunostaining intensity

Table 1. Main identified components of *Corchorus olitorius* extract

Rt	$(M-H)^-$	MS^2	Identified as
4.1	341	179, 161	Caffeoyl glucose
4.7	353	191, 179	3-Caffeoylquinic acid
9.9	463	299, 271, 255	Quercetin glucoside
10.9	505	299, 271, 255	Quercetin acetylglucoside
11.5	515	353, 191, 179, 173	3,5-Dicaffeoylquinic acid
12.1	515	353, 191, 179, 135	1,3-Dicaffeoylquinic acid
12.6	489	284, 255, 227	Luteolin/kaempferol acetylglucoside

Table 2. The intensity of caspase-3 and Ki-67 immunolabeling in hnFSSC spheroids treated with *Corchorus olitorius* extract at 50 µg/mL concentration for 36 and 72 h

	Corchorus olitorius extract group		Control group	
	36 h	72 h	36 h	72 h
Caspase-3	+	++	++	++
Ki-67	++	+++	+	-

hnFSSC: Human newborn foreskin stem cell

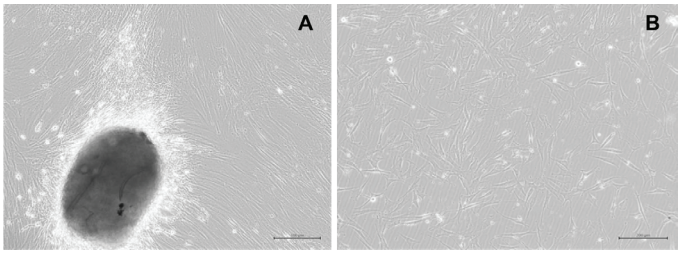

Figure 1. hnFSSCs. (A) Basal photomicrographic representation of cells on day 4 of isolation. (B) Mucosa-derived hnFSSC morphologies at passage 1. scale bars=200 µM

hnFSSCs: Human newborn foreskin stem cells

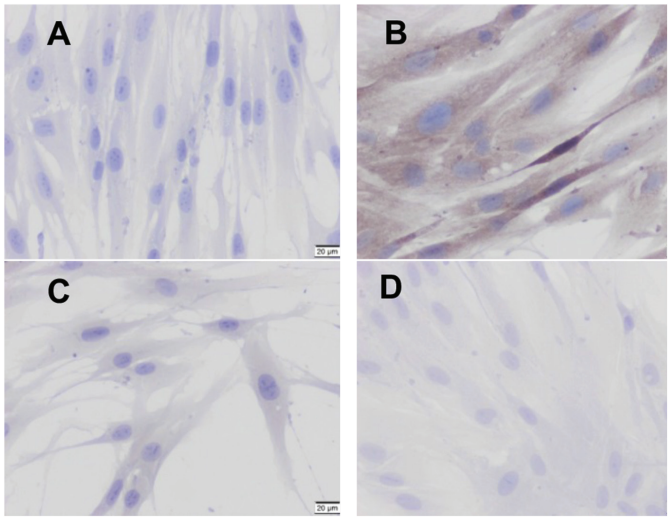

Figure 2. Immunochemical staining indicated the positive mesenchymal stem cell surface markers CD45 and CD34 and negative for CD90. Negative control (A), CD45 (B), CD34 (C), CD90 (D). Scale bars=20 µM

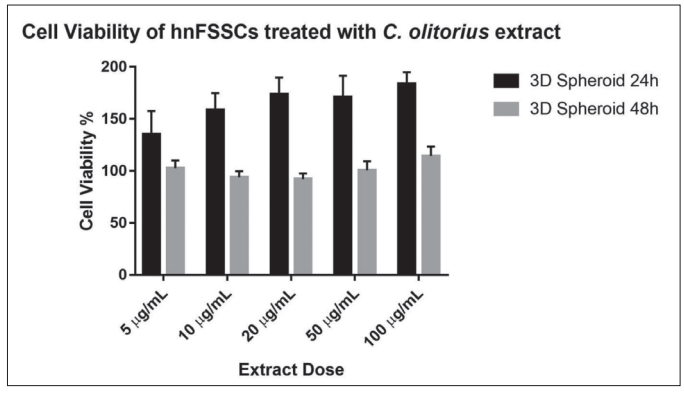

Figure 3. Effect of *Corchorus olitorius* extract on cell viability of hnFSSCs. The data are given as mean ± standard deviation

hnFSSCs: Human newborn foreskin stem cells

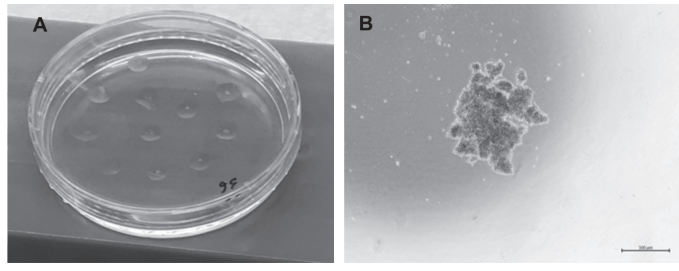

Figure 4. (A) Hanging drops (600 cells/20 µL) on the lid of a petri dish, (B) 7 day incubated spheroid. Scale bars=500 µM

for Ki-67 was moderate to strong for spheroids treated with extracts for 36 and 72 h (Table 2). As shown in Figure 6, Ki-67 immunoreactivity was weak or negative in the control groups (Figure 6).

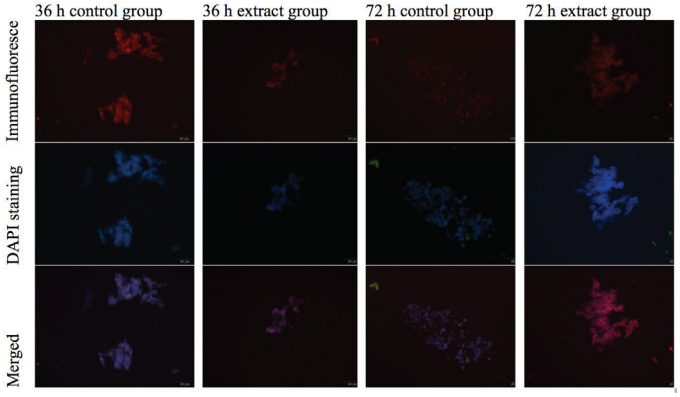

Figure 5. Immunofluorescence, DAPI staining, and merged photomicrographs of caspase-3 in 36 and 72 h hnFSSC spheroids treated with 50 µg/mL *Corchorus olitorius* extract for 48 h. Scale bars=50 µM
hnFSSC: Human newborn foreskin stem cell

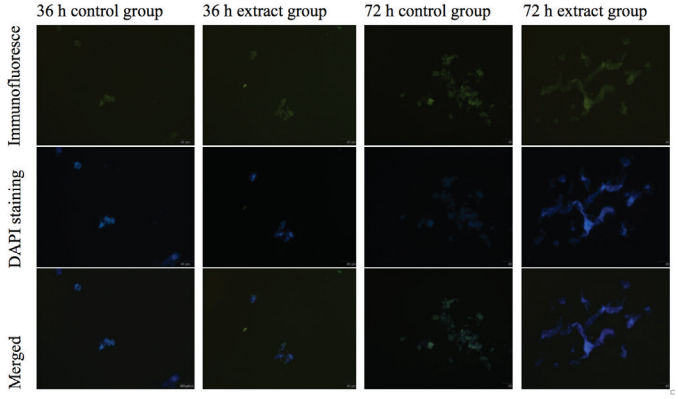

Figure 6. Immunofluorescence, DAPI staining, and merged photomicrographs of Ki-67 in 36 and 72 h hnFSSC spheroids treated with 50 µg/mL *Corchorus olitorius* extract for 48 h. Scale bars=50 µM
hnFSSC: Human newborn foreskin stem cell

DISCUSSION

Circumcision is a ritual that has been performed for centuries for medical, cultural, or religious reasons. The foreskin removed after surgery is usually discarded. It has been thought that the foreskin tissue might have the potential to be used as a source of stem cells, especially if the procedure is performed in early infancy and the tissue is collected from newborns.[4] The foreskin is usually more easily accessible than other tissues used for stem cell generation. In addition, as the tissue is usually discarded straight after the procedure, subjection to ethical issues might be negligible in terms of stem cell collection. If the collected foreskin tissue is from newborns, the differentiation rate and capacity are higher than in adults and nearly as high as in bone marrow.[4] Most studies' results suggested that hnFSSCs therapy is more beneficial than adult and embryonic stem cell therapies. The fact that positive markers were shown for hemopoietic and neural stem cells is also an indication that foreskin stem cells might be used in treatments for blood cancers, Parkinson's and Alzheimer's.[1] A very similar study found that hnFSSCs can also differentiate in myogenic cells.[3] In parallel to these studies, our results also revealed that hnFSSCs expressed MSC markers. In the current study, hnFSSCs positively expressed CD45 and CD34, which are known to be MSC surface markers. However, CD90 expression was negative. This might have been due to the hnFSSCs collected having originated from mucosal cells. Our results suggested that hnFSSCs are capable of differentiating into MSCs and have potential to be used in tissue renewal and repair.

MSCs play an important role in repairing damaged tissue through their anti-inflammatory properties. Recent studies showed that 3D spheroids of MSCs have high differentiation ability and cell survival when compared with 2D culture. Moreover, 3D MSCs' spheroid structure increases the anti-inflammatory proteins from immune cells.[15] MSC spheroids are widely used in oncology research as they synthesize more extracellular matrix than 2D culture. 3D culture also increases the therapeutic effects of intervention when compared with 2D formation.[12] MSC spheroids are solid aggregates due to upregulated cadherin expression.[16] In our study, hnFSSC spheroids were formed using the hanging drop technique. Our results showed that spheroids collected from hnFSSCs were not as compact as MSC spheroids. We obtained more loose and floating spheroids from hnFSSCs. However, even though the spheroid structure was loose, the cells were intact and interacted with each other.

In the current study, the apoptotic and proliferative effects of the plant *C. olitorius* on spheroids were studied. Caspase-3 is known as an executioner caspase and its trigger induces apoptosis, programmed cell death.[17] On the other hand, expression of Ki-67 is an indication of cell proliferation.[18] Caspase-3 immunostaining was observed in both cell groups, but this was expected in spheroid structures as the center is more compact and the nutrients are harder to diffuse to the center. However, caspase-3 staining intensity was less in extract-treated cells, which shows the plant might prevent stem cell apoptosis. In contrast to these results, Ki-67 immunostaining was higher in extract-treated cells than in the control group in both incubation periods, which indicates that *C. olitorius* might have the capacity to increase stem cell proliferation. The LC-MS/MS results indicated that *C. olitorius* contains polyphenolic compounds including quercetin and caffeoylquinic acid and their derivatives. Other studies also showed similar results, showing that the plant is rich in flavonols and hydroxycinnamic acids.[9,10] A 50 µg/mL dose was regarded as the treatment and optimal dose for further immunofluorescence analysis. In addition, other studies also stated that the plant has apoptotic effects in cancer cell lines via caspase-3 activation.[19] On the other hand, quercetin glucuronide has been shown to increase neural stem cell proliferation and promote migration.[20] Another study showed that quercetin enhanced bone marrow MSC proliferation and osteogenic differentiation.[21] This indicates that *C. olitorius* has the potential to increase stem cell proliferation by its rich polyphenolic content, which might be supportive for stem cell differentiation and better for mimicking *in vivo* structures and further tissue repair.

Study limitations

Flow cytometry could help in the identification of stem cell sources. In addition, the use of Western blotting could enhance Ki-67 and caspase-3 immunofluorescence staining results in terms of identification of protein expression of the antibodies.

CONCLUSION

In summary, the results indicate that hnFSSCs have great potential in stem cell differentiation and potential to be used in stem cell therapy. Moreover, spheroids were obtained from hnFSSCs and *C. olitorius* extract has the potential for enhancing their proliferation activity. All of these indicate that hnFSSCs and using spheroids may be used as a part of future clinical applications. These *in vitro* results also need to be evaluated with animal studies for further progression of hnFSSC spheroids in clinical applications.

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