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

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

METHODS: After routine circumcision procedure the cells were isolated and cultured in suitable medium. The plant leaves was extracted with ethanol and composition analysis was done by LC-MS/MS method. The foreskin stem cells were characterized immunocytochemically for distribution of CD 45, CD34, CD 90. hnFSSC spheroids were formed using the hanging drop technique. The obtained spheroids were then stained by immunofluorescence method for distribution of caspase-3 and Ki-67 after treated with C. olitorius extract for 48 h.

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

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

Keywords: Corchorus olitorius, spheroid, human foreskin, stem cell

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, where in vitro studies has shown that they have the potential to differentiate into adipocytes, osteoblasts and chondrocytes.¹ According to International Society of Cellular Therapy (ISCT) 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, Stro-1 and lack of expressions of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules.² Because of easy isolation and no subjection to ethical
issues, MSCs are among the first stem cell types to be used in the treatment of various conditions, including autoimmune diseases, orthopedic injuries, liver and cardiovascular diseases.\(^3\)

Skin is the largest organ of the human body and source of multipotent mesenchymal cells which have the capacity for multipotential differentiation. Human newborn foreskin tissue is part of the skin that is obtained by non-invasive techniques and can proliferate without cell differentiation over a long period.\(^4\) The recent studies reported that human foreskin isolated cells (hnFSSCs) have stem cell properties, multi-potent and pluripotent abilities. Somuncu et al (2016)\(^5\) showed that storage of hnFSSCs and newborn foreskin tissue might be very beneficial in case 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 spheroids creation, namely: hanging drop, spinner culture, non-adhesive 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 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 tissues structure, such as their micro-environments, spatial architecture, physiological responses, signaling cascades, gene expression patterns and drug resistance mechanisms. Thus, the behaviour of 3D-cultured cells is more reflective of in vivo cellular responses.\(^7\)

Corchorus olitorius L. is a plant which is highly consumed in Eastern Mediterranean and Middle Eastern countries. The plant is known to have medicinal properties showing anti-inflammatory, anti-cancer, anti-bacterial and anti-oxidant effects.\(^8\)\(^-\)\(^11\) It is also known that the plant content is rich in polyphenols, antioxidant vitamins and minerals which are part of endogenous antioxidant systems.\(^8\)\(^,\)\(^9\)\(^,\)\(^12\) C. olitorius contains quercetin and its derivatives and chlorogenic acid derivatives which is thought to provide C. olitorius its medicinal properties.\(^9\)\(^,\)\(^12\)\(^-\)\(^13\) Polyphenols are also tend to improve proliferation and has the potential to increase stem cell viability due to differentiation in stem cells.\(^14\)\(^,\)\(^15\)
The aim of the study was to obtain spheroid formation of hnFSSCs which is isolated from newborn human foreskin tissue. Furthermore, the authors would like to assess the proliferative and apoptotic effects of C. olitorius plant on hnFSSCs spheroids.

**Materials and Methods**

**Isolation and Culture of Human Foreskin Stem Cells**

Human newborn foreskin tissue obtained following routine circumcision. Foreskin sample was obtained from 4 to 40 weeks age donors at Near East University Hospital after obtaining informed consent from donor. The mucosa part of the foreskin was collected. Mucosa was digested enzymatically with 1 mg/mL collagenase type 1 (Sigma, C0130) for 1 hour at 37°C and 5% CO₂. Cells were collected and centrifuged to remove collagenase. The hnFSSCs were cultured in DMEM-F12 medium supplemented with 10% fetal bovine serum 1% penicillin-streptomycine and 25
µg/mL amphotericin B in a humidified atmosphere at 37°C and 5% CO₂. When the cultured cells reached 80% confluence state, they were sub-cultured using 0.25% trypsin-EDTA solution (Biochrom, L 2143) further studies.

**Characterization of Human Foreskin Stem Cells**

Human newborn foreskin stem cells were characterized immunocytochemically for distribution of CD 45, CD34, CD 90 (Thy-1 glycoprotein). The hnFSSCs were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) at 4°C for 30 minutes. For permeabilization, 0.1% Tween 20 (Sigma-Aldich) was added for 15 minutes on ice. The cells were washed with PBS and endogenous peroxidase activity was quenched by incubation with 3% H₂O₂ for 5 minutes at room temperature. After washing cells with PBS three times for 5 minutes, primary antibodies anti-CD 45 (sc-1178), anti-CD34 (sc-74499), anti-CD 90 (Thy-1 glycoprotein) (sc-19614) were added and incubated overnight at 4°C. Biotinylated secondary antibody and streptavidin-peroxidase (Histostain-Plus, IHC Kit, HRP, 859043, Thermo Fischer) were added, each secondary antibody was incubated for 30 minutes followed by PBS wash (x3) for 5 minutes. Cells were then stained with diaminobenzidine (DAB) for 5 minutes for enhancement of immuno-labelling. After washing with distilled water, they were counterstained with Mayer's hematoxylien for 5 minutes 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 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, United States of America, USA) and extracted with 80% ethanol while incubated overnight at room temperature with occasional stirring. The extract was vacuum filtered and concentrated to 200 mL by rotary evaporator (BUCHI Rotavapor R-210). Extract was evaporated and lyophilized (Christ Alpha 1-4 LD Plus, Germany) to yield 14.8 g of crude extract.
LC-MS/MS Analysis of *C. olitorius* leaves Extract

The extract composition of *C. olitorius* leaves was investigated by LC/MS-MS analysis. Liquid chromatography separation was performed using an Agilent 1200 HPLC system (Agilent, USA) equipped with an automatic degasser, a quaternary pump and an autosampler. Chromatographic separation was carried out on an Waters SunFireTM C18 column (150 mm × 4.6 mm, 5 m) at 40°C. The flow rate of mobile phase was maintained at 0.5 mL/min. The mobile phase 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 3200 Q TRAP LC/MS/MS System, 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 analyst 1.6 software.

Cell Viability and Growth Assay

Extract was dissolved with dimethylsulfoxide (DMSO, Sigma-Aldrich) to 100 mg/mL. The extracts were 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 medium. hnFSSCs were incubated for 24h and 48h.

The cell viability was estimated by MTT assay. MTT solution (Biotium, #30006) was heated to 37°C and then 10 µL were added to the each well. After 4h incubation at 37°C in 5% CO₂, 200 µL DMSO was added to dissolve the formazan salts. The absorbance was measured at 570 nm with spectrophotometer (Versa Max, Molecular Device, Sunnyvale, USA).

Preparation of 3D Spheroid Model and Determination Effects of *C. olitorius* leaves Extract

Human newborn foreskin stem cell spheroids were formed using the hanging drop technique with 600 cells per 20 µL droplet (Y5, Fermenne 2013). Cells were incubated in a humidified atmosphere at 37°C in 5% CO₂ for 36h and 72h. 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**

Spheroids were fixed with 4% paraformaldehyde at room temperature for 30 minutes and then washed three times with PBS. The spheroids were then embedded in OCT compound (Jung, 0201-08926) and cross-sectioned with cryostat at 8μm thickness. Sections were kept at -20°C until staining procedure. The sections were warmed at room temperature for overnight and washed with PBS for 2x30 minutes at 37°C. The sections were traced around with a PAP pen (Diagnostic BioSsytems, KO39). The blocking solution [10% sheep serum (sc-2488) in PBS with 0.05% Triton X-100] was added and incubated for 1h. 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, added for overnight in humidified camber at 4°C. The cells were then incubated with secondary antibodies (goat anti-rabbit TRITC sc-2091, goat anti-mouse FITC Milipore AP308F) for 2h 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) then evaluated under a fluorescence microscope (Olympus IX71, Tokyo, Japan).

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

**Results**

**Cell morphology**

Fibroblast-like and spindle-formed cells were isolated from mucosal part of the 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, proliferation rate was triggered and accelerated (Figure 1).

**Immunocytochemical Characterization Human Foreskin Stem Cells**
Immunostaining analysis showed that hnFSSCs were positive for CD45, CD34 and negative for CD90. Mucosa derived foreskin fibroblast-like stromal cells expressed mesenchymal stem cell surface markers at passage 1 (Figure 2).

**LC/MS-MS Results of *C. olitorius* Aqueous Extract**
The analysis of the extract of *C. olitorius* was done by liquid chromatography high performance coupled with mass spectrometry (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**

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

**Effects of *C. olitorius* Extract on 3D Culture hnFSSC Spheroids Model**
The cells started to aggregate after 24 hours (Figure 4). After 36 and 72h, two different group 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 7th day of incubation. Day 7 was therefore chosen as the time for collecting the spheroids for assays. Immunoreactivity of caspase-3 was detected in both control and extract treated groups. However, the intensity of caspase-3 was less in extract treated group than control group (Table 2). Immunoreactivity of caspase-3 was higher in 36h incubated control group than extract treated spheroid group (Figure 5). Immunostaining intensity for Ki-67 was moderate to strong for both extracts treated 36 and 72h spheroids, respectively (Table 2). As shown in Figure 5, Ki-67 immunoreactivity was weak or negative in control groups (Figure 6).
Discussion

Circumcision is a ritual which has been done for centuries either for medical, cultural or religious reasons. The foreskin removed after the surgery is usually discarded and thrown away. It has been thought that the foreskin tissue might have the potential to be used as a source of stem cell especially if the procedure is performed in early infancy and the tissue is collected from newborn.⁴ Foreskin is usually more easily accessible than other tissues which are 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 newborn, the differentiation rate and capacity is higher than adults, nearly as high as bone marrow.\textsuperscript{4} Most studies results suggested that hnFSSCs therapy is more beneficial than adult and embryonic stem cell therapies. Having shown positive markers for haemopoietic and neural stem cells, is also an indication of foreskin stem cells possibility to be used in blood cancers, Parkinson’s and Alzheimer’s treatments.\textsuperscript{1} A very similar study found out that, hnFSSCs can also differentiate in myogenic cells.\textsuperscript{3} In parallel to these studies, our results also stated that hnFSSCs had expressed mesenchymal stem cell markers. In this current study, hnFSSCs positively expressed CD45 and CD34 which are known to be the MSC surface markers. However, CD90 expression was negative. This might be due to the collected hnFSSCs being originated from mucosal cells. Our results suggested that hnFSSCs are capable of differentiating into MSCs and constitute a potential to be used as a part of tissue renewal and repair.

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

In the current study, apoptotic and proliferative effects of \textit{C. olitorius} plant on spheroids were studied. Caspase-3 is known as an executioner caspase and its trigger induces apoptosis, programmed cell death.\textsuperscript{17} On the other hand, expression of Ki-67 is an indication of cell proliferation.\textsuperscript{18} Caspase-3 immunostaining was present in both cell groups however; 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 lesser in extract treated cells which shows
the plant might prevent stem cell apoptosis. As oppose to these results, Ki-67 immunostaining was higher in extract treated cells than control group in both incubation time periods which states 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 stating the plant is rich in flavonols and hydroxycinnamic acids.\(^9\)\(^{-10}\) 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 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 repairment.

**Study Limitations**

Flow cytometry method could help the identification of stem cell source. In addition, using of Western blotting technique could enhance Ki-67 and caspase-3 immunoflorescense staining results, in terms of identification of protein expressions of the antibodies.

**Conclusion**

In summary, the results indicate that hnFSSCs has a great potential in stem cell differentiation and potential to be used in stem cell therapy. Also, 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 hnFSSCs spheroids in clinical applications.

**Acknowledgements**
The authors would like to thank Experimental Health Research Center of Health Sciences laboratory.

**Conflicts of interests**

The authors declare that there are no conflicts of interest.

References


### Tables

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

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<td>353, 191, 179, 173</td>
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**Table 2.** The intensity of caspase-3 and Ki-67 immunolabelling in hnFSSC spheroids treated with *C. olitorius* extract at 50 µg/mL concentration for 36 and 72h.

<table>
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**Figures**

A [image of spheroid]

B [image of cell culture]
Figure 1. Human foreskin stem cells (hnFSSCs). (A) Basal photomicrographic representation of cells at day 4 of isolation. (B) Mucosa derived hnFSSCs morphologies at passage 1. Scale bars = 200 μM.
**Figure 2.** Immunochemical staining indicated the positive mesenchymal stem cell surface markers CD45, CD34 and negative for CD90. Negative control (A), CD45 (B), CD 34 (C), CD 90 (D). Scale bars= 20 μM.

**Figure 3.** Effect of *C. olitorius* extract on cell viability of hnFSSCs. The data are given in mean ± SD.
Figure 4. A) Hanging drops 600 cells/20 µL on the lid of a petri dish, B) 7 days incubated spheroid. Scale bars= 500 µM.
Figure 5. Immunofluorescence, DAPI staining, Merge photomicrographs of caspase-3 in 36 and 72 hours hnFSSC spheroids treated with 50 µg/mL *C. olitorius* extract for 48h. Scale Bars=50µM.
Figure 6. Immunofluorescence, DAPI staining, Merge photomicrographs of Ki-67 in 36 and 72 hours hnFSSC spheroids treated with 50 µg/mL C. olitorius extract for 48h. Scale Bars=50µM.