

METHOD OF ISOLATING MESENCHYMAL STROMAL CELLS AND APPLICATIONS FOR TISSUE ENGINEERING

Field of Invention

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The invention relates to a method of isolating mesenchymal stromal cells, particularly for use in research following subsequent expansion and differentiation thereof.

Background

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Mesenchymal stromal cells are stem cells which are able to develop into a variety of cell types, although as they are pluripotent (as opposed to totipotent), not an embryo that could develop into an organism.

15 As a result, mesenchymal stromal cells are the subject of much research, and a good source of the same is highly desirable.

However, when isolating mesenchymal stromal cells from the source material there remain inconsistencies in the current best practise isolation techniques, which result in consumable wastage. This causes increased production cost. Microbial contamination during isolation is also a common occurrence as is the production of substandard mesenchymal stromal cells.

25 An aim of the invention is therefore to provide an isolation method which overcomes these difficulties.

Summary of Invention

30 In an aspect of the invention there is provided a method of isolating mesenchymal stromal cells comprising the steps of:

washing a tissue sample at least 3 times;

incubating the tissue sample in a solution comprising at least one antibiotic and at least one antimycotic at a temperature of around 28-38°C and atmosphere comprising about 4-6% CO₂;

5 washing the tissue sample with phosphate buffered solution to substantially remove antibiotic and antimycotic therefrom;

cutting the tissue sample into small pieces;

incubating the tissue sample in collagenase dissolved in DMEM-KO at a temperature of around 28-38°C and atmosphere comprising about 4-6% CO₂ to produce a solution of tissue fragments;

10 centrifuging the solution of tissue fragments to form a pellet, which is resuspended and transferred into MSC culture media for subsequent incubation;

characterised in that MSC culture media is added to the tissue sample during the collagenase incubation to quench the action of the collagenase such that the tissue sample is only partially digested.

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Advantageously this produces cells with better viability (83-89% prior to cryopreservation) compared to prior art methods involving complete digestion, whereby the resulting cells are obtained by straining the completely digested tissue - complete digestions result in poor viability and cells which are biologically stressed. An additional advantage is that significantly less consumables are required.

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In one embodiment the MSC culture media is added when the tissue sample is transparent but still substantially solid and not yet liquefied by the collagenase.

25 In one embodiment the initial washing of the tissue sample is with sterile distilled water.

In one embodiment the at least one antibiotic comprises penicillin and streptomycin in PBS.

30 In one embodiment Trypsin-EDTA is added to the solution of tissue fragments and incubated at 28-38°C, 4-6% CO₂ for around 5 minutes to detach cells from the matrix.

In one embodiment the tissue fragments in MSC culture media are incubated in a low oxygen hypoxia or CO₂ incubator for about 24 hours to promote growth of cells. Typically the MSC culture media is repeatedly replaced until the cells reach about 70-80% confluency.

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In one embodiment the cells are expanded through the following steps:

- washing with PBS;

- adding a Trypsin-EDTA solution and incubating at 28-38°C, 4-6% CO₂ to detach cells;

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- adding MSC culture media to the cells;

- centrifuging the cells then resuspending the pellet in MSC culture media;

- seeding the cells into MSC culture media

- incubating in a low oxygen hypoxia or CO₂ incubator until the cells reach 70-80% confluency, replacing the MSC culture media every 2-4 days or so.

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Typically the expansion steps can be repeated several times to progress the cells from passage 0 to passage 3, wherein a final product is formed on resuspending the pellet.

In one embodiment the final product undergoes cryopreservation.

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In one embodiment the tissue sample is derived from umbilical cord, foreskin or cornea.

Typically the arteries, veins and blood components are removed from the umbilical cord while minimising the loss of the Wharton's jelly therefrom. Advantageously this decreases contamination by erythrocytes, as well as any other cells, cell fragments or derivatives thereof from umbilical vein (eg HUVEC). The prior art method does not involve complete removal of vein and arteries and also results in accidental removal of the matrix substance which produces decreased/inferior cell yield.

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30 In one embodiment the umbilical cord is processed in about 3-5 separate segments. Advantageously this minimizes contamination, provides replicate samples, and reduces the consumables required due to the higher surface area:volume ratio. The prior art methods involve the whole umbilical cord being processed as a single sample whereby

erythrocytes, fibrinogen, fibrin, cell debris, cell contents etc., from a region of the cord can act as contaminants during tissue incubation which accelerates microbial contamination.

- 5 With regard to umbilical cord, the tissue sample is typically incubated in about 1-5% (w/v), preferably around 3% (w/v) collagenase type IV for approximately 10-12 hours.

With regard to foreskin, the tissue sample is typically incubated in about 1-5% (w/v), preferably around about 1-5% (w/v), preferably around 3% (w/v) collagenase type IV
10 for approximately 4-10 hours.

With regard to cornea, the tissue sample is typically incubated in about 1-5% (w/v), preferably around 3% (w/v) collagenase type IV for approximately 2-4 hours.

- 15 In one embodiment the tissue sample is dental pulp. Typically in this embodiment the initial washing of the tissue sample is with Dulbecco's phosphate buffered saline without calcium or magnesium.

In one embodiment the dental pulp is extirpated from a freshly extracted deciduous
20 tooth. Typically the tooth is sectioned horizontally at the cemento-enamel junction using a sterilized diamond disc while being irrigated with saline or distilled water.

In this embodiment the tissue sample is typically incubated in about 1-5% (w/v), preferably around 3% (w/v) collagenase type I for about 30 minutes.

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In one embodiment the incubation of tissue samples is conducted at a temperature of around 37°C and atmosphere comprising about 5% CO₂.

In a further aspect of the invention, there is provided a tooth holder comprising:

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a circular rotating chuck fixed to a base;

the chuck including three jaws and a collar, whereby rotation of the collar extends or retracts the jaws thereby adjusting the aperture size between the jaws to allow a tooth to be held thereby;

irrigation means above the chuck for dripping liquid onto the tooth;
characterized in that the chuck includes a central channel through which liquid may be pumped.

- 5 In one embodiment the jaws are provided with grooves or steps to allow for precise measurement and placement of the tooth.

In a further aspect of the invention there is provided a system for immunophenotyping mesenchymal stromal cells comprising the steps of:

- 10 extracting total RNA from a sample of cells;
synthesising cDNA from the RNA;
determining the expression of genes using semi-quantitative RT-PCT analysis by amplifying the cDNA using two or more primer pairs;
characterised in that the genes and respective primer pairs are selected from SEQ
15 ID Nos: 1 and 2; 3 and 4; 5 and 6; 7 and 8; and/or 9 and 10.

Expression of these genes, which are dopaminergic neuronal markers, indicates that the cells are mesenchymal stromal cells capable of creating a dopaminergic cell line that can be used a research grade product e.g. for Parkinson's disease research.

- 20 Typically the sample of cells is a final product.

In a further aspect of the invention there is provided a tissue or product made by differentiating mesenchymal stromal cells produced by the method as herein described.

- 25 In a further aspect of the invention there is provided a dopaminergic cell, neuron or derivative thereof made by differentiating mesenchymal stromal cells produced by the aforementioned method.

- 30 In a yet further aspect of the invention there is provided corneal tissue made by differentiating corneal limbal cells derived from mesenchymal stromal cells derived from a cornea tissue sample according to the aforementioned method.

Typically cornea tissue can be grown in different layers to form a cornea.

In a yet further aspect of the invention there is a provided skin tissue made by differentiating mesenchymal stromal cells derived from a foreskin sample according to
5 the aforementioned method.

This skin tissue may be used for foreskin restorative surgery or human skin grafts, e.g. for use in treating burn victims.

10 **Brief Description of Drawings**

It will be convenient to further describe the present invention with respect to the accompanying drawings that illustrate possible arrangements of the invention. Other arrangements of the invention are possible, and consequently the particularity of the
15 accompanying drawings is not to be understood as superseding the generality of the preceding description of the invention.

Figure 1 illustrates different types of mesenchymal stromal cells in the respective finished products.
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Figure 2 illustrates the ability of different types of mesenchymal stromal cells to form aggregates/neurospheres in NIM media.

Figure 3 illustrates the results of semi-quantitative RT-PCR analysis of the expression
25 of certain genes for immunophenotyping of mesenchymal stromal cells

Figure 4 illustrates immunocytochemistry of different types of mesenchymal stromal cells

30 Figure 5 illustrates a tooth holder according to an embodiment of the invention

Detailed Description

The techniques for isolating stromal cells from mammalian umbilical cord (Wharton's Jelly), dental pulp, foreskin and cornea are described below.

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ISOLATION OF WHARTON'S JELLY STROMAL CELLS

Transporting the umbilical cord upon delivery

Umbilical cord should be clamped ideally within 30 to 60 seconds following birth of the infant placed. The clamp is placed at or below the level of the placenta to allow optimal blood transfer from the placenta to the infant. Sterile surgical blades or any other sterile cutting instruments or implement are used to cut the umbilical cord cut into three to five segments and placed into 50ml polypropylene conical centrifuge tubes pre-added with 25ml of transport buffer stored at 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C. This entire process should be done in a labour room with temperature ranging from 20 °C to 25 °C and the umbilical cord must be placed into the transport buffer within 24 hours upon birth to maintain tissue viability. The umbilical cord is then transported to the laboratory for mesenchymal stromal cell isolation process. Components of transport buffer are as described in Table 1 below.

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Table 1

No.	Transport Buffer Components	Final concentration
1.	Sodium chloride (NaCl)	133 to 140mmol/L
2.	Potassium chloride (KCl)	2.4 to 3mmol/L
3.	Sodium phosphate (Na ₂ HPO ₄)	9.5 to 10.5mmol/L
4.	Potassium phosphate (KH ₂ PO ₄)	1.5 to 2.0mmol/L
5.	Antibiotic-Antimycotic*	5 to 12%
6.	Penicillin-Streptomycin*	5 to 12%
7.	Sterilized distilled water	0.8 to 1.2L
8.	Hydrochloric acid (36%)	0.2 to 1000ml until pH of transport buffer reach 7.2 to 7.5 at 20 °C to 25 °C

Umbilical cord cleaning process

- The umbilical cord is processed in a Class II Biological Safety Cabinet (BSC) with following environment conditions; room temperature ($25^{\circ}\text{C}\pm 5$), relative humidity ($65\%\pm 10$). The following process is carried out entirely in a BSC sterilized with 70% ethanol and 30 minutes UV radiation. The 50ml polypropylene conical centrifuge tubes containing segmented umbilical cords are wiped with 70% ethanol. The following apparatus and materials in Tables 2-3 below are used for the cleaning process.
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Table 2

No.	Apparatus/material	Sterilization method	Quantity/ Volume
1.	Surgical scissors (sharp/sharp)	Autoclave (121°C/20-30 minutes)	5
2.	Surgical scalpels	Autoclave (121°C/20-30 minutes)	5
3.	Surgical blades	Gamma radiated	15
4.	50ml polypropylene conical centrifuge tubes	Gamma radiated	32
5.	Micropipettor (10-100µL)	70% ethanol & UV radiation	1
6.	Micropipettor (100-1000µL)	70% ethanol & UV radiation	1
7.	Micropipette tips (2-200µL)	Autoclave (121°C/20-30 minutes)	96
8.	Micropipette tips (50-1000µL)	Autoclave (121°C/20-30 minutes)	96
9.	Surgical tray	Autoclave (121°C/20-30 minutes)	5
10.	Sterile serological pipettes (5ml)	Gamma radiated	15
11.	Sterile serological pipettes (10ml)	Gamma radiated	15
12.	Sterile serological pipettes (50ml)	Gamma radiated	15
13.	Sterilized distilled water (SDW)	Autoclave (121°C/20-30 minutes)	2L
14.	Pipettor	70% ethanol & UV radiation	1
15.	Phosphate buffered saline (PBS)	Autoclave (121°C/20-30 minutes)	2L
16.	Antibiotic-Antimycotic (100X)*	Sterile filtration (0.2µm)	10%v/v
17.	Penicillin-Streptomycin (100X)*	Sterile filtration (0.2µm)	10%v/v
18.	Collagenase type IV*	Sterile filtration (0.2µm)	3%w/v
20.	Ethanol	Not required	70%v/v
21.	Cell culture plate (60mm)	Gamma radiated	15
22.	DMEM-KO*	Sterile filtration (0.2µm)	97%v/v
23.	Trypsin-EDTA (0.25%)*	Sterile filtration (0.2µm)	2%v/v
24.	MSC culture media (see Table 3)	Sterile filtration (0.2µm)	1L
25.	Tissue forceps	Autoclave (121°C/20-30 minutes)	7

Table 3

No.	MSC culture media components	Final Volume (ml)	Concentration (%)
1.	DMEM-KO	880	80 to 90
2.	Antibiotic-Antimycotic	5	0.2 to 1
3.	Penicillin-Streptomycin	5	0.2 to 1
4.	Fetal Bovine Serum (FBS)	100	5 to 12
5.	Glutamax	10	0.5 to 1.5

- Upon transportation of the umbilical cord to the laboratory, the cord is segmented into 5 parts and the segmented cord is transferred into five individual 50ml polypropylene conical centrifuge tubes pre-added with 30ml SDW. Segmented cords are washed by inverting the tubes repeatedly to remove blood traces to prevent blood contamination. This step is repeated at least three times to ensure the cords are thoroughly washed using fresh SDW and 50ml polypropylene conical centrifuge tubes.
- 5 The segmented cords are transferred to the surgical tray to remove blood clots and traces of any components contained within blood including but not limited to neutrophils, lymphocytes, plasma, and erythrocytes. This is to prevent blood, blood related products, and any other products from causing contamination. Umbilical cord contains two umbilical arteries and one umbilical vein, both embedded within a specific
- 10 mucous proteoglycan-rich matrix which is known as Wharton's Jelly, which is then covered by amniotic epithelium. The umbilical arteries and vein are carefully removed by means of dissection without losing the matrix substance in which most of the Wharton's Jelly Stem Cells (WJSCs) are found. WJSCs can be isolated from three regions of the umbilical cord, namely, the perivascular zone, the inter-vascular zone and
- 15 the sub-amnion. The cord is then immersed in 70% ethanol for 25 to 30 second to sterilize the surface and immediately immersed into SDW for another 25 to 30 seconds to remove ethanol residues and create an immediate hypotonic environment on the cord's surface to eliminate microbes.
- 20 The cords are then transferred into five individual 50ml polypropylene conical centrifuge tubes pre-added with 25ml 10% v/v Antibiotic-Antimycotic and 10% v/v Penicillin-Streptomycin in PBS. The tubes are incubated at around 28-38°C, about 4-6%

CO₂ for around 2-16 hours, preferably 37°C, 5% CO₂ for 2 hours/overnight. To minimize contamination, the umbilical cord is processed as five individual segments until the end of the isolation process. The incubator is programmed at around 28-38°C, about 4-6% CO₂, preferably at 37°C, 5% CO₂ to provide an optimal environment for the
5 cord tissues/cells to maintain viability.

Wharton's jelly stromal cell isolation process

Prior to incubation, each segmented cord is washed with PBS twice for 30 to 60 seconds, to remove traces of Antibiotic-Antimycotic and Penicillin-Streptomycin. The
10 cords are cut into small pieces (2-5mm) and transferred to a fresh 50ml polypropylene conical centrifuge tubes containing 20ml of 3% w/v collagenase type IV dissolved in DMEM-KO. Cords are allowed to be partially digested at around 28-38°C, about 4-6% CO₂ for around 2-16 hours, preferably 37°C, 5% CO₂ for 10 to 12 hours. Then 400µl of 0.25% Trypsin-EDTA is added into the partially digested cord and incubated at 37°C,
15 5% CO₂ for 5 minutes to detach cells from the matrix. To neutralize the enzymatic action, 20ml of MSC culture media is added into each tubes and centrifuged at about 1500-2500rpm, 700-1000g force/RCF, for around 8-12minutes, at about 25-28°C, preferably about 1800rpm for 10 minutes at 25°C. It is crucial to neutralize the enzymatic action as cell viability will reduce if Trypsin is allowed to stay in contact
20 with the cells for too long. Prior to centrifugation, 50% of the supernatant is carefully removed by pipetting to eliminate excess MSC culture media and Trypsin.

Undigested semi-transparent umbilical cord fragments from the tubes are picked using sterile tissue forceps and transferred into 60mm cell culture plate. The process is
25 repeated till all umbilical cord fragments are transferred and left to air dry for 15minutes in the BSC. This step is to ensure the tissues are attached to the culture plate surface. Upon adherence of the cord tissue fragments onto the culture plate, 5-7ml of MSC culture media is added gently without disrupting the attached tissues. The MSC culture media provides sufficient nutrients and an optimal environment for the survival of the
30 tissues. Culture plates are then transferred to a CO₂ incubator or low oxygen hypoxia incubator and incubated for around 16-36 hours, preferably about 24 hours. After 24 hours incubation, 2/3 of the media together with some matrix substance are pipetted out gently without disrupting adhered tissues and replaced with fresh MSC culture media.

The culture plates are transferred back to the CO₂ incubator and an additional 5ml of fresh MSC culture media is added in the next 24 hours for continuous supply of nutrients for cell growth and also to remove dead or apoptotic cells from the culture. The culture is observed using an inverted microscope every day and 2/3 of media is replaced every 2 to 4 days until the cells reach 80% confluency until subcultured to passage 1 stock. Around 70-80%, preferably about 80% cell confluency is used as the end point for each passage to maintain cultures with optimal cell numbers and viability.

ISOLATION OF DENTAL PULP STROMAL CELLS

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Tooth structure can be divided into two parts anatomically, namely the crown and the root, separated by the cemento-enamel junction (CEJ). The CEJ is the crown part that consists of enamel, dentin and pulp chamber. The anatomic root is located below the CEJ that is covered with cementum and also has a pulp canal in the root area. The development of teeth begins embryologically as a series of interactions between the oral epithelium and neural crest-derived ectomesenchymal cells of the early jaw.

Dental pulp consists of loose connective tissue that is confined within the pulp chamber and root canals of the tooth. The composition of a dental pulp is by weight 75% water and 25% organic material comprised of a plentiful matrix of cells. Cells such as odontoblasts, fibroblasts, undifferentiated ectomesenchymal cells, macrophages and other immunocompetent cells are the principal fibres that can be found within the dental pulp. Cells within the pulp provide the mature pulp with odontogenic, nutritive, sensory and defensive functions that allow for the preservation of vitality during normal homeostatic maintenance and post injury repair and regeneration. Odontoblasts are highly specialized cells that are responsible for the secretion and mineralization of the fibrillar extracellular matrix of dentin. Fibroblasts are important in formation and maintenance of the pulp matrix consistency of collagen. Undifferentiated ectomesenchymal cells are found throughout the pulp core which is therefore a source of material for stromal cell applications.

Extraction of dental pulp from tooth

An intra-oral examination is done to assess the pulp status of the subjects. Samples are collected from the pulp tissue that is extirpated from freshly extracted deciduous teeth. The extracted deciduous tooth is kept in a specimen container filled with normal saline
5 to maintain the moisture content within the tooth. The cutting of the tooth and extirpation of the dental pulp tissue should commence as soon as possible after tooth extraction from the subject. Extracted teeth are sterilized externally with Povidone iodine (PVP-I, Sigma Aldrich, St Louis, USA) and washed with distilled water to minimize the contaminants from the tooth surface contaminating the extirpated dental
10 pulp tissue.

A tooth holder is used to hold the teeth securely, as illustrated in Figure 5. The tooth holder comprises a modified circular rotating drill chuck 2 with three jaws 4 fixed to a stainless steel base 6. The drill chuck includes a central channel 8 through which water
15 may be pumped via tube 10 to deploy saline or distilled water to the tooth and for cleaning the device. The jaws 10 may be extended or retracted via rotation of the collar 12, which also alters the aperture size at the tips of the jaws to allow a tooth 14 to be gripped firmly. The jaws may be provided with grooves 16 or steps to allow for precise measurement and placement of the tooth to optimize dental pulp extirpation. Water may
20 be also dripped from a tube 18 located above the chuck 2.

The tooth is sectioned horizontally at the cemento-enamel junction using a sterilized diamond disc straight hand piece to expose the pulp. Extirpation of the dental pulp tissue is done under aseptic conditions. Copious irrigation with saline or distilled water is
25 provided throughout the procedure which acts as a lubricant during and throughout the process, and also serves to reduce the amount of heat generated from the cutting process. This minimizes the destruction of tissue from heat. The dental pulp tissue is then transferred into a 1.5 ml tube containing DMEM Knock Out Basal Media (Gibco, Grand Island, NY). The extirpated pulp tissue sample is immediately sent to the
30 laboratory for isolation process.

Dental pulp stromal cell isolation process

Dental pulp tissues are processed in a Class II Biological Safety Cabinet (BSC) with the following environment conditions; room temperature ($25^{\circ}\text{C}\pm 5$), relative humidity ($65\%\pm 10$). The following process is carried out entirely in a BSC sterilized with 70% ethanol and 30 minutes UV radiation. The 1.5ml microcentrifuge tubes containing dental pulp tissues are wiped with 70% ethanol. The dental pulp tissues are washed three times in a washing buffer solution that contains Dulbecco's Phosphate-Buffered Saline without calcium and magnesium (DPBS - Ca^{2+} , - Mg^{2+} , Invitrogen, USA), 0.85% of penicillin-streptomycin and 0.85% of antibiotic-antimycotic. Then, the tissue is minced into small fragments prior to Collagenase Type I treatment (Gibco, Grand Island, NY). After that, the tissue is incubated at 37°C for 30 minutes. Next, the tissue is transferred into the 15 ml tube (BD Bioscience, Franklin Lakes, NJ, USA) containing 8 ml of MSC culture media. The tissue is centrifuged for 6 minutes at 1250 rpm. The supernatant is discarded and the pellet is gently resuspended with 10 ml of MSC culture media. Then, the cells are seeded in a T25 culture flask together with the growth media and incubated in a 5% CO_2 incubator humidified with 95% of air or using a low oxygen hypoxia incubator. The medium is replaced every three days and monitored for any signs of contamination. After the primary culture becomes confluent in approximately 10–20 days, cells are collected by trypsinization using 0.05% Trypsin-EDTA (Gibco, invitrogen) and processed for subsequent subcultures.

ISOLATION OF FORESKIN STROMAL CELLS

Transporting the foreskin upon circumcision

Foreskin fibroblast-like stromal cells (FDSCs) are progenitors isolated from human tissue that can differentiate into many cell types. These cells are mainly found on the dermis of the foreskin. Human foreskin is obtained from neonates, children, teenagers or adults of male sex during circumcision and placed into sterile 50ml polypropylene conical centrifuge tubes pre-added with 25ml of transport buffer stored at 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C. This entire process should be done in a room with temperature ranging from 20°C to 25°C and the foreskin must be placed into the transport buffer solution within 24 hours to maintain tissue viability. The foreskin is then transported to the laboratory for mesenchymal stromal cell isolation.

Foreskin cleaning process

Foreskin tissues are processed in a Class II Biological Safety Cabinet (BSC) with the following environment conditions; room temperature ($25^{\circ}\text{C}\pm 5$), relative humidity ($65\%\pm 10$). The following process is carried out entirely in a BSC sterilized with 70% ethanol and 30 minutes UV radiation. The 50ml polypropylene conical centrifuge tubes containing the foreskin tissues are wiped with 70% ethanol. The sample is then transferred into a 50ml polypropylene conical centrifuge tubes pre-added with 30ml SDW. The sample is washed by inverting the tubes repeatedly to remove traces of blood and to prevent any contamination. This step is repeated at least three times to ensure the foreskin is thoroughly washed using fresh SDW in a 50ml polypropylene conical centrifuge tube. The foreskin is then immersed in 70% ethanol for 25 to 30 seconds to sterilize the surface and immediately immersed into SDW for another 25 to 30 seconds to remove ethanol residues and create an immediate hypotonic environment on the skin's surface to eliminate microbes. The tissue is then transferred into a 50ml polypropylene conical centrifuge tubes pre-added with 25ml 10% v/v Antibiotic-Antimycotic and 10% v/v Penicillin-Streptomycin in PBS. The tubes are incubated at around 28-38°C, about 4-6% CO₂ for around 2-16 hours, preferably 37°C, 5% CO₂ for 2 hours/overnight. The incubator is programmed at 37°C, 5% CO₂ to provide an optimal environment for the cord tissues/cells to maintain viability.

Foreskin fibroblast-like stromal cell (FDSC) isolation process

Prior to incubation, the sample is washed with PBS twice for 30 to 60 seconds, to remove traces of Antibiotic-Antimycotic and Penicillin-Streptomycin. The foreskin is then cut into small pieces (2-5mm) and transferred to a fresh 50ml polypropylene conical centrifuge tubes containing 20ml of 3% w/v collagenase type IV dissolved in DMEM-KO. The sample is allowed to be partially digested at around 28-38°C, about 4-6% CO₂, preferably 37°C, 5% CO₂, for 4 to 10 hours. 400µl of 0.25% Trypsin-EDTA is added into the partially digested sample and incubated at 37°C, 5% CO₂ for 5 minutes to detach cells from the tissue. To neutralize the enzymatic action, 20ml of MSC culture media is added into the tube and centrifuged at about 1500-2500rpm, 700-1000g force/RCF, for around 8-12minutes, at about 25-28°C, preferably about 1800rpm for 10 minutes at 25°C. It is crucial to neutralize the enzymatic action as cell viability will be reduced if Trypsin remains in contact with the cells for too long (typically it should not exceed 15 minutes). Prior to centrifugation, 50% of the supernatant is carefully removed by pipetting to eliminate excess MSC culture media and Trypsin. Undigested semi-transparent foreskin tissue fragments from the tube are transferred using sterile tissue forceps into a 60mm cell culture plate. This process is repeated until all foreskin fragments are transferred and left to air dry for 15minutes in the BSC. This step is to ensure the tissues adhere to the culture plate surface. Upon adherence of the tissue fragments onto the culture plate, 5-7ml of MSC culture media are added gently without disrupting the attached tissues. The MSC culture media provides sufficient nutrients and optimal environment for the survival of the tissues. Culture plates are then transferred to a CO₂ incubator or low oxygen hypoxia incubator and incubated for 24 hours. After 24 hours incubation, 2/3 of the media is pipetted out gently without disrupting adhered tissues and replaced with fresh MSC culture media. The culture plates are transferred back to the incubator and an additional 5ml of fresh MSC culture media is added in the next 24 hours for continuous supply of nutrients for cell growth and also to remove dead or apoptotic cells from the culture. The culture is observed using an inverted microscope every day and 2/3 of media is replaced every 2 to 4 days until the cells reach 80% confluency until subcultured to passage 1 stock. 80% cell confluency is used as the end point for each passage to maintain cultures with optimal cell numbers and viability.

ISOLATION OF CORNEAL LIMBAL EPITHELIAL STROMAL CELLS

Corneal limbal epithelial stromal cells (CLSCs) is a non-keratinized epithelium composed of a cluster of cells with a capacity for self-renewal. These cells are localized at the basal cell layer of the limbus which is responsible for the regeneration of corneal epithelium and to maintain the cornea. CLSCs differ from the corneal epithelial cells due to the lack of corneo-specific differentiation keratins (K3/K12) expression, connexin 43-mediated gap junction intercellular communication, p63 nuclear transcription factor, cell cycle duration, and label retaining properties.

Transporting corneal tissue upon keratoplasty or from any other procedure

Human corneal tissue, obtained via any form of keratoplasty surgery done for patients with keratoconus, corneal scarring, Fuchs' dystrophy, Lattice dystrophy and other cornea related diseases which requires cornea transplantation, or human corneal tissue obtained in any other manner, is placed into a sterile 50ml polypropylene conical centrifuge tubes pre-added with 25ml of transport buffer stored at 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C. This entire process should be done in a room with temperature ranging from 20 °C to 25 °C and the corneal tissue must be placed into the transport buffer within 24 hours of the procedure to maintain tissue viability. The cornea tissue is then transported to the laboratory for stromal cell isolation.

Corneal tissue cleaning process

Corneal tissues is processed in a Class II Biological Safety Cabinet (BSC) with the following environment conditions; room temperature (25°C±5), relative humidity (65%±10). The following is carried out entirely in a BSC sterilized with 70% ethanol and 30 minutes UV radiation. The 50ml polypropylene conical centrifuge tubes containing the cornea tissues are wiped with 70% ethanol. The sample is then transferred into a 50ml polypropylene conical centrifuge tube pre-added with 30ml SDW. The sample is washed by inverting the tubes repeatedly to prevent contamination. This step is repeated at least three times to ensure the tissue is thoroughly washed using fresh SDW and 50ml polypropylene conical centrifuge tubes. The tissue is transferred into a 50ml polypropylene conical centrifuge tube pre-added with 25ml 10% v/v Antibiotic-Antimycotic and 10% v/v Penicillin-Streptomycin in PBS. The tube is

incubated at 37°C, 5% CO₂ for 2 hours/overnight. The incubator is programmed at 37°C, 5% CO₂ to provide an optimal environment for the cord tissues/cells to maintain its viability.

5 **Corneal limbal epithelium stromal cell (CLSC) isolation process**

Prior to incubation, the sample is washed with PBS twice for 30 to 60 seconds, to remove traces of Antibiotic-Antimycotic and Penicillin-Streptomycin. The tissue is then cut into small pieces (2-5mm) and transferred to a fresh 50ml polypropylene conical centrifuge tubes containing 20ml of 3% w/v collagenase type IV dissolved in DMEM-KO. The sample is allowed to be partially digested at around 28-38°C, about 4-6% CO₂, preferably 37°C, 5% CO₂, for 2 to 4 hours. 400µl of 0.25% Trypsin-EDTA is added into the partially digested sample and incubated at around 28-38°C, about 4-6% CO₂, preferably 37°C, 5% CO₂, for 5 minutes to detach cells from the tissue. To neutralize the enzymatic action, 20ml of MSC culture media is added into the tube and centrifuged at about 1500-2500rpm, 700-1000g force/RCF, for around 8-12minutes, at about 25-28°C, preferably about 1800rpm for 10 minutes at 25°C. It is crucial to neutralize the enzymatic action as cell viability will be reduced if Trypsin remains in contact with the cells for too long. Prior to centrifugation, 50% of the supernatant is carefully removed by pipetting to eliminate excess MSC culture media and Trypsin. Undigested tissue fragments and the remaining MSC culture media from the tube are aspirated into 60mm cell culture plate. An additional 5-7ml of MSC culture media is added into the culture plate. The MSC culture media provides sufficient nutrients and optimal environment for the survival of the tissues. The culture plates are then transferred to a CO₂ incubator or a low oxygen hypoxia incubator and incubated for 24 hours. After 24 hours incubation, 2/3 of the media is pipetted out gently without disrupting adhered tissues and replaced with fresh MSC culture media. The culture plates are transferred back to the CO₂ incubator and an additional 5ml of fresh MSC culture media is added in the next 24 hours for continuous supply of nutrients for cell growth and also to remove dead or apoptotic cells from the culture. The culture is observed using an inverted microscope every day and 2/3 of media is replaced every 2 to 4 days until the cells reach 80% confluency until subcultured to passage 1 stock. 80% cell confluency is used as the end point for each passage to maintain cultures with optimal cell numbers and viability.

EXPANSION OF MESENCHYMAL STROMAL CELLS (MSCs)

Expansion of MSCs from passage 0 to passage 1 stock.

Prior to subculture from passage 0 to passage 1 of MSCs derived from umbilical cord, dental pulp, foreskin and cornea, a Class II BSC with following environment conditions; room temperature ($25^{\circ}\text{C}\pm 5$), relative humidity ($65\%\pm 10$), is sterilized with 70% ethanol and 30 minutes UV radiation. The 60mm cell culture plates with 80% confluence cells are transferred from the CO₂ incubator to the BSC. Culture media is pipetted out completely and the culture plate is washed with 10ml PBS (without Magnesium and Calcium ions) twice. Each culture plate is gently swirled 10 to 15 times during washing process and the remaining PBS is pipetted out. 2ml of pre warmed TrypLE™ Express or Accutase or Trypsin-EDTA is added into each culture plates and incubated at 37°C, 5% CO₂ until the cells detach. Cell culture monolayers are observed under an inverted microscope for cell detachment from the surface of the culture plate and the culture plate is tapped gently to dislodge cells. 5ml of pre-warmed MSC culture media is added to the culture plate and swirled gently. MSCs suspension from culture plates are transferred through pipetting into 15ml polypropylene conical centrifuge tubes each. The tubes are then centrifuged at 1200rpm for 6 minutes at 25°C. The supernatant is discarded and cell pellets are re-suspended with 5ml of pre-warmed MSC culture media. After performing the cell count either by haemocytometer with 4% Trypan blue exclusion method or by using automated cell counter, MSCs cells are seeded into T175 culture flask at a 1500cells/cm² seeding density with the addition of 15ml of MSC culture media and the flasks are moved forward and back gently to cause the cells to spread evenly. The remaining cells at passage 0 are then cryopreserved as MSCs SC0. The flasks containing MSCs SC1, are then incubated at 37°C in 5% humidified CO₂ incubator or low oxygen hypoxia incubator. Every 3 days cells are observed under inverted microscope and 8ml of MSC culture media are replaced until cells reach 80% confluency. Upon reaching 80% confluency, WJSC SC1 cell cultures are sub-cultured into passage 2.

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Expansion of MSCs from passage 1 to passage 2 stock.

Prior to subculture MSCs from passage 1 to passage 2 a Class II BSC with following environment conditions; room temperature ($25^{\circ}\text{C}\pm 5$), relative humidity ($65\%\pm 10$) are

sterilized with 70% ethanol and 30 minutes UV radiation. The T175 flasks with 80% confluence cells are transferred from the CO₂ incubator to the BSC. Spent media is aspirated out completely and washed with 20ml PBS (without Magnesium and Calcium ions) twice. Each of the flasks is gently swirled several times during washing process and remaining PBS is aspirated out. 5ml of pre warmed TrypLE™ Express is added into each flasks and incubated at 37°C, 5% CO₂ until cells detach. Cell culture monolayers are observed under an inverted microscope for cell detachment from the surface of the flask and the flask is tapped gently to dislodge cells. 15ml of pre-warmed MSC culture media is added to the flask and swirled gently. MSCs suspension from T175 flasks is transferred through pipetting into 50ml polypropylene conical centrifuge tubes each. The tubes are centrifuged at about 1000-1250rpm, for around 5-7 minutes, at about 25-28°C, preferably about 1200rpm for 6 minutes at 25°C. The supernatant is discarded and cell pellets are re-suspended with 5ml of pre-warmed MSC culture media. After performing a cell count, MSCs are seeded into T175 culture flask at a 1500cells/cm² seeding density for optimal cell proliferation while reducing the chances of inducing biological stress into the cell population. 15ml of MSC culture media are then added the flasks are moved forward and back gently to cause and ensure that the cells spread evenly. Remaining cells at passage 1 are cryopreserved as MSCs SC1. The flasks containing MSCs SC2, are then incubated at 37°C in a 5% humidified CO₂ incubator or with a low oxygen hypoxia incubator. The cells are observed under inverted microscope every 2 to 4 days and 8ml of MSC culture media is replaced during each observation. This process is repeated until cells reach 80% confluency. Upon reaching 80% confluency, MSCs SC2 cell cultures are sub-cultured into passage 3 as a finished product.

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Expansion of MSCs from passage 2 to passage 3 as finished product.

Prior to subculture MSCs from passage 2 to passage 3 a Class II BSC with following environment conditions; room temperature (25°C±5), relative humidity (65%±10) is sterilized with 70% ethanol and 30 minutes UV radiation. The T175 flasks with 80% confluence cells are transferred from CO₂ incubator to the BSC. Spent media is aspirated out completely and the T175 flask is washed with 20ml PBS (without Magnesium and Calcium ions) twice. Each of the flasks are gently swirled several times during washing process and remaining PBS is aspirated out. 5ml of pre warmed

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- TrypLE™ Express is added into each flask and incubated at around 28-38°C, about 4-6% CO₂, preferably 37°C, 5% CO₂, until the cells detach (i.e. stop adhering to the flask wall). Cell culture monolayers are observed under an inverted microscope for cell detachment from the surface of the flask and the flask is tapped gently to dislodge cells.
- 5 15ml of pre-warmed MSC culture media is added to the flask and swirled gently. MSCs suspension from T175 flasks are transferred via pipetting into 50ml polypropylene conical centrifuge tubes each. The tubes are centrifuged at about 1000-1250rpm, for around 5-7 minutes, at about 25-28°C, preferably about 1200rpm for 6 minutes at 25°C. The supernatant is discarded and cell pellets are re-suspended with 5ml of pre-warmed
- 10 MSC culture media. Upon performing a cell count, cells at passage 3 (MSCs SC3) are prepared for cryopreservation.

CRYOPRESERVATION

- 15 **Cryopreservation of MSCs passage 3 (MSCs SC3) as finished product.**
- Prior to cryopreservation of MSCs finished product at passage 3 a Class II BSC with following environment conditions; room temperature (25°C±5), relative humidity (65%±10) is sterilized with 70% ethanol and 30 minutes UV radiation. Freezing media is used as cryopreservation media. The appropriate volume of freezing media is
- 20 prepared fresh according to the formula in Table 4 below:

Table 4

No.	Freezing media component	Concentration
1.	MSC culture media	90%
2.	Dimethyl sulfoxide (DMSO)	10%

- Freezing media is not allowed to be exposed to light and is kept at 4°C±2 – this is to negate the effects of light on DMSO. Cryovials are sterilized using 70% ethanol and labelled using a permanent cryo marker pen. MSCs SC3 pellets in 50ml polypropylene conical centrifuge tubes are gently exposed to desired volume of cold Freezing media by using a micropipette. Cells are carefully re-suspended to avoid gas bubble formation. The desired volume of cell suspension is transferred into pre-labelled cryovials.
- 30 Freezing density and required volume of freezing media are illustrated in Table 5.

Table 5

No.	Freezing density (cells)	Volume of Freezing media (μl)
1.	5 x 10 ⁶	800
2.	10 x 10 ⁶	1000
3.	20 x 10 ⁶	1200

Cryovials are immediately transferred to a -80°C freezer for 24 hours and finally kept in liquid nitrogen cryotank the following day. Cryovials are kept at -80°C freezer to allow for a gradual cooling process to occur (1°C/minute) till the vials reach -50°C prior to the vials being transferred into a liquid nitrogen cryotank. This is to reduce intracellular crystal formation thereby maintaining the optimal amount of viable cells. Location and details of each cryovial stored are recorded to ease the retrieval process.

OBSERVATIONS

Cell morphology

Cell morphology of WJSCs, SHED, FDSCs and CLSCs at 80 to 90% were observed at passage 3 using an inverted microscope. As illustrated in Figure 1, the adherent MSCs displayed typical fibroblast-like spindle shaped morphology which confirm established descriptions of stromal-like cell morphology.

Immunophenotyping of MSC markers

Immunophenotyping was carried out via flow cytometry at SC3. Upon reaching 80% confluency, the cells were harvested using 0.05% trypsin-EDTA (Invitrogen) and resuspended in Dulbecco's Phosphate Buffer Saline (DPBS; Invitrogen) at a cell density of 1.5x10⁶ cells/mL. Two hundred microliters of the cell suspension (3x10⁵ cells) were incubated with the labelled antibodies in the dark for 1 hour at 37°C. Antibodies conjugated to fluorochromes are susceptible to photobleaching and should be protected from light during all phases of an experiment. The following antibodies were used to mark the cell surface epitopes: CD90-phycoerythrin (PE), CD44-PE, CD73-PE, CD166-PE and CD34-PE, CD45-fluoroisothiocyanate (FITC), and HLA-DR-FITC (all from BD Pharmingen). All analyses are standardized against negative control cells incubated with

isotype-specific immunoglobulin (Ig) G1-PE and IgG1-FITC (BD Pharmingen). At least 10,000 events are acquired on BD FACS Calibur flow cytometer to analyse the total gated cell population. Purity and MSCs-like phenotype of the isolated cells were confirmed through flow cytometry result, wherein more than 90% of WJSCs, SHED, FDSCs and CLSCs expressed MSC markers (CD44, CD73, CD90) and >60% for CD166. The cells also expressed less than 0.5% for hematopoietic markers (CD34, CD45 and HLA-DR). These results are illustrated in Table 6 below:

Table 6

	WJSC	SHED	FDSC	CLSC
CD90	+	+	+	+
CD44	+	+	+	+
CD73	+	+	+	+
CD166	+	+	+	+
CD34	-	-	-	-
CD45	-	-	-	-
HLA-DR	-	-	-	-

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In vitro multilineage differentiation

The cultures of respective sources are initiated at a density of 1500 cells/cm² in 6-well plates and are grown to confluence and subjected to differentiation into adipogenic, chondrogenic, and osteogenic lineages. Adipogenic lineage is stimulated by inducing the cells with 10% FBS, 200 mmol/L indomethacin, 0.5 mmol/L 3-isobutyl-1-methyloxanthine (IBMX), 10 mg/mL insulin, and 1 mmol/L dexamethasone (all reagents from Sigma Aldrich). Lipid droplets are visualized by staining with oil red O staining (Sigma Aldrich). For chondrogenesis differentiation, cells are cultured in media supplemented with ITS+1 (Sigma Aldrich), 50 mmol/L L-ascorbic acid-2 phosphates (Sigma Aldrich), 55 mmol/L sodium pyruvate (Invitrogen), 25 mmol/L L-proline (Sigma Aldrich), and 10 ng/mL of transforming growth factor-beta (TGF- β) (Sigma Aldrich). Assessment of proteoglycan accumulation is visualized by alcian blue staining (Sigma Aldrich). The osteogenic differentiation is stimulated in a 3-week culture in media supplemented with 10% FBS, 100 μ mol/L dexamethasone, 10 mmol/L β -glycerol phosphate (Fluka, Buchs, Switzerland), and 100 mmol/L of L-ascorbic acid-2

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phosphates. Assessment of calcium accumulation is visualized by von Kossa staining. WJSCs, SHED, FDSCs and CLSCs were able to differentiate into chondrogenic, adipogenic and osteogenic lineages *in vitro*). Chondrogenic differentiation revealed production of extracellular matrix glycosaminoglycans when stained with Alcian blue and differentiation of adipocyte were confirmed with oil red O staining for presence of lipid droplets vacuole. In osteocyte differentiation medium, spindle-shaped dental MSCs metamorphosed into cubical cells with visible mineralized foci detected as black spotted areas with von Kossa staining.

10 Differentiation of MSCs into dopaminergic-like cell

Embryoid Body (EB) formation

Stage 1, EB formation

At 80% confluency, cells are transferred into a non-adherent petri dish containing a neural-inducing medium (NIM) composed of 92% D-MEM/ F-12 (1:1), 6000 mg/L D-glucose, 5% KnockOut SR, 2 mM L-glutamine, 1% MEM non-essential amino acids solution, 0.1 mM b-mercaptoethanol, and 1% N2 supplement (all from Gibco BRL) via hanging drop method. The EBs are well developed under these conditions in which cell aggregates form within 24 hours of incubation.

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Dopa media A

The EBs are aspirated to super-hydrophilic plates (Costar, Cambridge, MA) in Neurobasal A media (GIBCO/Invitrogen, Carlsbad, CA) and supplemented with B27 (Invitrogen, Carlsbad, CA), 20 ng/mL basic fibroblast growth factor (bFGF; Invitrogen) and 20 ng/mL epidermal growth factor (EGF; Invitrogen). Fresh culture medium including EGF and bFGF is added to the medium after 4–5 days of culture.

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Dopa media B

The EBs were induced with a cocktail of 200 ng/mL sonic hedgehog (SHH), 100 ng/mL fibroblast growth factor 8 (FGF8), 10 ng/mL glial cell line-derived neurotrophic factor (GDNF; R&D Systems, Minneapolis, MN), and 10 μ M forskolin (PeproTech EC, London, England). Cells are then cultured at 37°C with 5% CO₂ for 7 days. Half of the

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medium is replaced every 2–3 days. After 7 days of cultivation, the cells differentiate into neuronal cells and are tested for neural cell marker expression.

As illustrated in Figure 2, MSCs derived from WJSCs, SHED, FDSCs and CLSCs are
5 able to form cell aggregates/neurospheres in NIM media. However neurite outgrowth was only observed in SHED, FDSCs and CLSCs aggregates/neurospheres.

Quantitative reverse-transcription Polymerase Chain Reaction

The cell pellets from respective sources are collected and total RNA is isolated using
10 TRIZOL method (Invitrogen), as per the manufacturer's protocol. The RNA is then quantified using a spectrophotometer (Agilent, NanoDrop, Technologies Inc.) and the purity is assessed by the absorbance value at ratio of 260:280 nm. The RNA is stored at -80°C. The first-strand complementary DNA (cDNA) is synthesized using 1 mg RNA treated with RNase-OUT ribonuclease inhibitor and the Superscript II First Strand
15 Synthesis system (Invitrogen) according to the manufacturer's instructions. PCR is performed in 0.2 mL Eppendorf tubes (Axygen) with a final volume to 12.5 mL. cDNA amplification is performed in a thermocycler using Taq polymerase supplied with KCl buffer and 1.5mM MgCl₂ (Invitrogen) at 94°C for 1 min, 58°C for 30 s and 72°C for 1 min. PCR products are resolved on 1.5% agarose (Invitrogen) gel run in 1x Tris borate–
20 EDTA buffer. The primer sequences are listed in Table 7. As illustrated in Figure 3, the expressions of some genes in the semi-quantitative RT–PCR analysis are quantified in duplicate, using SYBR Green Master Mix (Applied Biosystems). PCR reactions are performed on an ABI 7900HT RT–PCR system (Applied Biosystems) and SDS v 2.1 software was used to analyse the results and to prove the immunophenotyping
25 characteristics. All measurements are normalized to 18s rRNA.

Table 7:

Gene	SEQ ID No.	Primers
Nestin	1	Forward: 5'-CAGCGTTGGAACAGAGGTTGG-3'
	2	Reverse: 5'- TGGCACAGGTGTCTCAAGGGTAG-3'
β -Tubulin III	3	Forward: 5'-CAACAGCACGGCCATCCAGG-3'
	4	Reverse: 5'- CTTGGGGCCCTGGGCCTCCGA-3'
GAD 65	5	Forward: 5'- ATGACACTGGAGACAAGGCC-3'
	6	Reverse: 5'- TTGGTAGCTGACCATTGTGG-3'
GAD67	7	Forward: 5'- TGGAAGTGGTGGACATACTCC-3'
	8	Reverse: 5'- AAGTACTTGTAGCGAGCAGCC-3'
Tyrosine hydroxylase (TH)	9	Forward: 5'-GGTTCCCAAGAAAAGTGTCTAG-3'
	10	Reverse: 5'-GGTGTAGACCTCCTTCCAG-3'

Immunocytochemistry analysis of the MSCs

Cells from respective sources are fixed for 20 min in 4% ice cold paraformaldehyde, treated with 0.1% Triton-X for optimal penetration of cell membranes, and incubated at room temperature (RT) in a blocking solution (0.5% BSA; Sigma Aldrich) for 30 min. Primary antibodies [Nestin (mouse, Abcam), β -tubulin (mouse, Abcam), GAD65/67 (rabbit, Abcam), TH (Rabbit, Abcam) with dilution of 1:400] are incubated overnight at 4°C, washed with DPBS, and then incubated with secondary antibodies (either fluorescein isothiocyanate [FITC]-conjugated IgG or rhodamine-conjugated IgG) at RT for 90 min. Slides were counterstained with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI, Chemicon, Temecula, CA, USA) for 5 min. Fluorescent images were captured using Nikon-Eclipse-90i microscope (Nikon, Tokyo, Japan, <http://www.nikon.com>).

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As illustrated in figure 4, the immunocytochemistry results show that WJSCs, SHED, FDSCs and CLSCs express Tyrosine hydroxylase (TH), β -tubulin, Nestin and Glutamate decarboxylase 65 and 65 (GAD65/67) protein post dopaminergic neuron-like differentiation. However, in undifferentiated SHED, expression of Nestin and GAD65/67 is observed while expression of β -tubulin, GAD65/67 and TH is observed in undifferentiated WJSCs. This finding shows that SHED and WJSCs are the best

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candidates for any therapeutic application for dopaminergic neuronal degenerative disease.

5 Cells obtained from the protocol described herein are able to retain 83% to 89% of viability (non-apoptotic) at passage 3. Prior art percentages achieved at passage 3 are around 70%-80% viability using 4% trypan blue exclusion method. However, usage of the 4% trypan blue exclusion method includes cell in late and early apoptosis as being viable, and as such, is not an accurate means of counting for cell viability. In this protocol the method used is more accurate, namely the Annexin V apoptosis detection
10 method by flow cytometry, to find out the exact percentage of non-apoptotic cells, excluding early and late apoptotic cells.

It will be appreciated by persons skilled in the art that the present invention may also include further additional modifications made to the system which does not affect the
15 overall functioning of the system.

Claims

1. A tooth holder comprising:
 - a circular rotating chuck fixed to a base;
 - the chuck including three jaws and a collar, whereby rotation of the collar
 - 5 extends or retracts the jaws thereby adjusting the aperture size between the jaws to
 - allow a tooth to be held thereby;
 - irrigation means above the chuck for dripping liquid onto the tooth;
 - characterized in that the chuck includes a central channel through which
 - liquid may be pumped.
- 10 2. A tooth holder according to claim 1 wherein the jaws are provided with grooves or steps to allow for precise measurement and placement of the tooth.
3. A tooth holder according to claim 1 for use in a method for isolating
- 15 mesenchymal stromal cells comprising the steps of:
 - washing a tissue sample at least 3 times;
 - incubating the tissue sample in a solution comprising at least one antibiotic
 - and at least one antimycotic at a temperature of around 28-38°C and atmosphere
 - comprising about 4-6% CO₂;
 - 20 washing the tissue sample with phosphate buffered solution to remove
 - antibiotic and antimycotic therefrom;
 - cutting the tissue sample into small pieces;

incubating the tissue sample in collagenase dissolved in DMEM-KO at a temperature of around 28-38°C and atmosphere comprising about 4-6% CO₂ to produce a solution of tissue fragments;

5 centrifuging the solution of tissue fragments to form a pellet, which is resuspended and transferred into MSC culture media for subsequent incubation;

 characterised in that MSC culture media is added to the tissue sample during the collagenase incubation to quench the action of the collagenase such that the tissue sample is only partially digested,

10 wherein the tissue sample is derived from dental pulp in which the initial washing of the tissue sample is with Dulbecco's phosphate buffered saline without calcium or magnesium, and

 wherein the dental pulp is extirpated from a freshly extracted deciduous tooth, sectioned horizontally at the cementoenamel junction, while being irrigated.