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Umbilical Cord Mesenchymal Stem Cells Derived Exosomes, Characterization and Therapeutic Application in Translational Medicine

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ABSTRACT
Regenerative medicine utilizes stem cells to repair or to re-establish the function of damaged or harmed tissues. Exosomes derived from umbilical cord mesenchymal stem cells have been studied as a vital component of regenerative medicine.Mesenchymal Stem Cell have captured great consideration in regenerative medicine for over a year by their differentiation capacity, potent immunomodulatory properties and their ability to be well refined. The use of human stem cells is a promising novel approach for the treatment of numerous diseases and wounds. Mesenchymal Stem Cell are multipotent cells which can be isolated from many sources including umbilical cord. Human umbilical cord represents a good source for Stem cells, because certain advantages such as they have fewer moral issues, have painless collection procedure and faster renewal properties. We aimed to isolate exosomes from human umbilical cord mesenchymal stem cells. Cord tissue was dissected using the explant strategy. The outgrowth of cells occurred within 7-8 days of explant, whereas 80-90% confluency was observed after 12-14 days from explants. The cell viability, cell count, morphology of isolated cells was assessed using inverted microscope. MSCs are described by the set of criteria, for example, they should be plastic adherent; express CD105, CD90, and lack expression of CD45, CD34. Further, exosomes were isolated from culture conditioned medium of human umbilical cord mesenchymal stem cells with the help of ultracentrifugation. Exosomes are nano-sized vesicles that are secreted by cells in culture medium and found in all body fluid. These vesicles, loaded with unique RNA and proteincargos, seem to have numerous biological functions. Exosomes participate in intercellular communications events, signalling within the human body and contribute to the healing of injured or diseased tissue or organs. MSC- derived exosomes can be applied for treatment of an assortment of diseases including heart, kidney, liver, and neurological diseases and cutaneous wound healing. The range of current scientific interest in exosomes is wide and goes from understanding their functions and pathways to using them in diagnostics, as biomarkers, and in the development of therapeutics.
KEYWORDS: *Regenerative Medicine, Mesenchymal Stem Cells, Multipotent, Human Umbilical Cord, Exosomes.*

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ABBREVIATION

°C	Degree Celsius
BSC	Biosafety Cabinet
CD	Cluster of Differentiation
CFU	Colony Forming Unit
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal Bovine Serum
gm	Grams
HLA	Human Lymphocyte Antigen
HUC	Human Umbilical Cord
IPA	Isopropyl Alcohol
LAF	Laminar Air Flow
Mins	Minutes
ml	Millilitre
mm	Millimetre
MVBs	Multivesicular Bodies
NB	Nutrient Broth
PBS	Phosphate Buffered Saline

pH Power of Hydrogen
Rpm Revolutions Per Minute
SCDM Soyabean Casein Digest Medium
UC Umbilical Cord
UCMSCs Umbilical Cord Mesenchymal Stem Cells
WJ Wharton's Jelly

I. INTRODUCTION

The term regeneration was hypothesized in ancient Greek mythology [34].Regenerative medicine has developed from earlier activities including surgery, surgical implants (artificial hips), and progressively refined biomaterial frameworks (skin grafts). The work that truly launched regenerative medicine into a substantial space of science started as cell treatment [40]. Regenerative medicine is interdisciplinary field that applies engineering and life science principles to promote regeneration; it has the potential to restore diseased, injured tissues of the body with intact tissues as well as whole organs. It also has the potential to heal or replace tissues and organs damaged by age, disease, or trauma, as well as to normalize congenital defects [22] [25].

It utilizes the stem cells to repair or to re-establish the function of damaged or harmed tissues. The principle of regenerative medicine is simple and basic which involves an assortment of cells (stem cells), refined of these cells, and transplantation of cells into the body with or without adjustment of their organic properties [24].

1.1Stem Cells

Stem cells are distinctive, non-specialized master cells of the body that can divide and proliferate indefinitely to produce more of the same stem cells and have a remarkable ability to self-restore and to give rise to particular specialized cell types[1] [17].

Under the right conditions, or given the right direction, stem cells can possibly turn into a developing cell that have characteristic shapes and specialized functions for use in regenerative medication, for instance, nerve cells, skin cells or heart cells [7].

1.2 Characteristics of Stem Cells

Three important characteristics of stem cells are as follow:

1) Stem cells are capable of dividing and renewing themselves:

Unlike most developed sorts of cells e.g., muscle cells, platelets, or nerve cells which don't typically reproduce, stem cells are capable of long-term self-renewal [8].

2) Stem cells are unspecialized.

One of the crucial properties of a stem cell is that it doesn't have any tissue-specific structures that permit it to perform specialized functions by giving rise to specialized cells, including heart muscle cells, blood cells, or nerve cells [8].

3) Stem cells can give rise to specialized cells.

The process by which unspecialized stem cells give rise to specialized cells is called differentiation. The interior and external signs are controlled by a cell's genes. The external signals for cell differentiation include chemicals secreted by other cells, physical contact with neighbouring cells, and certain molecules in the microenvironment [8].

1.3 Stem Cells Classification

Stem cells can be grouped based on their ability to divide and differentiate into various cell types. They are classified by various ways. Based on potency stem cells are classified into five main types: totipotent, oligopotent, pluripotent, multipotent, or unipotent. Based on source it has been classified as embryonic stem cells, adult stem cells, induced pluripotent stem cells, allogenic and autologous stem cells [12].

i. Totipotent:

Totipotent stem cells can differentiate into embryonic and extraembryonic cell types. Such cells can develop a complete, viable organism. These cells are produced from the combination of an egg and sperm cell. Totipotent stem cells give rise to somatic stem/progenitor cells and primitive germ- line stem cells [2].

Totipotent cells can form all the cell types in a body and are found in early undeveloped tissues. Examples are the zygote formed at egg fertilization and the first few cells that result from the division of the zygote [12].

ii. Pluripotent:

These cells have the ability to differentiate into almost all cell types that make up the body. They have the ability to differentiate into cells of all three essential germ layers: ectoderm, mesoderm, and endoderm. Examples include undeveloped stem cells and cells that are derived from the mesoderm (framing bone, ligaments, muscles), endoderm (forming gastrointestinal and respiratory tract), and ectoderm (bring about skin

and sensory system) germ layers that are formed in the early stages of embryonic stem cell differentiation [12][43].

iii. Multipotent:

Multipotent cells have the ability to differentiate into a firmly related group of cells. Examples include hematopoietic (adult) stem cells that can become red and white blood cells or platelets [14].

iv. Oligopotent:

Oligopotent cells have the ability to differentiate into a few cell types. Examples include (adult) lymphoid or myeloid stem cells [12]. The corneal epithelium is a squamous epithelium that is constantly renewing and is Oligopotent [2].

v. Unipotent:

Unipotent cells produce just one single cell type, their own. Examples include (adult) muscle stem cells[30] [41].

1.4 Mesenchymal stem cells (MSCs):

MSCs are multipotent adult stem cells that are found in various tissues. The most popular sources for MSCs are bone marrow, adipose tissue, peripheral blood, placenta, umbilical cord tissue or umbilical cord blood [13][16][23].

The International Society for Cell Therapy (ISCT) proposed a set of minimum criteria that are necessary for identifying MSCs such as plastic adherence with over 95% of the surface expression of CD105, CD73, CD90 biomarkers, negative biomarker expression in over 98% of the cells for CD45, CD34, CD14 or CD11b, CD79α or CD19, HLA-DR and have the ability to differentiate into various lineages including adipose tissue, bone, and cartilage [11][19].

MSCs are multipotent stem cells and can possibly separate into all cell types of the connective tissue, for example, chondroblasts, osteoblasts and adipocytes. They are utilized in regenerative medicine as a result of their immunomodulatory and anti-inflammatory properties and their capacity to treat tissue injury. These cells may be a proper source for tissue designing, regenerative medication, and organogenesis applications [15][37] [38][46].

Compared with other stem cell sources, we preferred UCT-MSCs because of their advantages such as they are viewed as clinical waste, minimal moral concern, non-invasive recovery, high proliferation capacity, low immunogenicity and their multi lineage differentiation capacity which make them an interesting candidate for tissue designing applications and cell-based treatment [26] [42] [48].

1.5 Morphology of Human Umbilical Cord

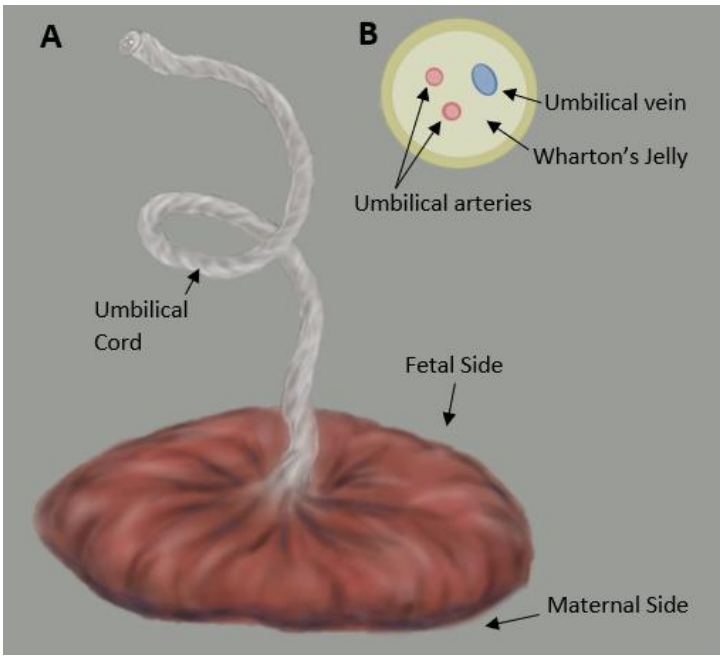


Figure.1. Human Placenta

(A) Represents placenta with umbilical cord. (B) Umbilical cord: cross section.

Umbilical cord contains two arteries which transport deoxygenated blood and metabolic by-products from foetus to placenta and one vein, which transport oxygenated and nourishes rich blood from placenta to foetus. All these vessels are surrounded by mucous connective tissue called Wharton's jelly, situated between the amniotic epithelium and umbilical vessels, which is the rich source of UCT-MSCs [6][13].This makes them an attractive candidate for allogenic cell-based treatments [26] [39].

1.6 Regenerative Medicine Today

With regenerative medication, the repair of undesirable tissue or restoration of bodily functions can be accomplished by a 'once and for all' therapy. In this way regenerative medication differ totally from the current clinical work using pharmacological or surgical procedures [33].

Regenerative medication contains two objectives: one is efficiently and safely transferring stem cells into injured organs and tissues, which may replace the transplantation of the whole organ in the near future; the other is to develop methodologies in order to improve the regenerative potential and capacity of stem cells residing in different organs [10].

The arising field of regenerative medication aims to address the unmet clinical requirement for organ and tissue replacement. Regenerative medication covers a wide range including techniques for the advancements of self-healing, cell-based treatment, tissue designing etc [20].

In the principal year of the 21st century, the accessibility of stem cells turned as front-line cellular treatment. A variety of cell types has been and is at present used in regenerative medicine. Before the isolation and identification of human stem cells, cells isolated from adult tissues were utilized. These were used in combination with natural or man- made materials as regenerative medication.[33].

1.7 Limitations of Regenerative Medicine

One reason why it took such a long time for stem cells to become a well-established research field is that in the early years a lot of time and energy were consumed in trying to define stem cells and in arguing about whether or not a particular cell was truly a stem cell. Additional putative characteristics of stem cells, like rarity, capacity for asymmetric division or tendency to divide infrequently, were incorporated into the definition, so that if a cell didn't display these extra properties, it would in general be excluded from the stem cell 'list [49].

The use of stem cells has a few drawbacks, like the requirement for a consistent supply of cells with stable phenotype, high costs and time delays for the generation and handling of these cells. Furthermore, issues identified with ectopic tissue development, in fusional toxicity brought by cells and cellular rejection or unwanted engraftment, have been reported [21].

Regenerative medicine treatments tend to be very expensive because they often need special production facilities and highly skilled staff. A significant worry that restricts the spreading of regenerative medicine is identified with various difficulties related to different challenges. e.g., live tissue handling and manufacturing [5].

1.8 Need of New Therapeutic Cell-Based Tools

Cells being the fundamental unit of tissues, organs and the body, it is not far from understanding that changes in the cells would result in change in the overall tissue, organ and finally the organism especially when it comes to Stem cells; an example of which is MSCs. They have been found to be very promising as an approach towards the treatment of several disorders which are currently incurable or rather untreatable by the modern medicinal and surgical methods. Nevertheless, it has to be clearly understood that numerous environmental and medicinal induced factors can adversely affect functioning of the SCs or progenitor cell leading to non desirable outcomes. Hence, complete understanding followed by characterisation of each individual influential component is essential to obtain precise pathways of treatment to induce cell-based therapeutic applications [28].The therapeutic effect of MSCs is believed to be mediated through the secretion of a variety of components; for example, cytokines or growth factors, extracellular vesicle such as micro vesicles (100-1000nm), exosomes (30-150nm) and apoptotic blebs (1-5µm). It also impacts the immunological framework by means of various pathways [44].

Exosomes being a component of cells provide multiple benefits over the traditional cell therapies as a regenerative medicine. Rather than using stem cells directly which may lead to pathological differentiation in the long term of the engrafted cells by giving rise to immune rejection of the viable cells; using exosomes which are nonviable, do not tend to elicit immune rejection and are far easier to manufacture and thus becomes a safer alternative [10].

1.9 Exosomes in UC-MSC

Exosomes are small extracellular vesicles that range from 30 to 150 nanometer in size. They originate from endosomes of different cell types and mediate cell-to-cell correspondence. Exosomes consist of DNA,

circular RNA, mRNA, long non coding RNA, micro-RNA, proteins etc. They are bundled with a conserved set of proteins, including tetraspanins (CD9, CD63 and CD81)and furthermore heat shock proteins (HSP70). These are the basic exosome specific markers. They take part in a variety of physiological and/or pathological processes [15]. Because of their nano meter-sized particles they can be easily transferred through blood and other natural biological fluids. Consequently, MSC-derived exosomes can mediate cell communication in both adjacent and distant regions through paracrine and endocrine signalling. The limit of MSC-derived exosomes to re-establish and maintain the homeostasis of the tissue microenvironment would rely upon the biochemical capability of their protein and RNA. MSC-derived exosomes have been utilized as drug delivery vehicles in tumour treatment and regenerative mediation in certain investigations [21].

Tetraspanins might be utilized for diagnostics of different tumours and infectious diseases. Specifically, CD63+ exosomes were demonstrated to be significantly expanded in patients with melanoma and other cancers. Along these lines, CD63 has been recommended to be a protein marker of disease. Likewise, CD81, another member of the tetraspanins family, plays a significant part in cell entry of hepatitis C infection [50].

Exosomes are secreted by a variety of cells and exist in almost all body fluids, including the blood, saliva, urine, cerebrospinal fluid, and milk. Exosomes obtained from human umbilical cord mesenchymal stem cells (HUC-MSCs) have higher neurolysin action than exosomes obtained from HBMSCs [45].

Exosome biogenesis occurs by means of a couple of steps:

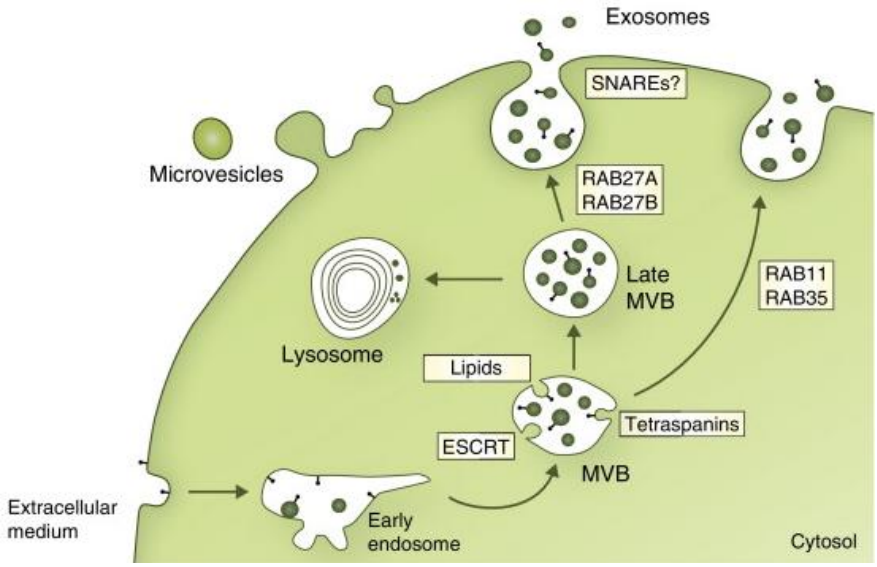


Figure. 2. Exosome Biogenesis

(1) Early endosomes go through a series of development steps framing late endosomes, named multivesicular bodies (MVBs),
(2) Fusion of endosome-derived MVBs with the cell membrane, and
(3)Release of exosomes into the extracellular environment through the exocytosis mechanism. Exosomes contain not just various proteins, including intracellular enzymes, surface receptors, transcription factors, and cytokines, yet in addition nucleic acids such as DNA, mRNA, miRNA, and other non-coding RNA, which can modulate the physiologies of the recipient cells [51].
Exosomes have received an enormous number of interests in research because of their roles in intercellular correspondence, immunomodulatory work, and their potential for use in identifying and treating infections. The composition and roles of the exosomes greatly rely on where MSCs are derived from, the physiologic state of MSCs, and the cell culture medium wherein MSCs are developed [47].

1.10 Exosomes: New Therapeutic Tool

As Exosomes are naturally-formed and are involved with numerous biological and pathological processes, they have various benefits when contrasted with other nanoparticles [3].

Exosomes have advantages over the corresponding MSCs: they are smaller and less complex than cells, so they are simpler to create and store, and have the potential to avoid some of the regulatory issues that face MSCs. Therefore, MSC-derived exosomes may address an optimal therapeutic tool for diseases in the near future [21].

Exosomes derived from stem cells have been widely used in various clinical applications. It has been reported that exosomes play a pivotal role in communication among cells and play a significant role in biological processes, including intercellular communication, development and differentiation of stem cells, and immune function. Stem cells and their paracrine factors, such as TGF-β, platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) are potential therapeutic drugs for the treatment of infection as well as tissue regeneration and wound healing. A few examinations have revealed that exosomes are associated in playing a significant part in regeneration of their functions and wound healing [4].

The organization of MSC-inferred exosomes has yielded advantageous impacts in an assortment of creature models of liver infection, including drug-incited intense liver injury, liver fibrosis, and hepatocellular carcinoma (HCC) [21].

Exosomes have drawn enormous research interest in view of their promising clinical application. They serve as diagnostics tools because they are carriers of molecular markers of many diseases and as a prospective delivery system for various therapeutic agents [9].

Exosomes can be released by different kinds of stem cells and are able to modify the function of the receptor cells and tissues. Compared with stem cells, which may cause strange differentiation and tumour formation, the exosomes mediated treatment harbours a more encouraging future [10].

1.11 Therapeutic Application of Exosomes

The growing information on the roles of exosomes in physiologic and pathologic processes has prompted a promising interest in their use as therapeutic vehicles. The role of exosomes in cell-to-cell communication, immune modulation, and movement of neurodegenerative infections and disease has given various dimensions for prospective therapeutic applications. Besides their physiologic and pathologic capacity, exosomes also allow for a channel for the delivery of therapeutic agents including drugs and biomolecules [47].

The therapeutic application of exosomes is now being investigated in malignant growths, CNS illnesses, immune-mediated conditions, and a variety of different fields including specific roles in drug and biomolecules delivery. Exosomes play a role in the invasion, metastasis, and angiogenesis of tumours', and also in the safe response against tumours [47].

Exosomes were utilized to re-establish the dynamic and cellular homeostasis conditions by disposing excess or harmful particles from cells, for example, as by eliminating transfer receptors during reticulocyte development and removing harmful DNA from the cytoplasm to avoid the senescence or apoptosis of normal human cells. In recent years, exosomes have been attributed to various biological functions by cell-to-cell correspondence through direct membrane fusion with the plasma layer, endosomal layer, endocytic pathways, or ligand-receptor communications. Exosomes have been shown to assume vital roles in various cellular processes, like inflammation, immune regulation, tissue recovery, senescence, and malignant [18].

Exosomes as drug delivery systems have various benefits including their ability to target explicit tissues, capacity to cross various barriers, including the blood–brain barrier, and their tolerance inside body fluids. These properties may permit upgraded delivery of drugs to locations where they are needed and avoid unnecessary side effects when drugs are distributed throughout the body. It can also carry numerous compounds including proteins and nucleic acids and even anti-inflammatory and anticancer agents. All these properties may permit exosomes to be brought into clinical practice in a variety of disease fields [35].

1.11.1 Exosomes as a Drug Delivery System

The approach of exosomes and their functions in biology are one of the most exciting breakthroughs in the medical field in recent years. Exosomes, a nano-vesicle created by most cell types, plays an exceptional role in cell-cell communication and can deliver different cargos of drug molecules, proteins, and nucleotides. As a relatively new carrier system, exosomes have different benefits such as unique structure, distinct physicochemical characteristics, low immunogenicity and toxicity and inherent targeting ability. Recent literature shows continued exploration and promise of exosomes as drug delivery carriers for various diseases including strong tumours, bone recovery, heart diseases, Parkinson's amongst others [31].

An exosome-based delivery system has particular benefits like specificity, safety, and stability. By their homing characteristic, exosomes can deliver their cargo to specific targets over a significant distance. Exosomes can also be used to deliver interfering RNA (siRNA) or pharmaceutically active substances. As exosomes are small and local to creatures, they are able to avoid phagocytosis, fuse with the cell membrane, and bypass the engulfment by lysosomes. Exosomes also can exhibit increased stability in the blood that permits them to travel significant distances inside the body under both physiological and pathological conditions. Moreover, exosomes have a hydrophilic core, which makes them suitable to have water-soluble drugs [3].

Research interest in exosomes has developed significantly during the last few years because of their unique properties. Transport inside exosomes permits concurrent intercellular communication by delivering various signals simultaneously. In contrast to free circulating soluble factors, they have the ability to release large amounts of functional molecules to recipient cells. In addition, cargos enclosed inside exosomes are

protected against degradation by enzymes and other processes by their lipid bilayer, conferring them stability and safety. Also, exosome properties permit cargo to travel long distances, have good biocompatibility, are non-immunogenic, specific-targeted and cross many physical barriers. Thus, exosomes are safe and stable endogenous nanocarriers and one of the best drug delivery systems options, with an increasing variety of applications [29].

II. METHODOLOGY

2.1 Collection of Human Umbilical Cord

The collection was performed in accordance with the ethical standards of the local ethic committee.

2.2 Preparation of Cell Culture Medium

Culture medium used in this study was DMEM/F12 basal medium supplemented with 10% FBS, 1% antibiotic–antimycotic solution.

2.3 Isolation of MSC from UC

The umbilical cord was collected in phosphate-buffered saline (PBS) and transferred to the laboratory where it was washed with saline and cut into 1 cm^2 segments. The segments were cut longitudinally and blood vessels were removed completely. The segments were transferred to cell culture dishes and cultured in DMEM media. The plates were incubated at 37℃ in a humidified atmosphere with 5% CO_2 and were left undisturbed for 7 days.

2.4 Passaging Cell Culture

After the initial week-long incubation period, the medium was changed for the first time and thereafter, it was changed every 3-4 days. After 2 weeks, the UC was removed and the adherent cells were allowed to expand until day 21 where the cells were detached using 0.25% trypsin-EDTA. The cells were then quantified by haemocytometer using the trypan blue and cells were sub cultured.

2.5 Morphology of HUCMSCs

The morphology of cells at each passage, at P0, P1, P2, P3 isolated from human umbilical cord tissue were observed using an inverted microscope.

2.6 Sterility Test

The study was carried out on solid media and different agars were used to study if there is any growth of bacteria. Nutrient agar, SCDM (Soyabean Casein Digest Medium) agar were used for examination.

Sterility Testing of Sample on Nutrient Agar:

Nutrient agar (NA) was prepared by assorting 5gm of peptone, 5gm of sodium chloride, 1.5gm beef extract, 1.5gm yeast extract and 15 gm of agar along with 1000ml of DW in a flask. The flask was heated till all the media components dissolved and the final pH was adjusted to 7.4+0.2 and sterilization was done by autoclaving. The agars were then poured onto sterile petriplates and allowed to cool and solidify. The NA plates were then streaked with sample (i.e., culture medium of different passages) and incubated for 24 hours at 37□ C. Next day plates were visually checked for any bacterial growth.

Sterility Testing of Sample on SCDM:

SCDM was prepared by assorting 17gm of tryptone, soya peptone 3gm, sodium chloride 5gm, dextrose 2.500gm, dipotassium hydrogen phosphate 2.500gm along with 1000ml of DW in a flask. The flask was heated till all the media components dissolved and the final pH was adjusted to 7.3±0.2 and sterilization was done by autoclaving. The agars were then poured onto sterile petriplates and allowed to cool and solidify. The plates where then streaked with sample and incubated for 24 hours at 37□ C. Next day plates were visually checked for any bacterial growth.

2.7 Cell Count

Microscopic examination of the cell sample was done with the help of a trypan blue solution to understand the count of cells and the observations were noted of each passage. i.e., (P0, P1, P2, P3)

2.8 Cell Viability

10 μL of the cell suspension is transferred to a sterile 1.5 mL tube. An equal volume of 0.4% Trypan blue is added to the cell suspension. A glass coverslip is placed on the counting chambers of an improved Neubauer haemocytometer, and the mixture is loaded onto 1 chamber so that the mixture exactly fills the chamber. The 4 large corner squares of the chamber are observed under the inverted microscope, and the viable/live (clear) or non-viable/dead (blue) cells are recorded for each square.

2.9 Proliferation Analysis of HUCMSCs

Cells between passages 0-3 were selected for proliferative analysis. The cells were passage at 80-90% confluence. Cells were incubated and cultured for 4-5 days between each passage. At each passage cells were counted with the help of a haemocytometer. The number of cells in each passage was recorded to generate a growth curve.

2.10 Characterization of UCMSCs

The cells were collected and washed twice in a DPBS buffer and centrifuged at 1500 rpm at 4°C for 10 minutes. After centrifugation, pellets were observed. Supernatant was decanted without disturbing the pellet. Cell count was taken of cell suspension using a trypan blue solution. Thereafter, the cells were resuspended at the concentration of 1.0 x 10^6 cells/mL. For staining, 20 µL of each of the following human antibodies was added to 50 µL of cell suspension. The samples were incubated at 37°C for 30 min, centrifuged, and washed twice with PBS. Cells were then detected by flow cytometry.

Table 1. Antibodies and Fluorochromes used to Analyse UCT-MSCs.

Marker	Category	Fluorochrome dye	Channel
CD 105	Positive marker	PE	FL2
CD 90	Positive marker	PE Dazzle	FL3
CD34	Negative marker	FITC	FL1
CD45	Negative marker	PerCP	FL4

Table 2. Sample Tube Preparation Protocol

Sample Tube No.	Contents
1	Unstained cells- 50µl
2	1 x 10^6 cells in 45µl of DPBS + 5µl CD 105(final volume 50µl)
3	1 x 10^6 cells in 45µl of DPBS + 5µl CD 90(final volume 50µl)
4	1 x 10^6 cells in 45µl of DPBS + 5µl CD 34 (final volume 50µl)
5	1 x 10^6 cells in 45µl of DPBS + 5µl CD 45(final volume 50µl)
6	1 x 10^6 cells in 30µl of DPBS + 5µ 7AAD + 5µl CD 34 + 5µl CD105 + 5µl CD90 + 5µl CD45 (final volume 50µl)

2.11 Isolation of Exosomes from UC-MSCs:

As per the method described by Richard J Lobb et al., exosomes were isolated from UC-MSC cell culture conditioned medium. In brief, UC-MSC cell culture conditioned medium was collected and were centrifuged at 300g at 4°C for 10 mins to remove detached cells. Supernatant was then filtered through a 0.22µm filter and then ultracentrifuged 100,000g for 90 mins and then 100,000g for another 90 mins. The resulting exosomes pellet was resuspended in PBS for further use.

Exosome were processed for flow cytometry as per the kit protocol (exosome isolation and analysis kit-flow cytometry, plasma (CD63/CD81), abcam # ab267479).In brief, 50 µl of capture beads were added in a round bottom tubes containing 10-15µl of isolated exosomes. The tubes were incubated in the dark overnight at RT. Next day the sample was wash with 1ml of 1X assay buffer. The beads were collected on magnetic rack or by centrifugation. The supernatant was removed, taking care not to disturb the microspheres. Five µl of primary detection antibody (CD81-PE) was added to the tubes and incubated in the dark for 60 mins at 4°C. The above washing step was repeated. The beads were collected on magnetic rack. The sample was resuspended in 350µl of 1X assay buffer and acquire on a flow cytometer.

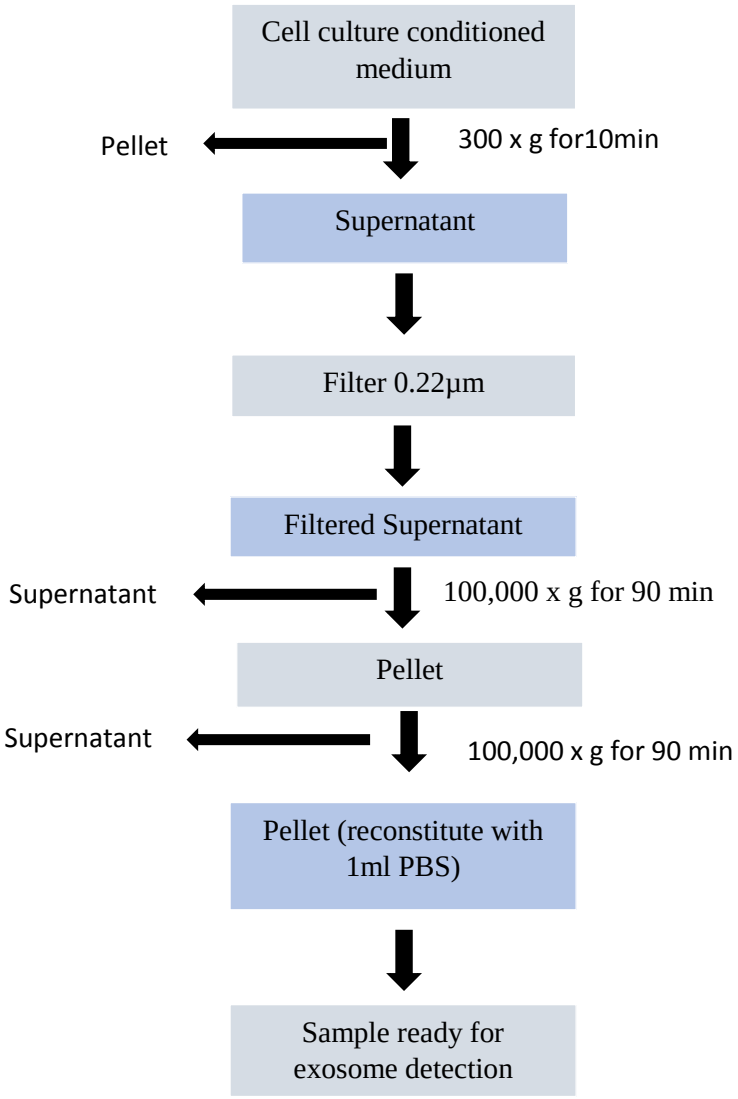


Figure. 3. Workflow for exosome detection

III. RESULT AND DISCUSSION

3.1 Umbilical Cord Collection and Preparation

Fig. 3.1 A. Collection of fresh human umbilical cord

The human umbilical cord was acquired from women with healthy pregnancies under the research institute and ethics guidelines. The cord was collected within 5 hours from birth. The sample was stored at 4°C until it was transported to the lab by placing it on ice in a Styrofoam box. The sample was processed within 2 - 4 h of collection. Later on, the cord was cut from placenta with a sterile surgical blade and was collected in a sterile tube containing phosphate buffered saline and was transported to the biosafety cabinet.

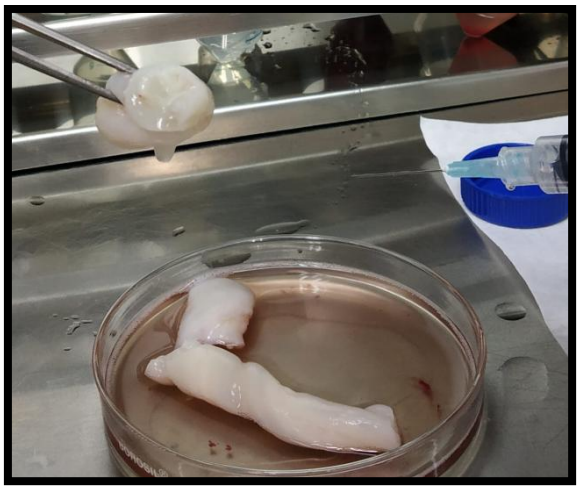


Fig. 3.1 B. Rinsing veins and artery to remove blood clots

The UC was washed several times with sterile saline to remove the blood completely. The veins and artery were washed with the help of a needle and syringe to remove the traces of blood.

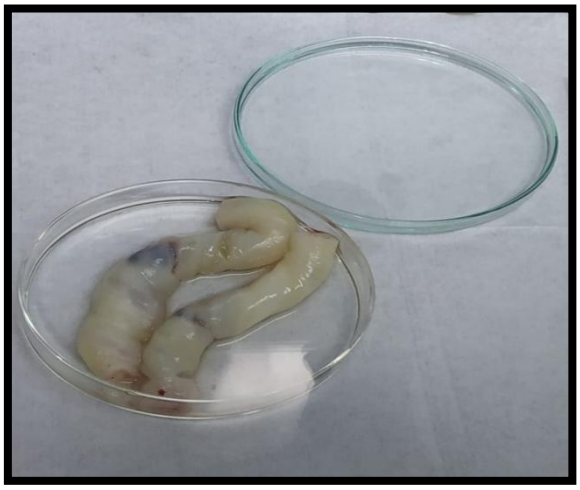


Fig. 3.1 C. Washed umbilical cord on petriplate

The cord tissue was handled and processed aseptically in a sterilized container using phosphate buffered saline in order to remove blood residues. The cord tissue was carefully washed without any rupture. Sterility was maintained throughout the process.

Fig. 3.1 D. Umbilical cord cut into 1-2 cm long pieces

Isolation of the human umbilical cord was carried out by the explant method. In the explant method, the UC was cut into small parts of about 1-2cm with a sterile scalpel. Blood vessels were completely removed and afterward the Umbilical cord was cut longitudinally with the help of scissors and forceps in a midline direction, completely exposing the blood vessels and surrounding WJ without disturbing the epithelium.

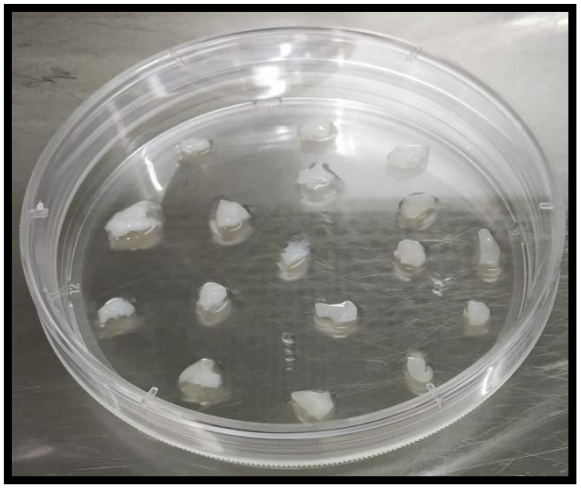


Fig. 3.1 E. Plating small explants of human umbilical cord

Each piece of cord tissue was then placed in a sterilized dish. The cord piece was individually positioned in the centre of a dry plate. Pieces of cord tissue were placed in a cell culture dish.

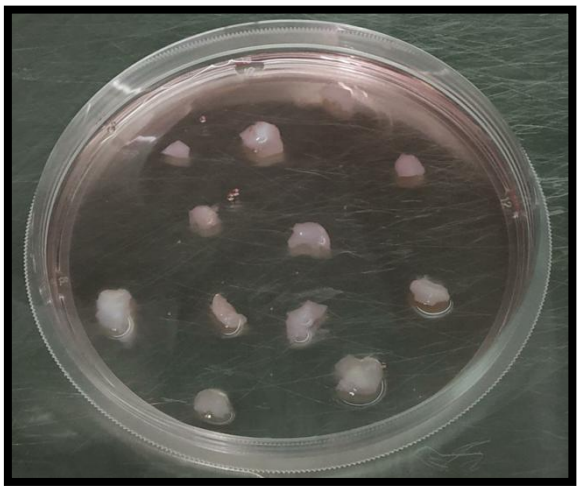


Fig. 3.1 F. Tissue supplement with culture medium

The UC fragments are placed into a culture-treated dish supplement with mediums where they are attached to the bottom. The media was added without disturbing the tissue by slow dispensing along the edge of the well. The tissue was left undisturbed for 7 days in order to avoid the detachment of the adherent tissue pieces. The plates were kept for incubation at 37°C, 5% CO_2.

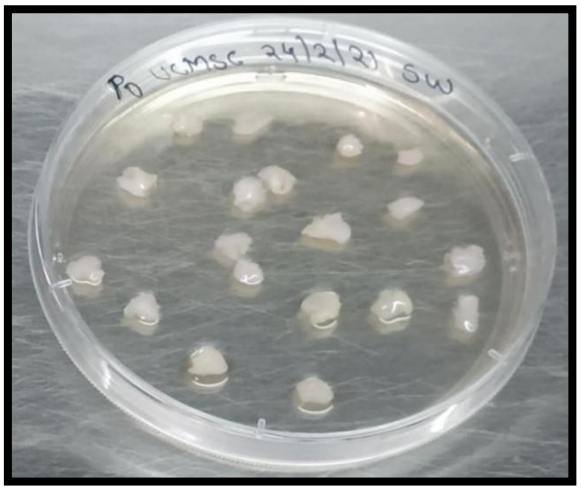


Fig. 3.1 G. Day 3 Incubation

Media was added when required, as per physical monitoring and visually changing conditions. Change in colour of media observed due to pH changed. The culture medium was replaced every 3-4 days for 2-4 weeks after seeding until a high-level confluence obtained. When a sufficient number of cells are obtained, tissue pieces are removed and a fresh new medium is added to permit these cells to proliferate for a couple of more days. Consistently, the plate was checked for the appearance of outgrowth of cells from the explants.

3.2 Morphological Observation of Isolated UC-MSCs:

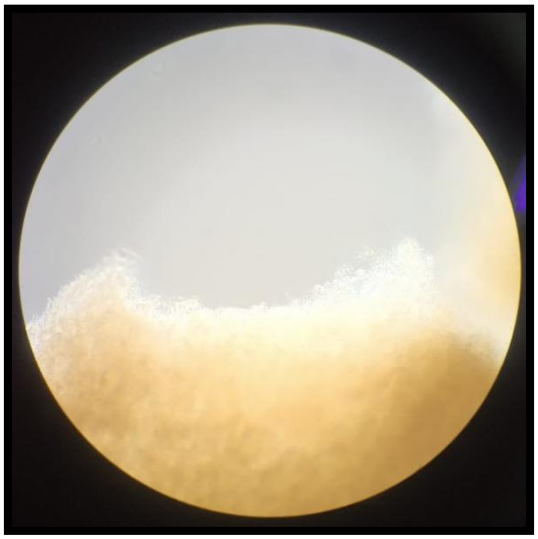


Figure 3.2 A. One day after plating

After plating, cell cultures were maintained at 37°C and 5% CO_2 in humidified atmosphere. The cord tissue was completely attached to the plastic surface of the culture dish. The fragments are undisturbed for 10 days to permit the stem cells to come out.

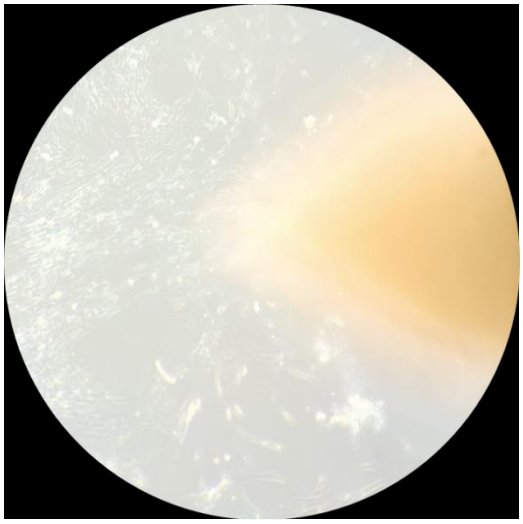


Figure 3.2B. MSCs at passage 0

At P0, the cells began to migrate from the explants following culture of tissue for 6-7 days. The cells reached 80% confluence after 12 days. Cells were attached to the tissue structure and outgrowing of cells. Adherent cells appeared at edges of umbilical cord explants after 12–15 days of culture. These outcomes showed that cells isolated from human umbilical cord tissue display morphology of MSC and expand rapidly without apparent changes in morphology. The initial growth of MSC at passage 0 (P0) consisted of adherent cells with a heterogeneous population; one of them was spindle-shaped fibroblast-like cells and another one had epithelial-like morphology.

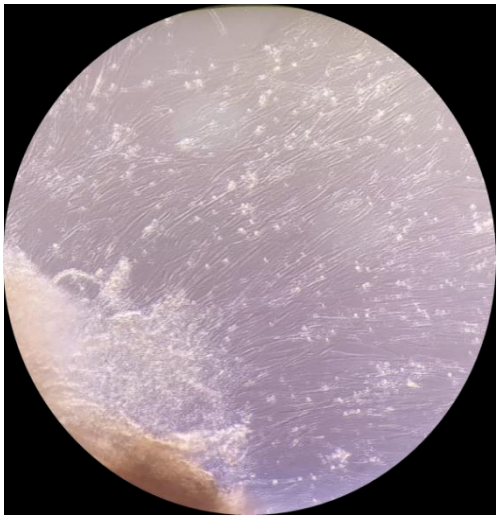


Fig. 3.2 C. MSCs at passage 1

Primary cells were cultured after two week and then 2-3 subcultures were generated. After 14 days, the cells reached 80-90% confluence and were ready for passage. These cells then became long and thin with small cell bodies. MSCs contain small raises with fibroblast-like appearance in the early phase.

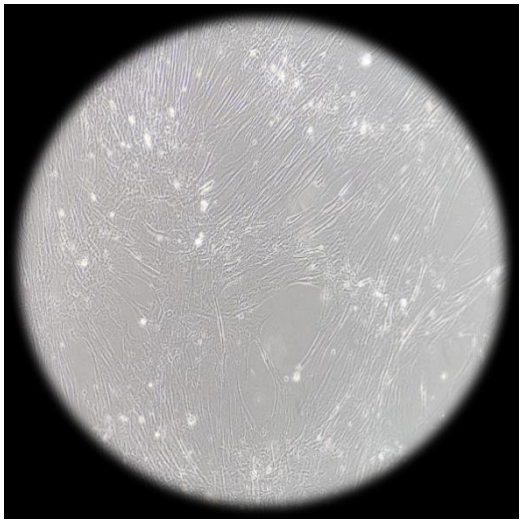


Fig. 3.2 D. MSCs at passage 2

Cells become larger and more elongated and also some cells gain an irregular and flat morphology in the middle phase. After trypsinization and subculture, the epithelioid cells disappeared from the culture. Cells formed a homogeneous monolayer of adherent, spindle shaped fibroblast-like cells. They grow and proliferate quickly.

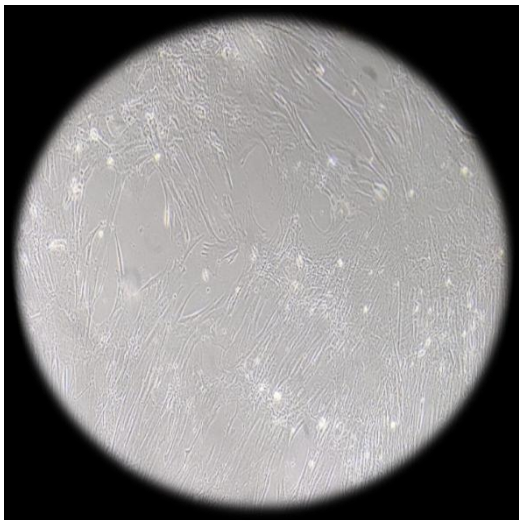


Fig. 3.2 E. MSCs at late passage 3

The MSCs were sub-cultured and expanded until 3rd passage. At early passages, the cells proliferate rapidly with small sized spindle shaped cells. In later passages, cells were evenly scattered and became large. MSCs had grown on the bottom wall of the culture flask, and the cells were uniform in shape with a long spindle shape and were fibroblast-like morphology. Each passage has been cultured until the 80-90% confluence was observed.

3.3 Phase Contrast Imaging of Isolated Cells from Human Umbilical Cord Tissue

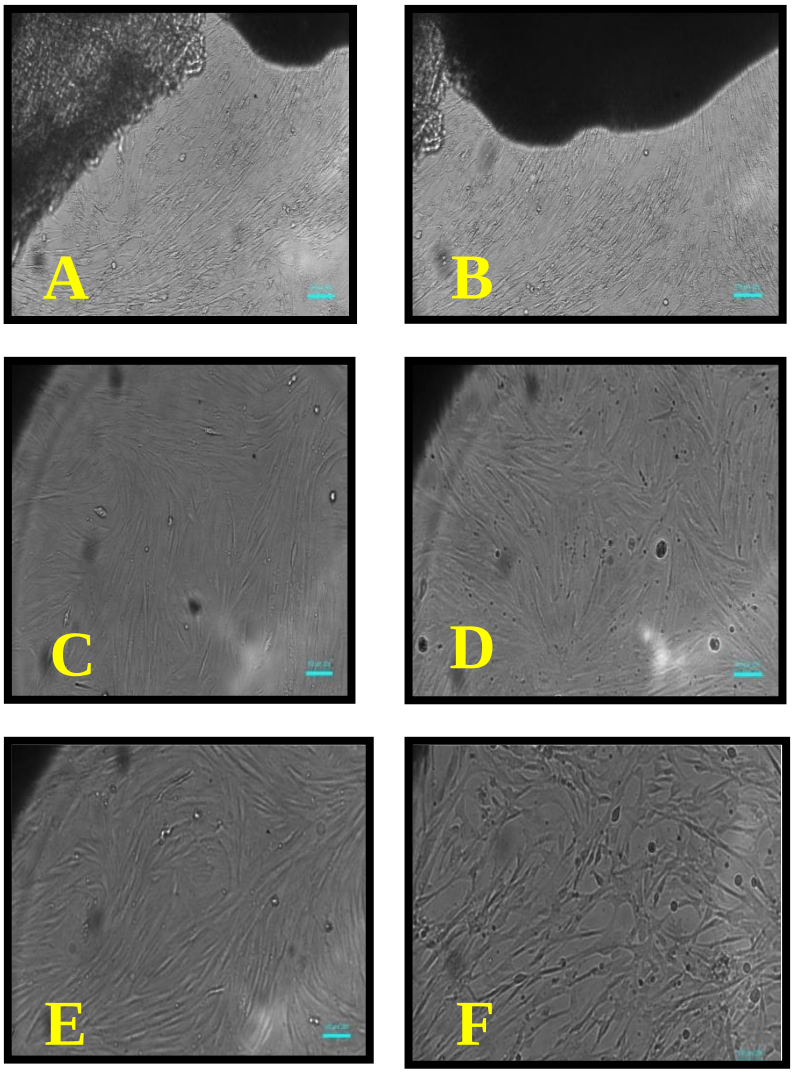


Fig. 3.3 Observations of Phase Contrast Imaging of Human Umbilical Cord in Primary and Passaged Cultures

A. Cells started to grow from the edges of the explants.
B. Cells are adhered to the plastic dish and start to proliferate.
C. Cells after two weeks of culture.
D. Cells reached 80-90% confluence and are ready for passage.
E. Cells maintained their spindle shaped morphology during passages.
F. MSCs isolated from umbilical cord using explant method exhibiting a mesenchymal like shape with a flat polygonal morphology, magnification:100x

MSCs display long spindle shaped fibroblast morphology when cultured in suitable culture media. Primary cultures of MSCs; however, may exhibit heterogeneous morphology with various shapes that become homogenous in successive cell passages.

3.4Sterility Testing of Culture Medium

Sterility testing is done so as to ensure that the culture is free from any sort of microbial contaminants. The tests were based upon the principle that if microorganisms are present in a medium which gives nutritive material and water, and kept at a favourable temperature, the organisms will develop and their presence can be indicated by turbidity in the initially clear medium.

3.4.1 Sterility Testing on Nutrient Agar

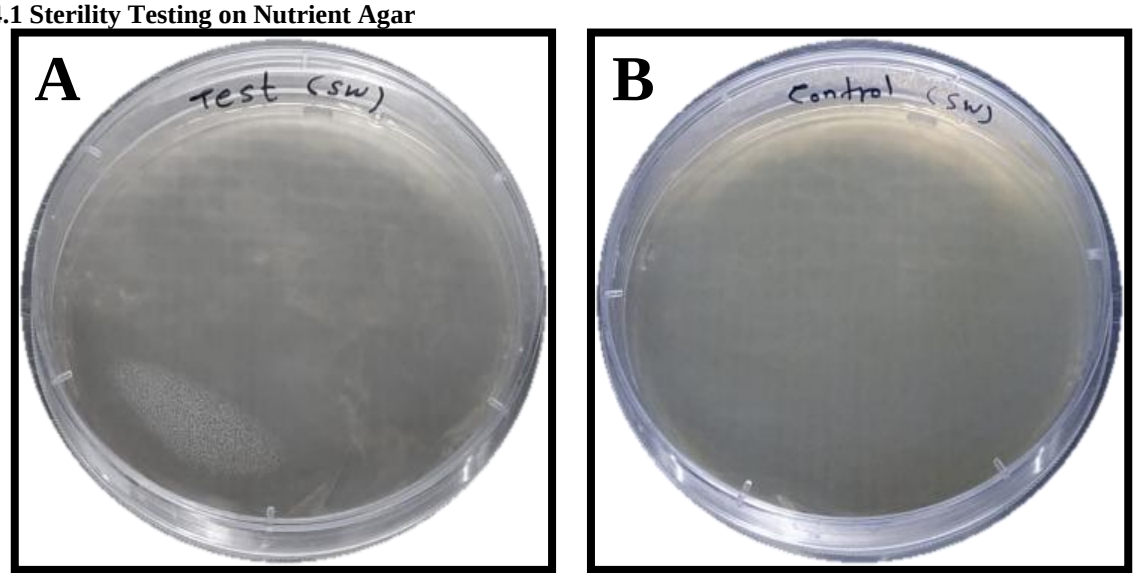


Fig. 3.4.1 (A): Test, Plates with Culture Medium; (B): Control, Without Culture Medium.

Sterility testing was required to ensure viable contaminating microorganisms are not evident in a product. This testing was conducted by a direct inoculation method. After inoculation, both media plates were incubated for 14 days. Intermittent observations as well as a final observation at the end of the testing period are conducted to detect any evidence of microbial contamination. Plates were visually examined and results showed that no growth was observed in any plate, i.e., control and test. The culture medium to be examined for the test complies with the test for sterility.

3.4.2 Sterility Testing on SCDM Agar

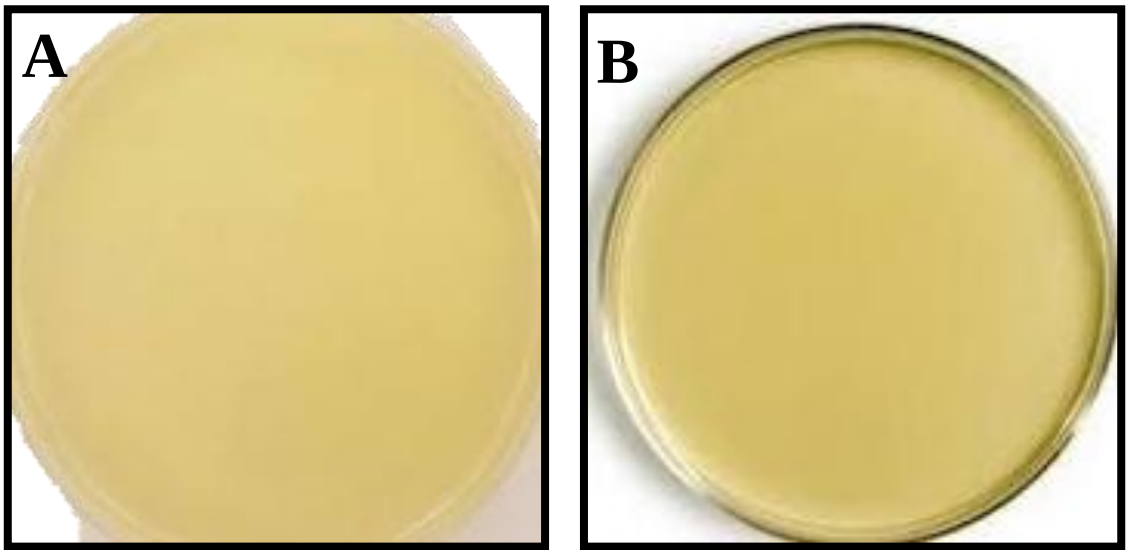


Fig. 3.4.2 (A): Test Plate with Culture Medium; (B): Control, Without Culture Medium.

Soyabean Casein Digest Medium is recommended for sterility testing. This medium is a highly nutritious medium used for cultivation of a wide variety of organisms. The combination of Tryptone and soya peptone makes the medium nutritious by providing nitrogenous, carbonaceous substances, amino acids and long chain peptides for the growth of microorganisms. Dextrose/glucose serves as the carbohydrate source and dibasic potassium phosphate buffers the medium. Sodium chloride maintains the osmotic balance of the medium. Plates were visually examined and the result showed that no growth was observed, the media was clear and transparent. The culture medium to be examined for the test complies with the test for sterility.

3.5 Cell Count of Culture Suspension

Trypan Blue is recommended for counting cells because it penetrates the cell membrane; thus, it enters the cytoplasm of cells with compromised membranes (dead cells) to stain them blue. The live cells remain intact and can be distinguished from dead cells by their ability to exclude the blue dye. The cell count is derived from the formula (# of live cells in 4 corner squares/4) × dilution factor (i.e., 2) × 10^4 cells

3.6 Cell Viability Determination

Trypan blue is one of several stains recommended for use in dye exclusion procedures for viable cell determination. This method is based on the principle that live (viable) cells possess intact cell membranes that exclude certain dyes, such as trypan blue, eosin, or propidium, whereas dead cells do not. When a cell suspension is simply mixed with the dye and then visually examined to determine whether cells take up or exclude dye. A viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm. Cell viability was calculated using the ratio of total live/total cells (live and dead). The viability is recorded for each corner square using the formula (# of live cells/total # of cells counted) × 100, and the overall viability is the mean of the 4 viabilities derived from the 4 corner square counts.

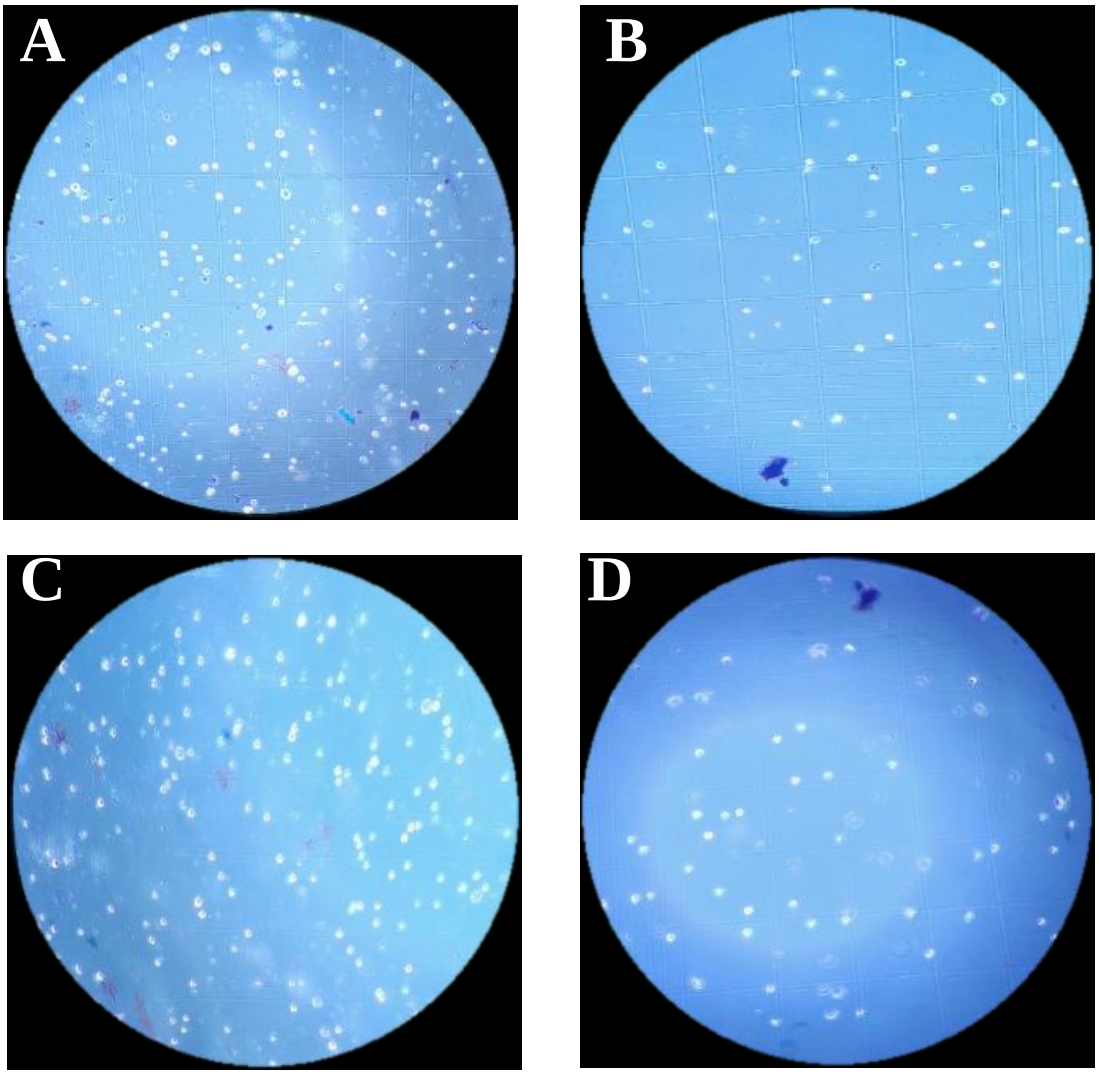


Fig. 3.6 A, B, C and D representing Cell count and Viability

Table 3. Cell Count & Cell Viability Determination

Passage No.	Viability	Cell Count	No of plates/ flask
P0	92%	10×10^6	5
P1	93%	90×10^6	15
P2	93%	270×10^6	45
P3	95%	810×10^6	135

MSCs in primary culture were 10,000,000 during the first 15 days (five culture plates each with 2,000,000 MSC in 90% confluence). Then they were sub cultured after every 6 days in the ratio of 1 to 3.

90,000,000 MSCs on day 21(first passage at 90% confluence), 270,000,000 MSCs on day 27 (second passage at 90% confluence), and 810,000,000 MSCs on day 33 (third passage at 90% confluence).

3.7 Proliferation Analysis

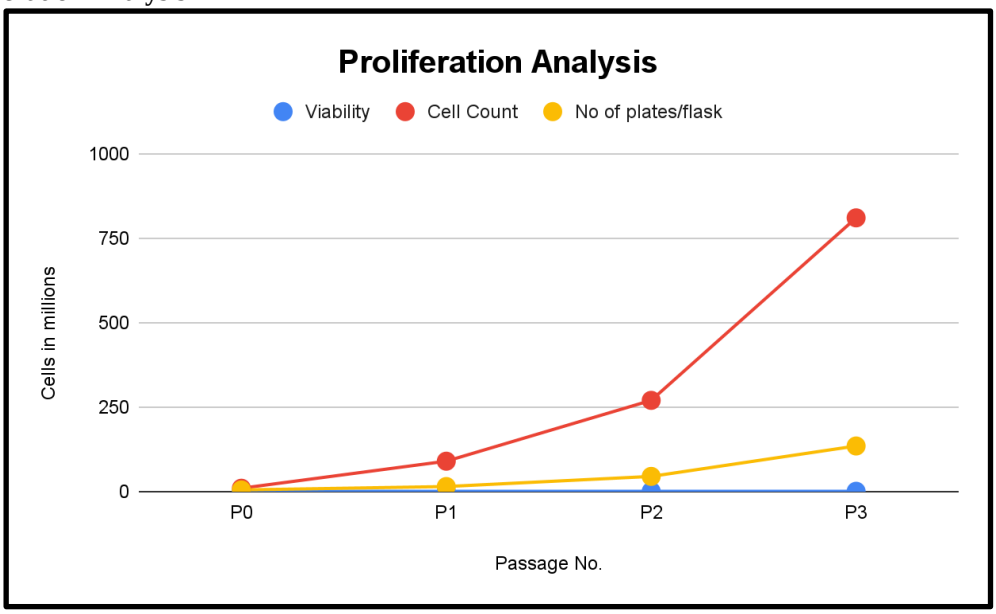


Fig. 3.7 Cell Number Obtained at Passage P0, P1, P2, P3 for UC-MSCs Sample.

Human umbilical cord mesenchymal stem cells show high proliferative ability. The total cell number was determined at each passage between P0 to P3 in order to measure the proliferative activity. The outcomes revealed that the number of cells was $10x10^6$ at P0, 90 at P1, $270\ x10^6$ at P2 and $810\ x10^6$ at P3. The total numbers of cells were recorded at each passage between P0- P3 and a growth curve was constructed. The growth curve demonstrated a clear increase in cell number between P0 to P3. The cells began to proliferate gradually and then entered the logarithmic growth phase which continued and reached the cell growth plateau.

3.8 Flow Cytometry Analysis

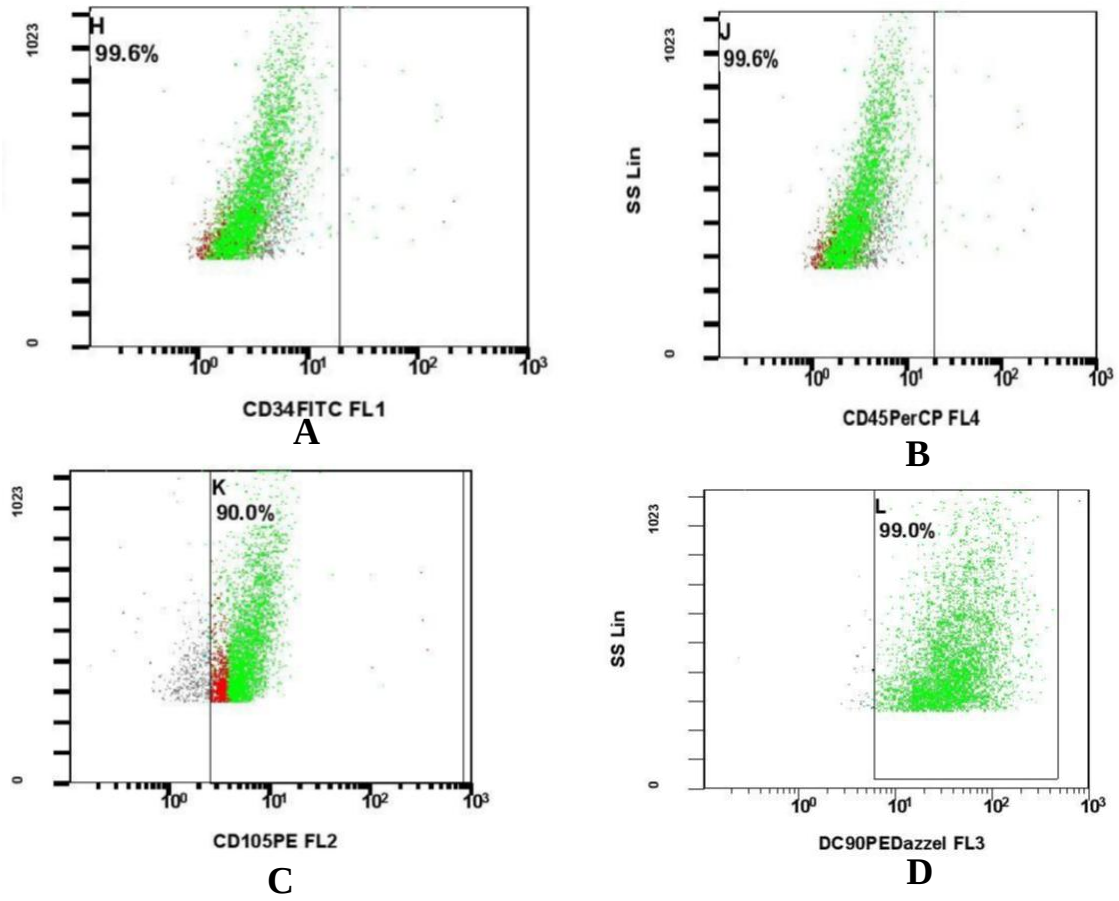
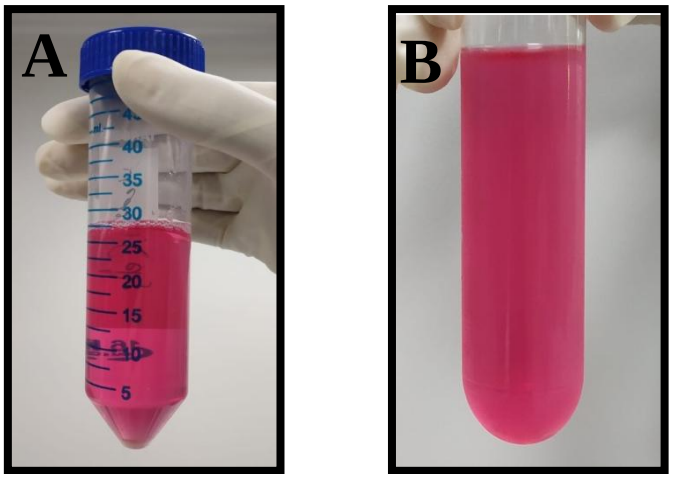


Fig. 3.8 A, B, C and D represent identification of UC-MSC

The immunophenotypes of human mesenchymal stem cells were analysed by flow cytometry. The shaded area shows the profile of the negative control. CD90, CD105, CD34 and CD45 were used to characterize the immunophenotypes of isolated MSCs from explant methods. Cultured MSCs are known to be strongly positive for the expression of CD90, CD105 and negative for the expression of CD34, CD45. The results are shown in figure 3.8.

The expressions of isolated MSCs P3 through the explant method were 99.02% for CD90, 90.0 % for CD105, 99.6 % for CD45 and 99.6 % for CD34. These results indicated that the isolated cells were purified MSCs.

3.9 Isolation of Exosomes from UC-MSCs



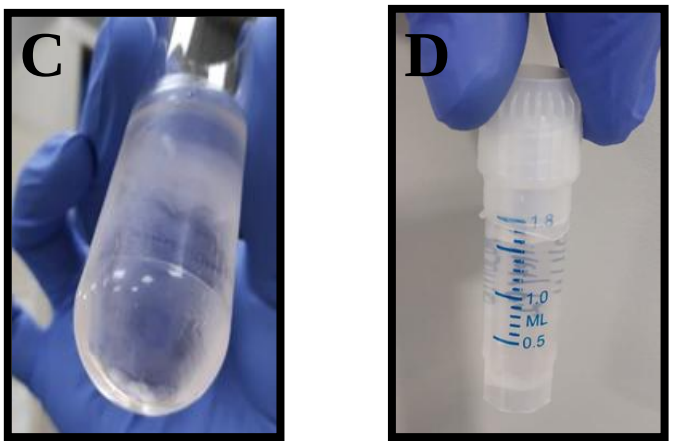


Fig. 3.9 Procedure for Purification of Exosomes by Ultracentrifugation.

A. Culture conditioned medium of passage 3 was centrifuged at low speed. Supernatant was collected and pellets discarded.
B. Supernatant was centrifuged at 100,000g
C. The sample is subjected to a second ultracentrifugation at 100,000 ×g to yield purified exosomes.
D. Exosome pellet is kept at -80□ C for further use.

3.10 Flow Cytometry Analysis of Exosomes:

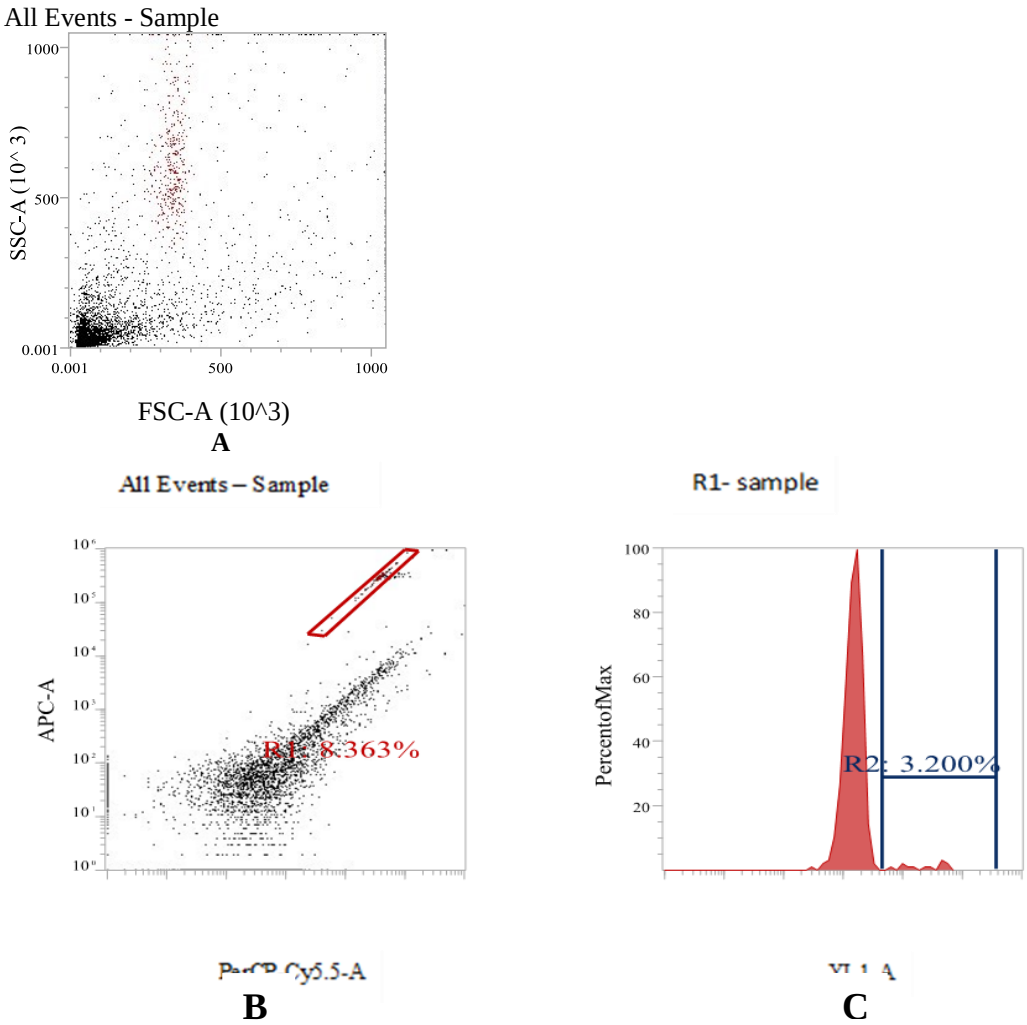


Fig. 3.10 Flow cytometric analysis of capture beads.

(A) Scatter Dot- Plot of beads, FSC vs SSC. (B) Gating strategy on FL3 vs FL4 for flow cytometry acquisition and analysis. (C) Representative histograms for detection of exosome quantity (3.200%) with flow cytometry kit.

IV. DISCUSSION

HUC-MSCs and HUC MSC-Exosomes participate in numerous biological processes and may function as novel targets for medical treatment. HUC MSC-based therapeutics hold great promise for the development of therapy aimed at repairing damaged human organ tissues. Over the previous many years, it has been highlighted that HUC MSC-exosomes primarily participate in promoting tissue repair and regeneration by transferring proteins, lipids and nucleotides, which boost the development of "cell-free therapy".

With the increase of our knowledge about the role of exosomes in infections, there has been a rapid surge of interest in this field. A significant prevention in exosome research is the difficulty in isolating pure samples of exosomes and quantifying them directly, due to their small size.

Current techniques employed for isolation include ultracentrifugation, ultrafiltration, size exclusion chromatography, precipitation, immunoaffinity-based capture. These strategies have undergone expeditious development which has made isolation easier and faster with larger and purified yields of exosomes. The development of a rapid, cost-efficient, and simple technique for the exosome isolation from clinical samples, followed by their exact quantification, would greatly advance research into their role as disease biomarkers and therapeutic.

In the present study ultracentrifugation was preferred for isolation of exosomes because it is the most widely applied and most basic method for the isolation of exosomes from human samples. This method depends on the sequential separation of particles by sedimentation dependent on their size and density using a series of centrifugal forces and duration. The principle of this method is to separate out the exosomes from the other materials present in the sample based on their volume and physical properties. It is a gold standard method for exosomes extraction and separation. Also, it uses high centrifugal speed which gives enough time to travel individual exosomes to form a pellet.

The percentage of exosomes obtained in this study was approx. 3% in 30 ml of conditioned media. We have detected exosomes using a kit which is composed of an exosome capture reagent consisting of 6.5-micron size magnetic beads bound anti- CD63 capture antibody and a PE conjugated anti-CD81 detection antibody by flow cytometry analysis as it is a conventional, easiest analytic method for biomarker characterization.

However, given all previous researches on HUC MSCs and HUCMSCs-exosomes, the therapeutic mechanisms mediated by HUCMSCs and HUC MSCs -exosomes still need to be further explored, especially when the molecules which play effective roles in diseases have not been identified. The exact molecular mechanism involved in the effect of HUC MSCs and HUC MSCs -exosomes on tissue repair and regeneration, requires further investigations before it is actually made available for clinical use. HUC MSCs and HUC MSCs -exosomes promise to be efficiently applied to the clinic in future. Our work provides a basis for further evaluating the potential of HUC MSCs -exosomes as therapeutic agents.

V. CONCLUSION

Umbilical cord-derived stem cells offer a great promise of regenerative medicine. The cells obtained from all umbilical cord compartments show characteristics of MSCs. They are plastic-adherent, have self-renewal ability, differentiation potential. UC represents an ideal source due to the ease of collection and lack of complications associated with cell isolation.

MSCs from the umbilical cord tissue were isolated by the explant culture technique. This method is inexpensive and gives a pure MSC population. Cell outgrowth was observed within a week after the initial culture. Following 2-3 weeks of culture, an adequate number of cells was obtained, which allowed for further analyses. According to the minimal criteria for defining MSCs by the international society of cellular therapy, MSCs adhere to plastic surfaces and express classical cell surface markers. We confirmed that isolated MSCs from umbilical cord tissue pieces displayed plastic adherent growth, spindle shaped morphology. They were also highly positive for mesenchymal stem cell markers CD105, CD90 positive and negative for cell differentiation markers of CD45 (a hematopoietic marker) and CD34. In this study, exosomes were confined from human umbilical cord mesenchymal stem cells with the assistance of ultracentrifugation. Exosomes were effectively isolated from the MSC.

CONFLICT OF INTEREST

None.

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