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Optimizing aseptic and serum milieu for the isolation of human whole umbilical cord tissue-derived mesenchymal stem cells

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Abstract

Background: Mesenchymal stem cells (MSCs) have become an attractive tool for tissue engineering and targets in clinical transplantation due to their regeneration potential and immune-suppressive capacity. The human umbilical cord, which is discarded at birth, can provide an inexhaustible source of stem cells for therapy. They are reported to contain immune privilege cells which may be suitable for allogenic-based therapies. However, the use of MSCs for therapeutic application is based on their subsequent large-scale in vitro expansion. A fast and efficient protocol for the generation of large quantities of MSCs is required to meet the clinical demand and biomedical research needs.

Results: MSCs were isolated from the umbilical cord by explants and enzymatic digestion and cultured in the appropriate growth medium resulted in the propagation of more than 1X 10^8 cells within 15 days from the single umbilical cord.

Conclusion: The isolation efficiency, cell yield, colony-forming unit fibroblast (CFU-F), growth kinetics, phenotypic characteristics of UCMSCs were determined.

Keywords: Stem cells, Umbilical cord, Mesenchymal stem cells, Explant, Differential potential

1 Background

The umbilical cord-derived multipotent mesenchymal stem cells (UCMSCs) could be a major source of multipotent stem cells that can be derived from umbilical cord (UC) tissue (whole/ Wharton's jelly). Research evidence suggests that UCMSCs and its derivatives have found to possess abundant positive clinical benefits in a wide range of investigations [1]. Stem cells derived from UC provide high accessibility and promising source for autologous/heterologous cell therapy. The tissue-specific adult UC stem cells therapy could be safe due to its incapability to form a teratoma [1], a drawback in tissue therapy, like pluripotent embryonic stem cells. Furthermore, UC-derived adult stem cells are ethically less controversial than embryonic stem cells [2]. Bone marrow and umbilical cord blood offer major sources of hematopoietic stem cell with several limitations, for example, invasive aspiration procedure decreased frequency and differentiation potential with the age of hematopoietic stem cells [3].

Obtaining stem cells from umbilical cord matrix thought to be a methodologically tranquil but avoiding contamination during culturing is very challenging. The successful isolation of UC-derived stem cells is daunting, for example, only three of ten umbilical cords have been cultured by many researchers [4–6]. The stem cell cultivation largely depends on the serum supplementation [7]. The ill-defined nature of Fetal Bovine Serum (FBS) provides inconsistent lot-to-lot performance that

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renders difficult conditions in downstream research and therapeutic applications [8]. The use of serum in cell culture sometimes leads to incorporation of bovine viruses and bovine-derived proteins that results in viral infection and bovine spongiform encephalopathy [9, 10]. There is a need to develop a rapid and effective protocol for the isolation of sufficient amount of UC-derived SCs to meet clinical and biomedical research demands and avoid complexity generated through the use of a different type of serum and contamination. It is also necessary to maintain apposite methods for collection, transportation and processing of the cord tissue that are required to retain stem cell recovery and maintain tissue integrity and viability. [11].

In this paper, we have generated qualitative data under optimized conditions for the transfer of the umbilical cord from the labor room right after delivery to the laboratory. The scaling up of umbilical cord-derived adult stem cells has also been observed. The reduced rate of contamination while opting for most suitable multiple decontamination steps of washing of cord with antibiotics solution was also seen.

2 Methods

2.1 Sample collection and processing

Full-term healthy human umbilical cord (UC) samples were collected according to the policy of Drugs Controller General Of India (DCGI) under Ministry of Health and Family Welfare, Government of India with reference number: File No.ECR/1678/Meerut/Indt/UP/2017 and Institutional Ethics Committee (IEC) Ref.No.IEC/Miet/A-003 9th May, 2018 at Meerut Institute of Engineering and Technology, Meerut, Uttar Pradesh, India from Silver Jubilee Hospital of Baramati, Maharashtra, Agarwal Hospital, TP Nagar, Meerut and Jeevan Hospital, Modi Nagar, Uttar Pradesh, India. Written informed consent of individual participants and doctors was collected from all donors for the study. Collected UC samples were first washed in normal saline before submerging in 1X phosphate-buffered saline (PBS) containing antimycotic (Penicillin and Streptomycin) solution 1 ml/100 ml and a dipped into 70% alcohol for 30–40 s. The process was repeated twice to remove blood cells. The treated UC cord sample was processed within 5 h post-delivery.

UC sample was dissected into small pieces with help of scissor and washed with Dulbecco's phosphate-buffered saline (DPBS, Gibco, Grand Island, NY, USA) containing 1% antibiotic. After washing, tissue was minced into small pieces (5–10 mm) using a sterile scalpel and kept in T-flask containing 3 ml of Dulbecco's Modified Medium F-12 (DMEM-F-12) and maintained in 5% CO_2 at 37 °C in a humidified atmosphere and fresh medium was changed every other day.

The enzymatic treatment of obtained UC sample was done by placing tissue into a petri plate using 1 mg/ml of collagenase type I and hyaluronidase. The plates were kept in a 5% CO_2 humidified atmosphere environment at 37 °C for 1 h. The solution was collected after incubation and centrifuged at 1800 rpm, resuspended in PBS, and again centrifuged for 10 min to form a pellet. The cells were seeded in tissue culture T25 Flask having 20 ml of DMEM-F-12 media and incubated in a humidified chamber at 37 °C with 5% CO_2 for 7 days. Media change was given at the third day but only half of the medium was replaced. A full medium change was given on every 7th day. The passaging was performed using 0.25% trypsin–EDTA solution and each passage was cultured for 2 weeks to expand until 60–80% confluence. Floating cells and media were aspirated from T25 Flask and monolayer was rinsed using 5–10 ml DPBS solution. Pre warmed trypsin EDTA (1X) was added to T25 Flask while coating the white surface. Flask was then incubated for 5–8 min at 36 °C to 38 °C or until cells have fully detached. An aliquot of detached cells was mixed to a single cell solution for the observation under the IX73 microscope (Olympus, Japan).

2.2 Adipogenic, chondrogenic and osteogenic differentiation of UCMSCs

Before induction, cultured UCMSCs were harvested and cultured under the 5% CO_2 humidified conditions to attain 60–80% confluence at 37 °C. The cells were cultured for 14 days and a change of medium was given every 3rd–4th day. Adipogenic, chondrogenic and osteogenic differentiation experiments were performed as per manufacturer instructions of human mesenchymal stem cell function identification kit (StemPro® ThermoFisher, India) and summarized differentiation methodology is presented in Fig. 1.

For adipose differentiation, 2.5 ml of adipose differentiation basal medium containing adipogenesis supplements (GIBCO) was nourished to 3.7×10^6 cells/well seeded on a 24-well plate for inducing UCMSCs to be committed to enter adipogenesis pathway for the generation of adipocytes. The cells were maintained to achieve 70–80% confluence within 3 weeks. Oil red staining was performed for detecting lipid droplets in the sample.

For chondrogenic differentiation, 2.5×10^5 cells were suspended in chondrocyte differentiation basal medium (GIBCO) and chondrogenesis supplements (GIBCO) for inducing UCMSCs to undergo chondrogenesis pathway for the generation of chondrocytes. The solution was centrifuged at 1800 rpm, resuspended in PBS, and again centrifuged for 10 min in a 15 ml conical tube (Corning) to collect pellet. The chondrogenic pellet was incubated, harvested after 3 weeks and fixed in 4%

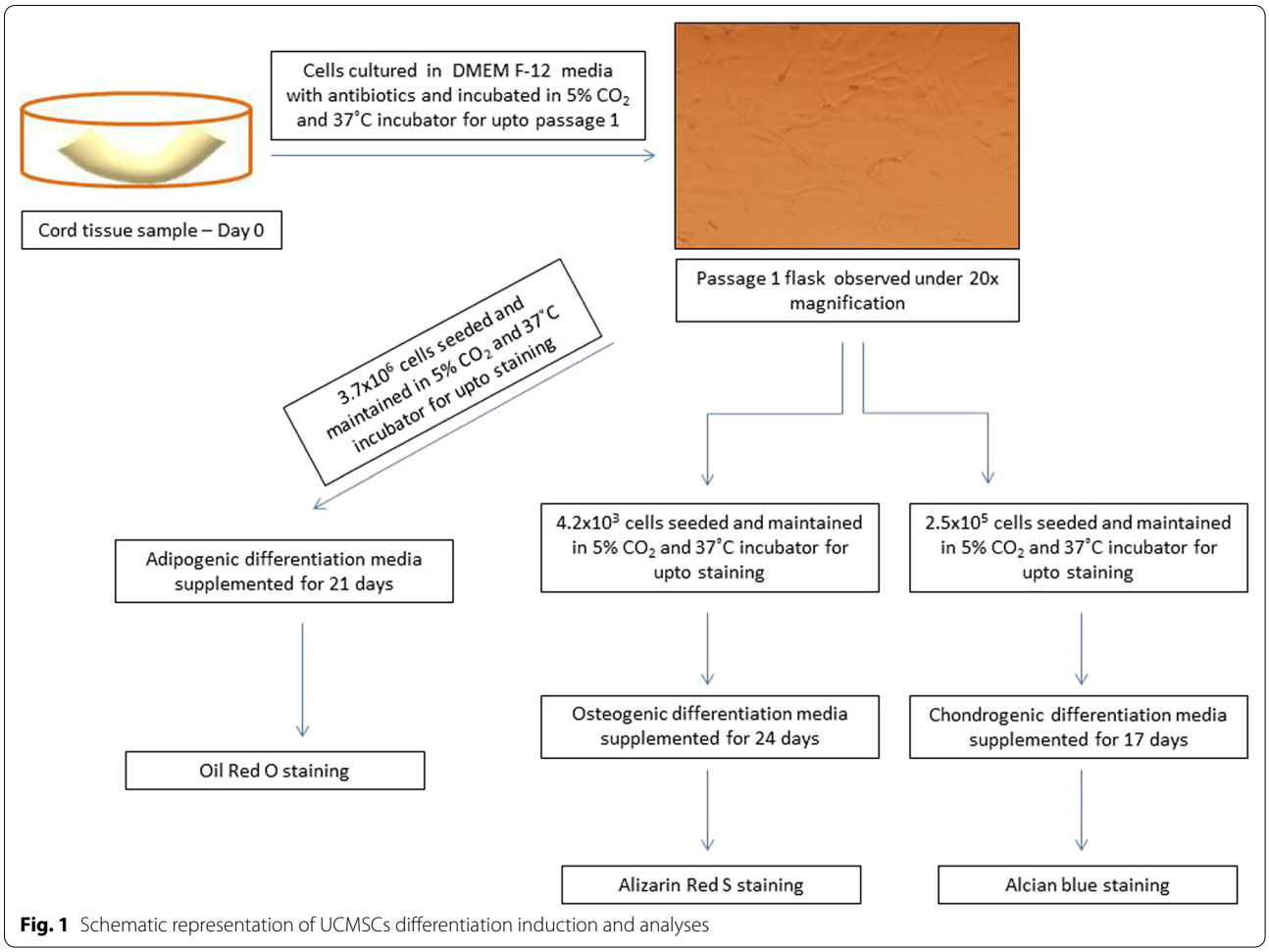


Fig. 1 Schematic representation of UCMSCs differentiation induction and analyses

paraformaldehyde (PFA). Chondrocytes obtained were identified using Alcian Blue (Sigma-Aldrich).

Furthermore, for osteogenic differentiation, 4.2×10^3 UCMSCs cells were seeded into osteocyte differentiation basal medium supplemented with osteogenesis supplement (GIBCO) for inducing to commit cells to generate osteocytes. The osteogenic differentiation of cells after 3 weeks was confirmed by Alizarin Red S staining for the calcium-rich extracellular matrix.

2.3 Colony-forming unit (CFU) assay

To assess the self-renewable capacity of UCMSCs, 1×10^6 viable cells were seeded onto the petri plate and left undisturbed for 10 min to allow adherence. 20 ml DMEM-F-12 media was added and incubated in 5% CO_2 humidified atmosphere at 37 °C for 7 days after which media change was given. Following the cultivation for around 14 days (before colonies began to merge), the monolayer was rinsed with DPBS/MeOH, fixed with 4% PFA for 10 min and then stained with crystal violet at room temperature. The stain was discarded, rinsed with deionized water and colonies were observed under an inverted microscope.

2.4 Flow cytometry analysis of UCMSCs

The immunophenotype of mesenchymal stem cells was analyzed for the expression of MSCs cell surface markers using flow cytometry after reaching 80% confluence at Stem Cell and Regenerative Medicine Laboratory, Vidya Pratishthan's School of Biotechnology, Baramati, Maharashtra (India) and Department of Biotechnology, Meerut Institute of Engineering and Technology, Meerut, India. 1×10^6 cells were divided into aliquots in 1.5 ml microcentrifuge tubes, and then, samples were centrifuged at 1800 for 5 min. The pallet was washed twice with PBS supplemented with growth medium. The cells were resuspended in 50 µL of PBS with 1% bovine serum albumin (BSA). MSCs surface markers dynamics was studied by FITC-conjugated primary antibodies CD90, CD105, PE-conjugated CD73 and CD105 as per the manufacturer's protocol. Corresponding isotype-matched antibodies/ or unstained cells were used as controls. The analysis

was done by BD FACSCalibur flow cytometer (BD Bio-Science, California, USA) using BD CellQuest software.

3 Results

The UC explant from whole umbilical cord was isolated and cultured in vitro. The cells were allowed to proliferate to a large number of UC-derived mesenchymal stem cells (UCMSCs) under aseptic conditions. UCMSCs cells exhibited a high degree of morphological heterogeneity and most of the cells have a rendered typical fibroblastic like phenotypic surface, spindle-shaped morphology and displaced a high capacity to adhere to plastic when maintained under standard culture conditions in tissue flasks (Table 1).

[Table 1. near here] Furthermore, these cells developed and flattened to cover the whole surface area of the petri plate. Our results showed a high degree of mesenchymal stem cells isolated using multiple washing steps with antibiotics and alcohol before processing of UC cord for culture. First 3 days, the cell culture showed spot-like cells of UCMSCs that started to grow from the UC explant. Explant tissue culture was proliferated to scattered and long spindle-shaped cells originating around the explant by 5–7 days of primary culture. Two weeks later, the culture grew and attained uniform spindle shape. In approximately 3 weeks, cells reached 80% confluence (Fig. 2).

For CFU-F assay, three plates with DMEM-F-12 medium with varying concentration of Hyclone's FCS were taken and incubated at 37 °C with 5% CO_2. After 7–8 days, formed incubation colonies were observed and it was found that the frequency of the colony increased with increasing concentration of FCS. 15% FCS containing plate showed a high frequency of colonies as compared to 5 and 10%. FBS/FCS is an essential component for cells growth and maintenance (Fig. 3). It contains low and high molecular weight biomolecules and a variety of factors that promote or inhibit growth.

The proliferation assay was done by growth curve, using the Trypan blue exclusion method. The amount of cells 2.48×10^8 cells was recovered from P1 where 1×10^6 cells were seeded and the doubling time reduced with increase in days. The primary culture was followed by second expansion step P1 (Fig. 4).

Isolation and culture with Gibco's NBCS of stem cells from cord tissue were observed for confluence within four weeks after primary culture and sub-culturing was performed. Upon observing under an inverted microscope, after 27 days of primary culture showed spindle-shaped fibroblast-like cells (Fig. 5a). Sprouting and cell growth were observed after 22 days of re-expansion P1 (Fig. 5b). Fourth passaging P4 leads to the elongated fibers of the MSCs from the tissue explant. Figure 6a and b shows the stem cells derived from UC explant growing in tissue culture medium with HiMedia's FBS.

Flow cytometry immune profiling demonstrated that all preparations remained immune-positive for markers characteristics of MSCs (CD73, CD90 and CD105) (Fig. 7a–d).

Table 1 UCMSCs has grown in culture media with Hyclone's FBS

Primary culture		**Passage**	
Initial seed	Harvest at P0	Plate at P1	Final cells at P1
Isolated cells from a single cord plated on T75 Flask	1×10^6 cells MSCs	1.27×10^7 MSCs in 2-cellSTACK chamber	2.48×10^8 MSCs
Day 0	Day 9–10		Day 15–16

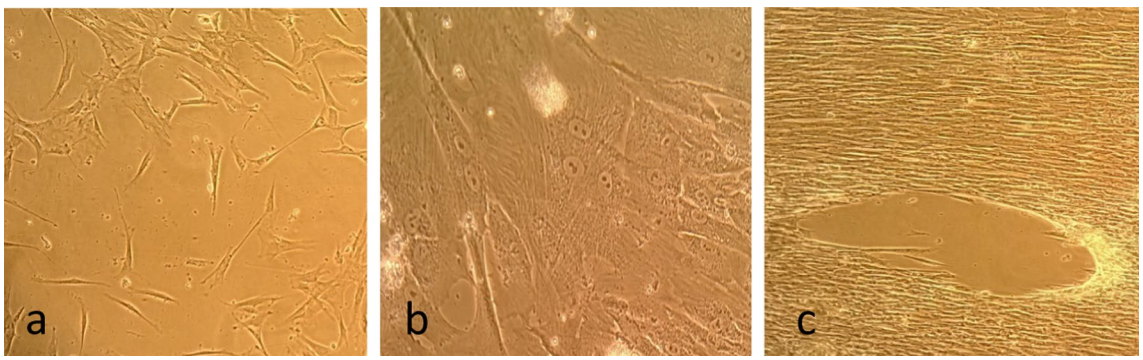


Fig. 2 Cord tissue-derived cells on (**a**) 7 days (**b**) 10 days (**c**) 20 days, respectively, as observed under 20X magnification under inverted microscope Olympus 1X73

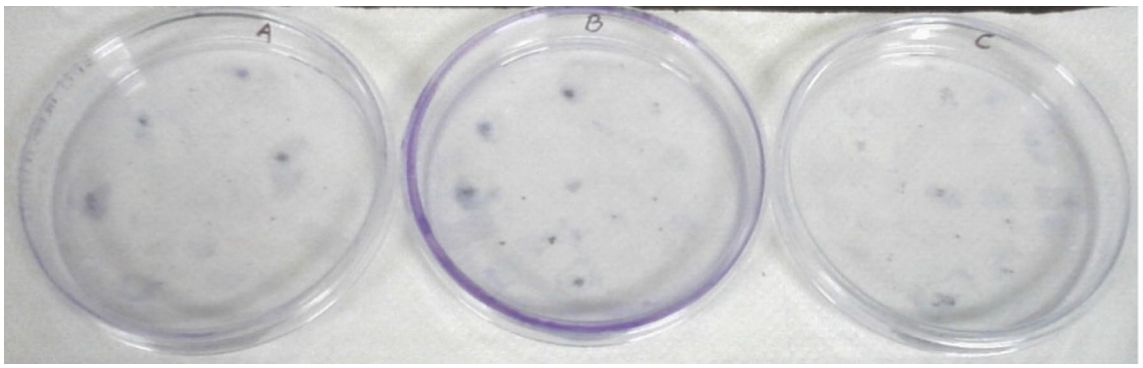


Fig. 3 CFU-F Colony formation (day 7) initiated. Human umbilical cord-derived stem cells expanded in **A** 5%, **B** 10% and **C** 15% FCS from Hyclone, respectively

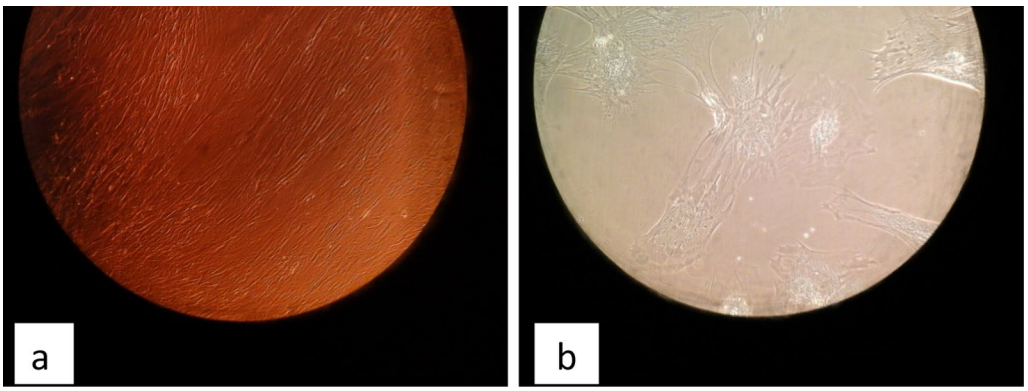


Fig. 4 Whole umbilical cord-derived stem cells were plated to obtain a growth curve: **a** After 3 weeks 100% confluent in DMEM-F-12 medium with Hyclone Serum under 20X. **b** After 10 days 40% confluent in DMEM-F-12 medium with Hyclone Serum under 40X as observed under Nikon Ti-S phase contrast microscope

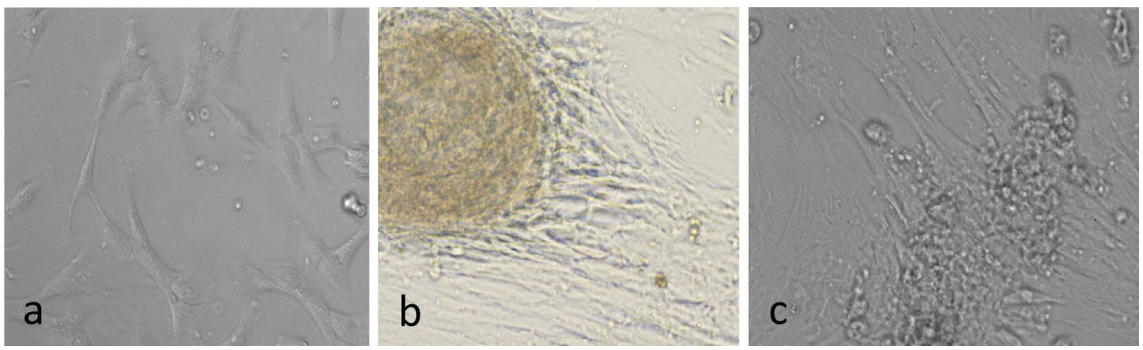


Fig. 5 Morphological features of stem cells grew in culture medium with Gibco's NBCS: **a** After 10–12 days of primary culture, spindle-shaped fibroblast-like cells appear as seen under phase contrast microscope Olympus 1X73 under 20X magnification. **b** Sprouting and cell growth after 4 weeks and above observed under 20X magnification **c** On 6–8 weeks observed under 20X magnification

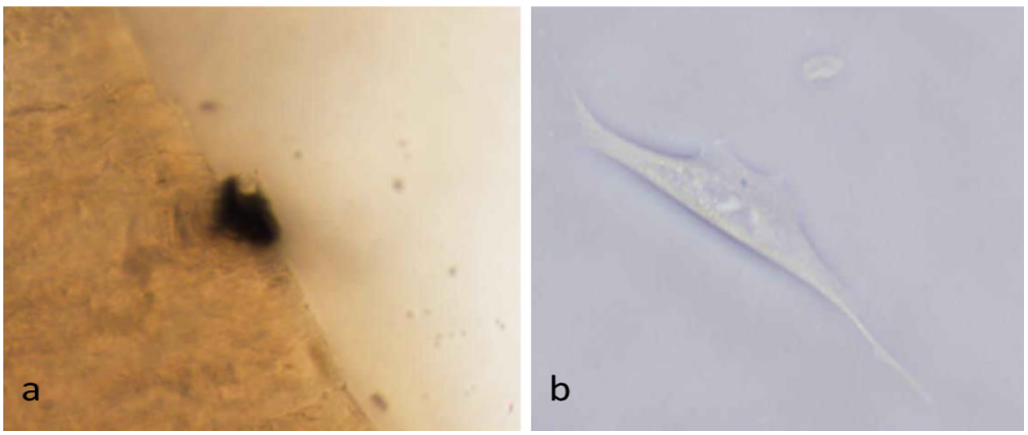


Fig. 6 Morphological features of stem cells grew in culture medium with HiMedia's FBS: **a** sprouting from umbilical cord explant under 10X **b** Stem cell-derived from explant under 40X as observed under inverted microscope Olympus 1X73. Comparatively and drastically low growth was observed with HiMedia FBS

Flow cytometry immune profiling demonstrated that all preparations remained immunopositive for markers characteristic of MSCs (CD73, CD90, and CD105). The unstained cells were used as control.

FACS analysis was done and generated histogram (Fig. 8) showed the major shift in the plot (Data.002) in comparison with control (Data.001), after gating, confirmed the presence of stem cells. CD105 is a specific surface marker expressed by human umbilical-derived adult stem cells. Hence, the presence of CD105 expressing cells confirmed the presence of human UC-derived mesenchymal stem cells.

Stem cell differentiation (chondrogenic, osteogenic, and adipogenic) was performed on the umbilical cord-derived tissue-specific stem cells (adult stem cells) which grew in culture medium supplemented with Gibco's NBCS. Chondrogenic differential media when supplemented to the flask after 30 days of the primary culture. For reference, control flask of 30 days of P1 culturing was used. Staining was performed after the supply of differential media for 17 days in culture flask with alcian blue stain which binds with sulfated glycosaminoglycan chains present in chondrocyte matrix and gives blue color (Fig. 9a–d).

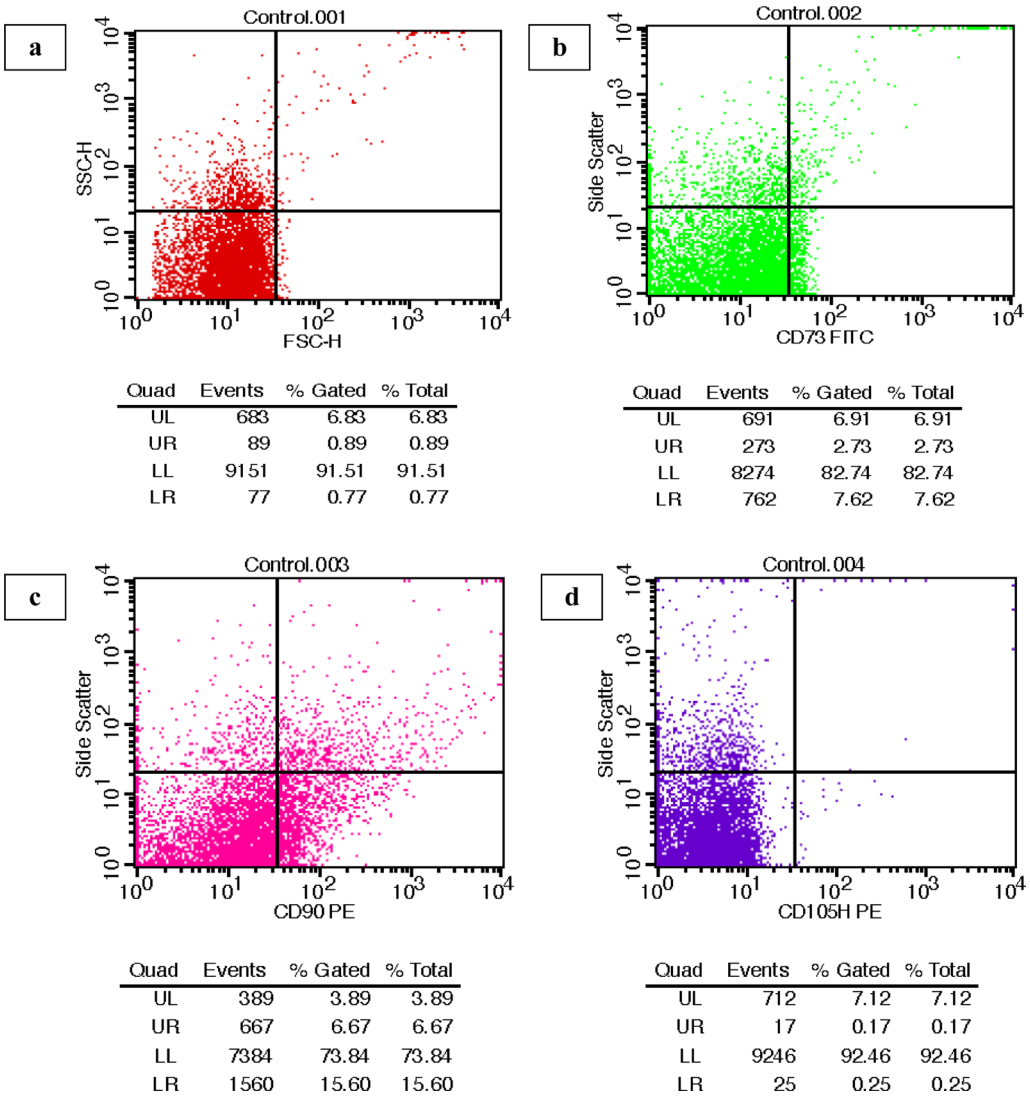
For osteogenic differentiation of the experimental cells, osteogenic differential medium was administrated to the flask with 37 days of primary culturing. Control flask of 14 days of P2 culturing was used. Staining was done after supplying differential media for 24 days in an experimental flask with alizarin red S which represents the calcium deposition and bone matrix formation (Fig. 10a–d).

Adipogenic differentiation was performed and adipogenic medium was given and oil red 'O' staining was performed which showed induction of adipogenic differentiation in the cells (Fig. 11a, b).

Thus, the differentiation of stem cells into adipocytes confirmed the presence of stem cells in the culture. This phenomenon can be further executed as regenerative applications [12]. Results showed that following the proper aseptic and growth media conditions, tissue-specific adult stem cells with chondrogenic, osteogenic and adipogenic differentiation ability raised from the small explant of umbilical cord tissue.

4 Discussion

Low toxicity and growth-promoting properties of FBS/FCS make it a common supplement for in vitro culture of most mammalian cells. However, animal sera are a potential source of viral contaminants, particularly mycoplasma, bovine viruses, and other pathogens that's why the only introduction of a standardized volume of serum in culture media is essential [13]. FBS is a type of blood serum which contains most of the plasma materials including glucose, hormones, carbon dioxide, electrolytes (mainly Na^+, Ca^{2+}, Mg^{2+}, HCO^{3-}, Cl^-) and proteins like globulin, albumin and fibrinogen except clotting factors. FBS is important for induction of some adhesive molecules expression [14]. FBS could also activate the immune system of a patient upon repeated administration and increases the risk of transmitting zoonotic viral or prion. As the level of various growth factors and other biomolecule plethora depend upon the environment, there is no defined and fixed quality of FBS between different batches. Also, there is no consistency in the quality of FBS between lots or batches. This inconsistency leads to the difficulty in controlling the cell culture and affects the differential potential of the cells [15, 16]. In this study,



Quad	Events	% Gated	% Total
UL	683	6.83	6.83
UR	89	0.89	0.89
LL	9151	91.51	91.51
LR	77	0.77	0.77

Quad	Events	% Gated	% Total
UL	691	6.91	6.91
UR	273	2.73	2.73
LL	8274	82.74	82.74
LR	762	7.62	7.62

Quad	Events	% Gated	% Total
UL	389	3.89	3.89
UR	667	6.67	6.67
LL	7384	73.84	73.84
LR	1560	15.60	15.60

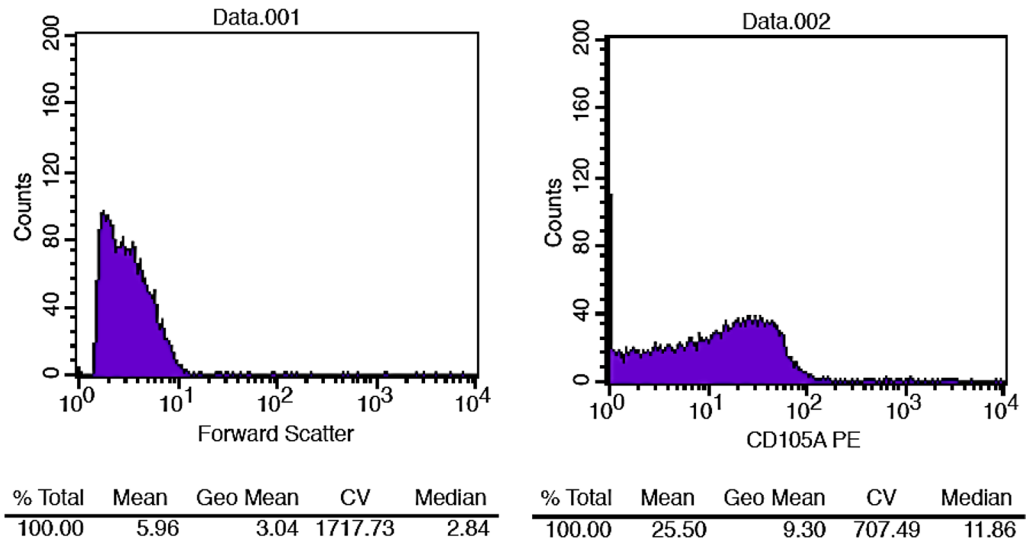
Quad	Events	% Gated	% Total
UL	712	7.12	7.12
UR	17	0.17	0.17
LL	9246	92.46	92.46
LR	25	0.25	0.25

Fig. 7 Dot blot results for the stem cell-specific markers: **a** control; **b** CD 73 FITC; **c** CD 90 PE; **d** CD 105 PE

various serums from different pharmaceutical firms were used and it was found the HiMedia FBS showed less potential and had no potency for the quality cell culture and differentiation, while Gibco's New Born Calf Serum (NBCS) and Hyclone's FCS showed excellent cogency in the whole umbilical-derived mesenchymal stem cell culturing and in chondrogenic, adipogenic and osteogenic differentiation.

5 Conclusion

Being a rich source of tissue-specific adult stem cells, the umbilical cord is the future of the success of cell-based therapies like regenerative medicine and tissue engineering. Collection of the umbilical cord and its processing for further cell culture under defined sterile environment/conditions, growth factors like serum and media plays an essential role in the success of the umbilical cord-derived mesenchymal stem cell culturing. The attainment of our study is majorly due to following the rules to avoid microbial load from the very start of the procedure, i.e., collection of the placenta in the labor room. UC is discarded tissue so using this discarded tissue has far sight application with less ethical controversy. Beside that application of adult stem cell reduces the risk of tumor formation in cell therapy due to lack of teratoma formation capacity. Repeated washing with antibiotic solution alleviated the microbial load and using the high-quality serum and culture media led to the good differentiation capability of the human umbilical cord-derived stem cells. Expansion



% Total	Mean	Geo Mean	CV	Median
100.00	5.96	3.04	1717.73	2.84

% Total	Mean	Geo Mean	CV	Median
100.00	25.50	9.30	707.49	11.86

Fig. 8 Single parameter histogram to evaluate the relative expression of a marker, in this case, the shift of the histogram peak for CD105 PE allows easy identification of the CD 105 positive cells indicates stem cell character

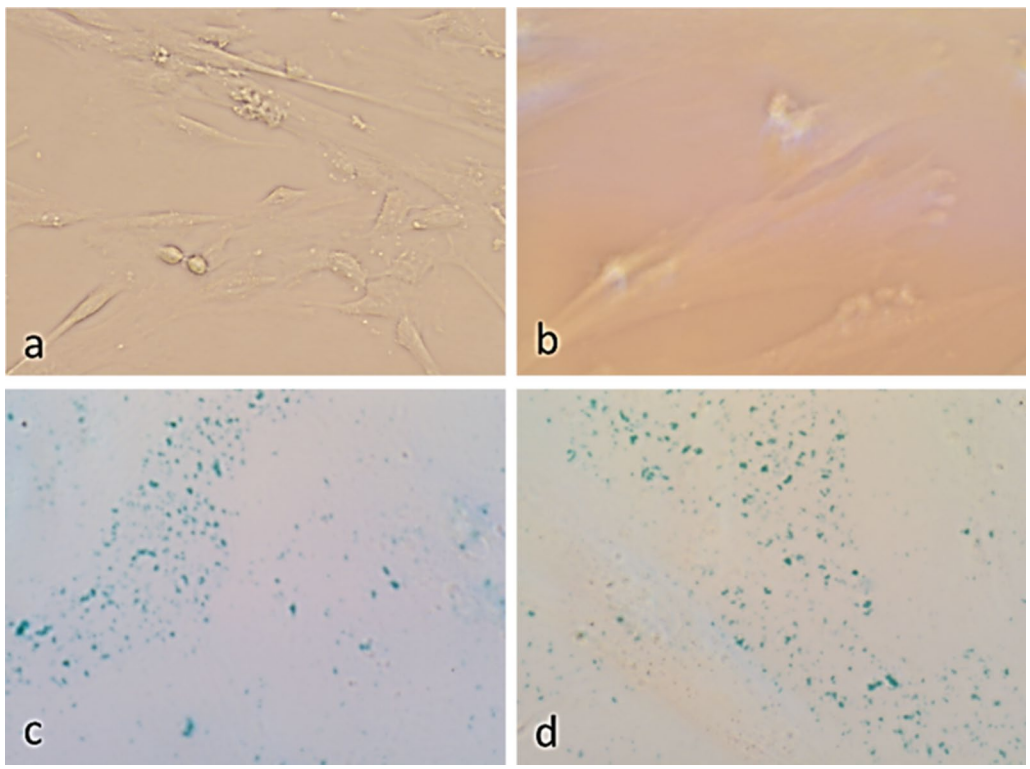


Fig. 9 After staining: **a** Control flask (20X Magnification, Phase contrast 2); **b** Control flask (40X Magnification, Phase contrast 3); **c** and **d** Glycosaminoglycan presence in experimental flask shown by the blue stains (40X Magnification, Phase contrast 3) as observed under inverted microscope Olympus 1X73

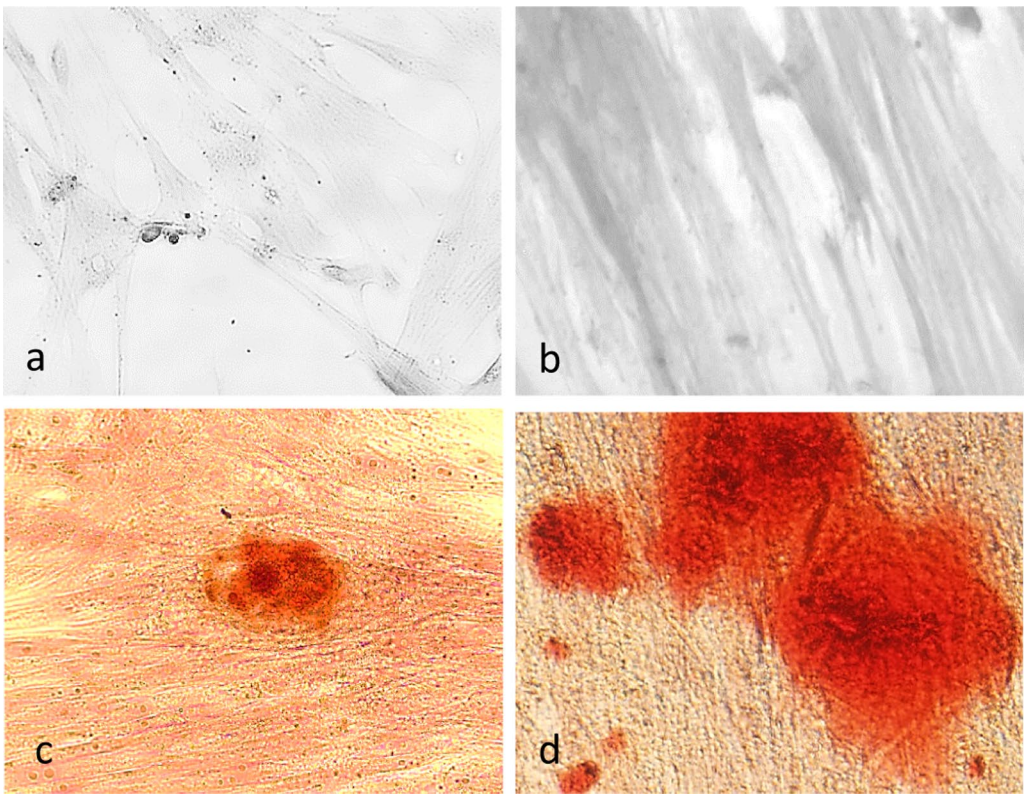


Fig. 10 After staining: **a** Control flask (20X Magnification, Phase contrast 2); **b** Control flask (40X Magnification, Phase contrast 3); **c** Calcium deposition represented by the red stain in the experimental flask (20X Magnification, Phase contrast 2); **d** Calcium deposition represented by the red stain in the experimental flask (40X Magnification, Phase contrast 3) as observed under inverted microscope Olympus 1X73

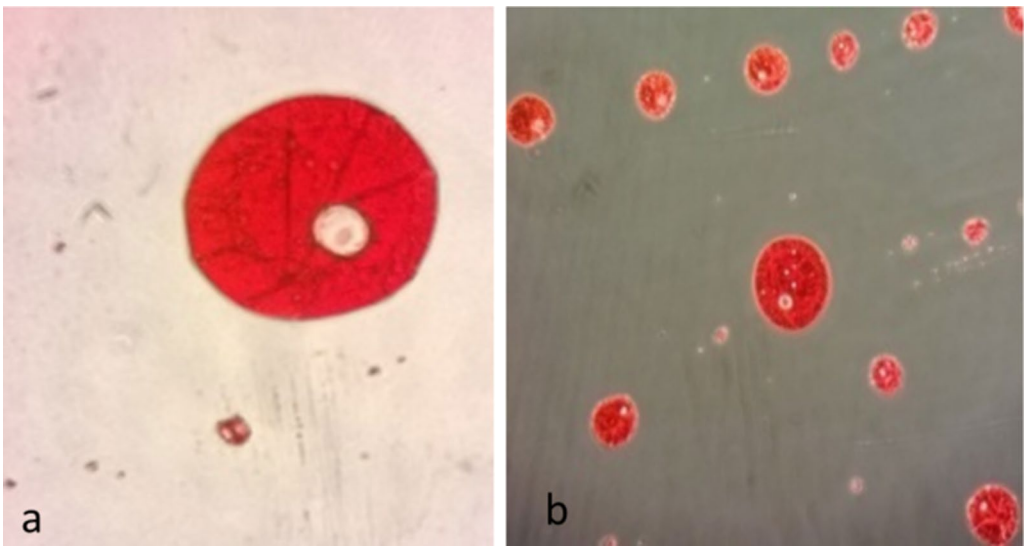


Fig. 11 Stained adipocyte under **a** 40X magnification and **b** 10X magnification as observed under inverted microscope Olympus 1X73

of high grade of these hUC-derived stem cells is essential for the successful clinical applications.

Abbreviations
MSCs: Mesenchymal stem cells; UCMSCs: Umbilical cord-derived Mesenchymal Stem Cells; FBS: Fetal bovine serum; BSE: Bovine spongiform encephalopathy; FCS: Fetal calf serum; IEC: Institutional ethics committee; UC: Umbilical cord; SC: Stem cells.

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Author contributions
YS provided the idea and performed characterization stem cells by exploiting Flow cytometry. SC, SS, JJ, NT, and KM performed stem cell isolation experiments, differentiation and result analysis. EC performed optimization of whole umbilical cord-derived stem cell isolation at her VSBT lab Baramati as well as provided guidance to run this entire project. All authors read and approved the final manuscript.

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Availability of data and material
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
Human umbilical cord (UC) samples were collected according to the policy of Drugs Controller General Of India (DCGI) under Ministry of Health and Family Welfare, Government of India with reference number: File No.ECR/1678/Meerut/Indt/UP/2017 and Institutional Ethics Committee (IEC) Ref.No.IEC/Miet/A-003 9th May, 2018 at Meerut Institute of Engineering and Technology, Meerut, Uttar Pradesh, India from Silver Jubilee Hospital of Baramati, Maharashtra, Agarwal Hospital, TP Nagar, Meerut and Jeevan Hospital, Modi Nagar, Uttar Pradesh, India. Written informed consent of individual participants and doctors were collected from all donors for the study.

Consent for publication
Not applicable.

Competing interests
The author(s) declare(s) that there are no conflicts of interest.

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