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EFFECT OF ANTIBIOTICS ON MESENCHYMAL STROMAL CELLS UNDER XENO-FREE CULTURE CONDITIONS FOR CLINICAL USE

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ABSTRACT

Recent advances in regenerative medicines indicate that adult Mesenchymal Stromal Cells are promising candidates for cell-based therapies because of their large in vitro expansion potential. During this expansion one of the main questions is obtaining a perfect combination of antibiotics that can be used safely on cultures with minimum interference on cellular levels. Considering this basic need current investigation is designed to propose antibiotic combination with optimum concentration having minimum effect on cells characteristics. For the analysis we used four types of antibiotics with three different concentrations each; Amikacin, Gentamycin, Cefoperazone Sodium, Vancomycin. It is observed that Amikacin showed slight alterations in cell viability and morphology as compared to other antibiotics. Out of four antibiotics Vancomycin proved to be the best antibiotic followed by Gentamycin and Cefoperazone Sodium. Hence Vancomycin can be used along with the Gentamycin; Cefoperazone sodium being a third generation antibiotic can be used alone to avoid detrimental effect of other antibiotic on cell lines.

KEYWORDS: Mesenchymal Stromal Cells, Amikacin, Gentamycin, Cefoperazone Sodium, Vancomycin

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INTRODUCTION

At the beginning of twentieth century, in vitro tissue culture methods were developed and were limited to applications that involved primary explant as the only source to study cells in vitro. One hundred years later after the discovery of Stem cells and their in vitro expansion researchers are heading towards their next goal ; The Cellular Therapy. They are exploiting these immortalized human stem cell lines as a relevant and well characterized experimental model system for various drug screening assays to develop novel therapeutics[1]. These types of cell culture model systems have been proved to be advantageous due to ease of use, exactly mimicking normal physiological condition, potentially limitless sample size and relative inexpensiveness. Progress in implementing more effective strategy for in vivo- in vitro extrapolation and human risk assessment depends upon significant advances in tissue culture technology. The level of complexities of these new technologies require adequate facilities, qualifying personnel with experience in cell culture techniques, assessment of quality and clear protocols for cell production. The rules for the implementation of cell therapy centers involve national and international standards of good manufacturing practices. However the universal challenge most researchers are facing in cell culture is bacterial contamination due to which it is difficult to obtain reliable results. Moreover they are common with poor aseptic techniques and can prove devastating in research settings. These contaminations can be transient; mediating a variety of interactions within and between organisms by gaining entry and multiplication in the host organisms. While they may steal nutrients from cell lines, they can also pray on cells themselves which will eventually lead to alteration of experimental results because of unwanted activation of cells. Due to the evolution from past 500 million years these bacteria have developed diverse mechanism showing resistance against different antibiotics[2,3]. Hence it is of utmost need to develop the best suitable antibiotic combination to observe healthy and normal growth of these cell lines. The bacteria which has so far detected in stem cell culture are both gram positive and gram negative; giving chance to scientist for the use of wide spectrum of antibiotics for complete prevention of infection. It has been known from the literature that cultivation parameters are critical for improved activity of antibiotics and hence for the enhanced growth of cells. Even small changes in cultural media may interface with the general metabolic profile of cells. Manipulating nutritional and environmental factor will thus influence promoted activity of antibiotics due to biosynthesis of certain natural compounds.

We, in our laboratory are collecting umbilical cord samples from different healthy donors for the isolation of mesenchymal stromal cells. These cells are characterized for their stemness and then grown on a large scale basis for therapeutic application. These cultures are at a risk of most of the bacterial & fungal contaminations due to mode of delivery of sample[4] which is through the vaginal track leading to significant loss of material, time and money and ultimately the loss of valuable cell lines. We studied antibiotics and their concentrations to establish standardized testing condition on growth of stem cells. We also tried to figure out the best suitable antibiotic combination for good and healthy cell growth. In the study for endpoint analysis on the concentration effect we selected four different classes of antibiotics which are Amikacin- An aminoglycoside antibiotic; commonly used against gram negative bacteria, Gentamycin- An aminoglycoside most commonly used against all gram negative pathogens, Cefoperazone Sodium- A third generation antibiotic for numerous gram positive as well as gram negative pathogens, and Vancomycin- A glycopeptide antibiotic used in the prophylaxis of gram positive bacteria[5,6].This work will be useful in the development of controlled strategies for large scale, effective and contamination free production of stem cells as a novel drug .So that in future a balance approach between sample throughput and biological relevance would

provide a better in vitro tool which will be complementary with animal testing and assist in providing more predictive human risk assessment.

MATERIALS AND METHODS

Isolation and culture of WJ-MSCs

(i) Raw Material Collection and Transportation.

Human umbilical cords (n = 5) irrespective of the sex of baby were collected from full-term births after either cesarean section or normal vaginal delivery with informed consent using the guidelines approved by the Institutional Committee for Stem Cell Research and Therapy (ICSCRT) at Unistem Biosciences Pvt ltd, India. The cord was clamped and approximately 15 cms of it was cut and transferred into a labeled tube containing F12 Dulbecco's modified Eagle's medium (DMEM/F12) without antibiotics. A specialized validated temperature controlled container (2–15℃) was given to those parents who had chosen to donate their child's MSCs for standardization. Cords were transported to the processing facility within 36 hrs.

(ii) Cord Tissue Processing.

The cord was washed with Dulbecco's Phosphate-Buffered saline (DPBS). Approximately fourty tissue explants of about 1mm size each were plated in tissue-culture-grade T-25 flasks containing Stempro MSC SFM Xeno free medium (Invitrogen). All flasks were left undisturbed in a 5% CO2 incubator maintained at 37°C for 4-5 days after which fresh culture medium was added to the flasks. Adherent cells were allowed to expand for 15–18 days by changing media at an interval of 4 days. Cells were harvested at 70–80% confluency using 0.125% trypsin (Gibco), a cell count was performed and 0.5 million cells with antibiotic were replated into T-75 tissue culture flasks for further expansion. These cells were observed for 8 days under microscope. MSCs were then removed from the flask with 0.125% trypsin-EDTA solution and washed twice with Phosphate-Buffered saline (PBS).

(iii) Antibiotic addition.

Amikacin (JR Scientific, INC) 12.5 mg/mL was added to cultured stem cells at a final concentration of 12.5, 25 and 50 µg/mL; Cefoperazone sodium (MP Biomedicals, LLC) 25 mg/mL was added to cells at a final concentration of 0.1, 0.125 and 0.25 mg/mL; Gentamycin (JR Scientific, INC) 50 mg/mL was added to cells at a final concentration of 60, 120 and 240 µg/mL and Vancomycin (Applichem) 5 mg/mL was added to cells at a final concentration of 2.5, 5 and 10 µg/mL. All concentrations used were higher than the Minimum Inhibitory Concentration (MIC) of antibiotics studied and used against the majority of microorganisms encountered in a hospital environment[7]. In all experiments we used a control flask without antibiotic. Cultures were incubated at 37 °C for 8 days.

(iv) Microscopic Observation and Viability determination.

All cultures including control were photographed in CKX41 Olympus inverted microscope (Olympus, Japan).Viable cells were counted microscopically by trypan blue dye exclusion method using a hemocytometer. Percentage viability was measured[8]as dividing number of viable cells by the total number of cells (Live and Dead) multiplied by 100.

(v) Phenotypic Characterization.

Harvested cells were subjected to immune phenotypic analysis. 1 x 10^5 Cells were incubated with specific mouse anti-human antibodies conjugated to flurochrome. Antibodies used were CD90 (FITC), CD73 (APC), CD45 (FITC), CD34 (PE), & CD 105 (PE). All antibodies were procured from BD Pharmingen, USA. After incubation for 20 mins at 4℃, cells were washed with PBS and acquired using a FACS Canto II flow cytometer (Beckton Dickinson). Approximately 10,000 events were acquired and data analysis was performed using the FACS Diva software.

RESULTS

Microscopic Observation

All cultures were photographed after eight days of culture with one control without antibiotic addition as shown in Figure 1

Photograph of Mesenchymal Stromal cells

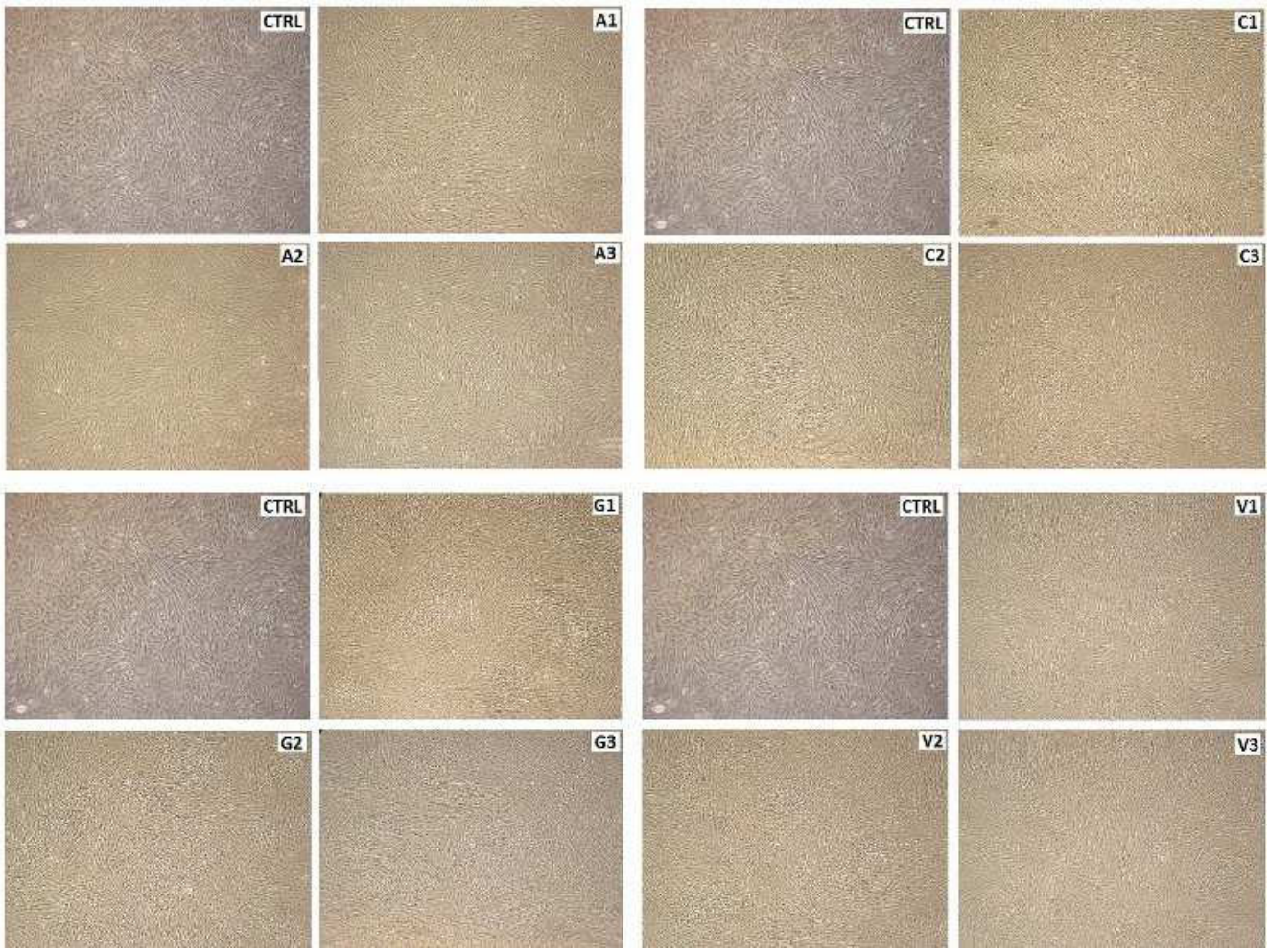

Figure 1

Control without antibiotic addition (CTRL), and after Amikacin 12.5 µg/mL (A1), 25 µg/mL (A2) and 50 µg/mL addition (A3); after Cefoperazone sodium 0.1 mg/mL (C1), 0.125 mg/mL (C2) and 0.25 mg/mL addition (C3); after Gentamycin 60 µg/mL (G1), 120 µg/mL (G2) and 240 µg/mL (G3) addition; after Vancomycin 2.5 µg/mL (V1), 5 µg/mL (V2) and 10 µg/mL (V3) addition.

Effect of antibiotics

The effect of in vitro administration of Amikacin, Cefoperazone sodium, Gentamycin and Vancomycin on cell count and viability was analyzed at different concentration as expressed in Table 1 and Table 2.

Effect of in vitro administration of Amikacin, Cefoperazone sodium, Gentamycin and Vancomycin on number of MSCs.

	Amikacin	Cefoperazone sodium	Gentamycin	Vancomycin
Control (Without antibiotic)	5.8 ± 0.09			
T1	4.35 ± 0.16	5.45 ± 0.19	5.56 ± 0.33	6.70 ± 0.12
T2	4.95 ± 0.16	6.07 ± 0.09	6.08 ± 0.08	7.28 ± 0.19
T3	3.92 ± 0.32	5.17 ± 0.15	4.90 ± 0.16	6.10 ± 0.12

Table 1

Data is expressed as mean x 10^6 cells ± SD. All groups contain 5 MSCs cultures. Amikacin: T1 = 12.5µg/mL, T2 = 25µg/mL and T3 = 50µg/mL; Cefoperazone sodium: T1 = 0.1mg/mL, T2 = 0.125mg/mL and T3 = 0.25mg/mL; Gentamycin: T1 = 60µg/mL, T2 = 120µg/mL and T3 = 240µg/mL; Vancomycin: T1 = 2.5µg/mL, T2 = 5µg/mL and T3 = 10µg/mL.

Effect of in vitro administration of Amikacin, Cefoperazone sodium, Gentamycin and Vancomycin on viability of MSCs.

Antibiotic	Without antibiotic (%)	With antibiotic (%)
Amikacin (25 µg/mL)	95.2 ± 1.48	89.82 ± 0.62
Cefoperazone sodium (0.125 mg/mL)		93.66 ± 1.02
Gentamycin (120 µg/mL)		93.92 ± 1.21
Vancomycin (5 µg/mL)		96.4 ± 1.14

Table 2

From Table 1, selected only one concentration of each antibiotic as optimum depending upon cell count for further investigation. The % viability of cell with chosen concentration of antibiotic is given in table 2. The data is expressed as % Mean Viability +/- SD. All groups consisted of 5 Mesenchymal stromal cell cultures.

Flow Analysis

To check interference of added antibiotic with surface receptors, flow analysis was carried against Mesenchymal stromal cells specific antibodies as shown in Figure 2.

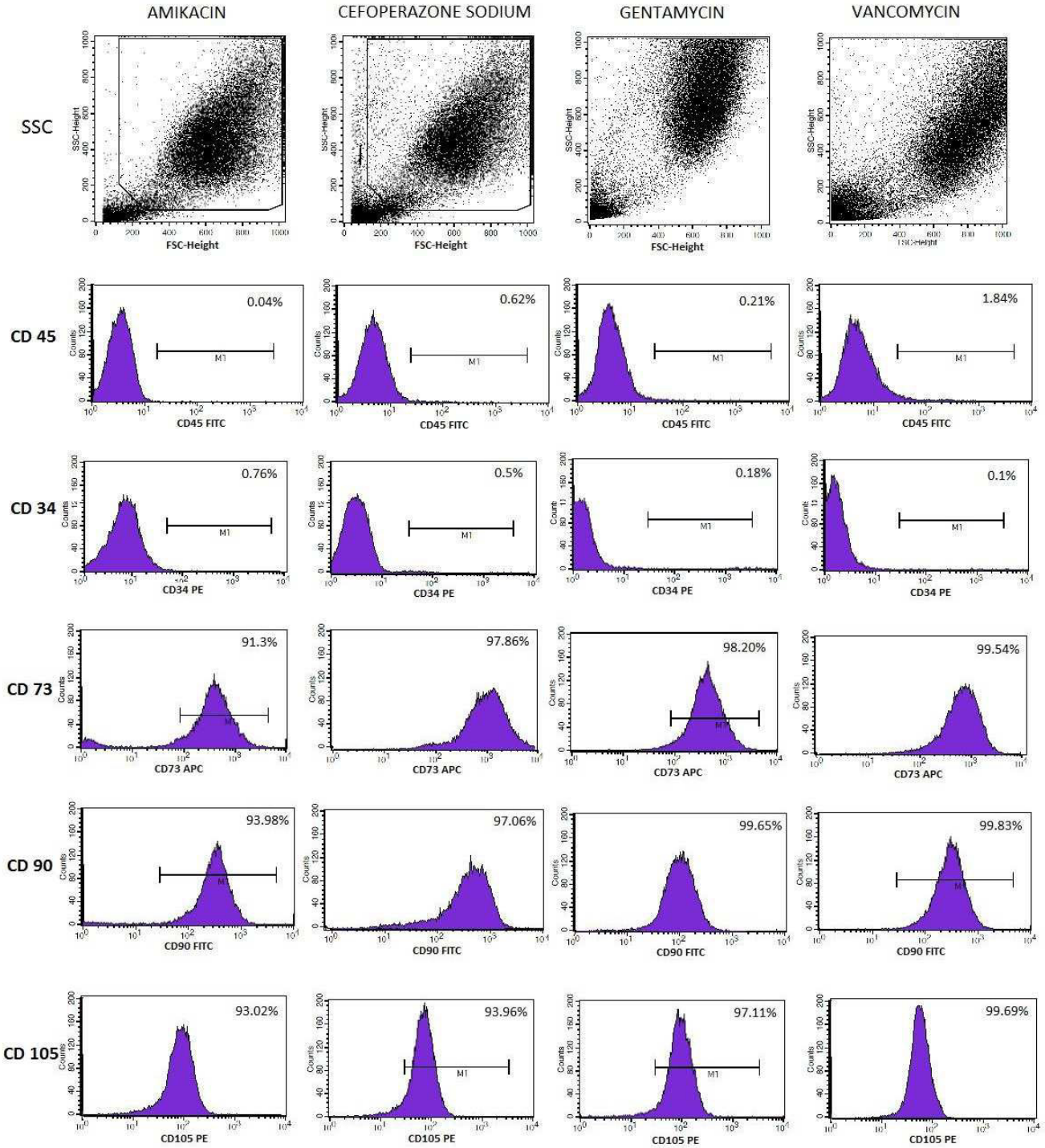

Figure 2

Flow result for optimum chosen concentration of all four antibiotics, Markers used for analysis were CD 34(PE) and CD 45(FITC) as negative markers and CD 73(APC), CD 90(FITC) and CD 105(PE) as Mesenchymal specific markers.