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Use of Human Primary Stem Cell Cultures for Screening Cell Proliferation Stimulatory and Inhibitory Effects of Botanical Preparations: an Alternative to Animal Models

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Abstract

Animals are widely used in scientific research as highly specific 'models' of humans. Primary cell culture is a widely used model to reduce and replace the use of animal models, as per the 3R concept. We investigated cell stimulatory effects of selected botanical preparations on primary human stem cell cultures, in place of animal models. Primary human fibroblast stem cell (hFSC), human mesenchymal stem cell (hMSC), and human haematopoietic stem cell (hHSC) cultures, were established in-house, and characterized by immunophenotyping. Varying concentrations of selected botanical preparations (mature leaf concentrate of Carica papaya Sri Lankan wild type cultivar [MLCC], distillates of Vernoina/ Mallotus [VMD], and Ficus benghalensis [FBD]) were tested ex vivo for cell proliferation stimulation on these cell platforms, using the MTT assay. Compared with untreated controls, significant proliferative effects were demonstrated by MLCC on female (at 0.2% concentration) and male (0.6%) hMSCs, and on male and female hHSCs (0.6-0.2%) ($P<0.05$). FBD (0.05-1.2%) stimulated significant proliferation of both male hMSCs, and male hHSCs, but inhibited proliferation of female hMSCs ($P<0.05$), with no significant effects on female hHSCs. Conversely, VMD (0.2-1%) showed significant inhibitory effects on both male and female hMSCs, yet at 0.6% showed significant proliferative effects on female hHSCs ($P<0.05$). Selected VMD concentration exhibited approximately 1.5-folds higher % cell stimulation than the positive control ($p < 0.01$). Thus, effective cell proliferative concentrations of botanical preparations were identified using primary stem cell lines, aligning with "replacement" as per the 3R concept.

Keywords: Human stem cells; botanical preparations; cell proliferation stimulants; 3R concept; Animal models

1. Introduction

Animals widely used in scientific, especially biomedical research as highly specific 'models' for humans have helped in the development of life saving cures for both humans and animals. In the 3 R concept, introduced by Russel and Burch "replacement" elaborate on 'alternatives' to substitute the use of animal models in research (Pollo *et al*., 2004). Primary cell cultures are one such widely used model to minimize and replace the use of animal models (Hussain *et al*., 2023). Stem cells, which are a cohort of cell populations residing in different tissues, have the ability to self-renew and differentiate into different mature cell types (Zakrzewski et al, 2019). These are widely used in therapy, in current treatment regimens against a wide range of diseases. Stem cell stimulants are important pharmaceutical reagents,

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which are used to stimulate stem cells in *vivo* and *in vitro.* As the available synthetic and recombinant stimulants are toxic and pose side effects, along with their exorbitant prices, pharmaceutical innovations are now directed towards inexpensive, nontoxic, phyto-stimulants (Udalamaththa *et al*., 2016). Sri Lanka, as a country with a rich biodiversity, and time-tested traditional medicine practices, possess numerous cues directed towards the possibility of stem cell therapy using botanical preparations. The current study aimed at investigating cell proliferation stimulatory effects of selected botanical preparations on primary human stem cell cultures; an inexpensive screening platform from biological waste, in place of animal models, as a novel approach in search of effective natural stimulants (Udalamaththa et al., 2021).

Research Hypothesis – The selected botanical preparations would exert significant proliferative stimulation effects on in-house established human stem cell cultures, proving the potential for replacing the animal models in drug development pipelines.

2. Materials and Methods

2.1 Ethical Approval

Ethical approval for the studies was obtained from the Ethics Review Committee, Faculty of Medicine, University of Colombo (ERC/15/133), and the Ethics Review Committee of the Lady Ridgeway Hospital for Children, Colombo 8 (ERC-LRH/DA/01/2016).

2.2 Preparation of botanical extracts

Mature leaves of Carica papaya of the Sri Lankan wild type variety were collected from plants maintained without insecticides from a home garden at Borelasgamuwa, Colombo district (longitudes 6.8412° N; latitudes 79.9025° E). The mature leaf juice of Carica papaya Sri Lankan wild type variety (MLCC) was prepared essentially following the procedure described by Gammulle et al. (2012).

The aqueous distillate of Vernonia/Mallotus (VMD) was prepared using fresh mature leaves, collected as practiced in Sri Lankan Traditional Medicine from two individual plants from a home garden in Gampaha (longitude- 80◦ 0′ 51.7176″ E, latitude- 7◦ 5′ 14.3160″ N), according to the method described by Ratnayake et al (2024).

The aqueous distillate of Ficus benghalensis was prepared using fresh leaflets and aerial roots collected from a home garden in Gampaha (longitude- 80◦ 0′ 51.7176″ E, latitude- 7◦ 5′ 14.3160″ N). It was prepared according to the traditional medicine practices by homogenizing 250g of fresh leaflets and 250g of fresh aerial prop roots in 1500 ml of distilled water for 10 min, and filtering through eight layers of muslin cloth. The resultant filtrate was distilled using a Liebig condenser following the standard distillation process.

All botanical preparations were sterilized using 0.45 µm syringe filters prior to use in cell culture protocols.

2.3 Human biological sample collection

Prior approval was obtained from the Director, de Soysa Maternity hospital, Colombo 08 to collect human cord blood and cord tissue samples. Samples were collected into sterile containers with transport medium from voluntary participants with informed consent, who underwent elective C-section for term delivery. Human neonatal foreskin samples obtained from routine circumcision surgeries at Lady Ridgeway Hospital were collected into sterile containers with transport medium following informed consent.

All collected samples were transported to the cell culture facility in the Combinatorial Research Laboratory at the University of Colombo and were processed immediately.

2.4 Establishment and characterisation of primary human stem cell lines

Explant method was used to isolate human mesenchymal stem cells from umbilical cord tissue samples, and human dermal fibroblast stem cells

from neonatal foreskin samples according to methods described by Venugopal et al., (2011), and Vangipuram et al., (2013), respectively. For characterisation of stem cell lines, cell surface markers were selected according to the ISCT guidelines (Dominici et al., 2006), and immunophenotyping was carried out according to the manufacturer's instructions (BD stemflow human Mesenchymal Stem Cell [hMSC] analysis kit, BD Biosciences, USA). Trilineage differentiation was carried out according to previously described methods by Venugopal and co-workers (2011).

Collected cord blood samples were processed according to the protocol recommended by EasySep™ Human Cord Blood CD34 Positive Selection Kit II, and hHSCs were cultured, maintained, sub-cultured, and cryopreserved according to protocols used by Broxmeyer and Colleagues (2003).

2.5 Evaluation of stem cell proliferative stimulatory activity of botanical preparations using the MTT assay

hMSCs, and hFSCs were plated in 96 well plates (Corning, USA) at a seeding density of 5000 cells/well, whereas hHSCs were plated at 15,000 cells/well, and incubated at 37 °C in a CO2 incubator for 24 hours in the standard complete cell culture medium. Next, the cells were treated in triplicates, for 48 hours with appropriate percentages of botanical preparations diluting the crude juice/distillate in distilled water and incubating under standard cell culture conditions. The positive control for hMSCs and hFSCs was the synthetic growth factor - β fibroblast growth factor [bFGF at 4 ng/µl, Sigma-Aldrich, USA]), while for hHSCs it was the Synthetic Cytokine Cocktail - Stem cell factor; 50 ng/ml, Thrombopoietin; 10 ng/ml, Flt3L; 50 ng/ml, Interleukin 6; 10 ng/ml). The positive and normal (sans treatment) controls were maintained in culture medium. MTT assay was carried out according to Bellagamba and co-workers (2016). Optical density (OD) measurements were obtained using a microplate reader (BIO-RAD, Model 680, USA) using a 540 nm filter. OD values for each percentage of botanical preparation were compared with normal and positive control OD values to calculate the percentage of cell proliferation or inhibition (proliferation stimulatory or inhibitory effects) as follows:

Percentage of proliferation/inhibition = ([Test OD – Control OD)/Control OD] x 100

2.6 Statistical Analysis

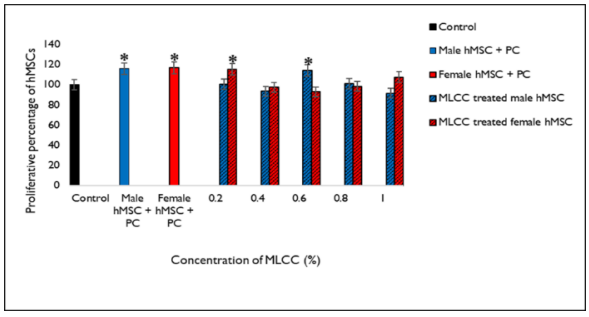
Statistical analyses were performed using SPSS 29.0 (IBM, USA). All ex vivo experiments were performed in triplicate and presented as mean ± SD. Results were generated from three independent experiments.

3. Results and Discussion

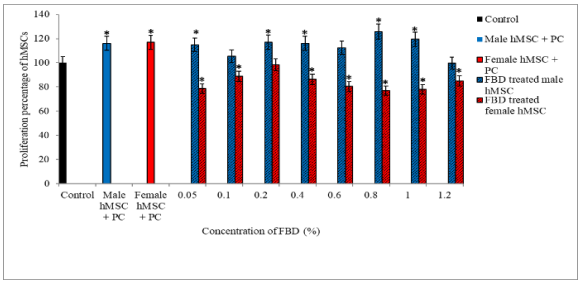
3.1 Effects of botanical preparations on hMSCs

Figure 1 illustrates the effects of different botanical preparations on male and female hMSCs. Figure 1A shows significant proliferation stimulatory effects at 0.2% and 0.6% of MLCC concentrations on female and male hMSCs respectively, whereas FBD (Figure 1B) shows significant proliferative effects on male hMSCs at 0.05,0.2,0.4,0.8 and 1% but significant inhibitory effects on the female hMSCs, at all tested concentrations ($P<0.05$). Conversely VMD (Figure 1C) showed significant inhibitory effects on both male (at 0.6 and 1.2%) and female hMSCs (0.1 – 0.8%) ($P<0.05$).

1A



1B



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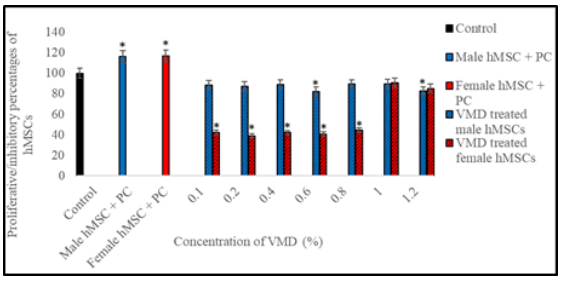
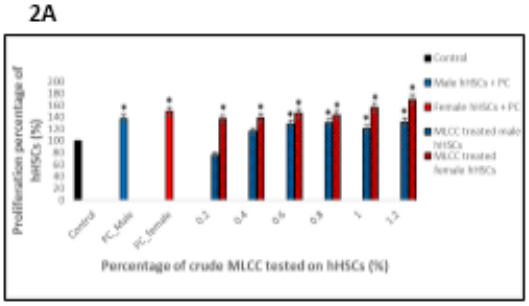


Figure 1. Proliferative stimulatory effects of different botanical preparations on male and female hMSCs. Effects of 1A) MLCC, 1B) FBD, and 1C) VMD on male and female hMSCs. Significant proliferative/ inhibitory percentages are denoted *(P<0.05).

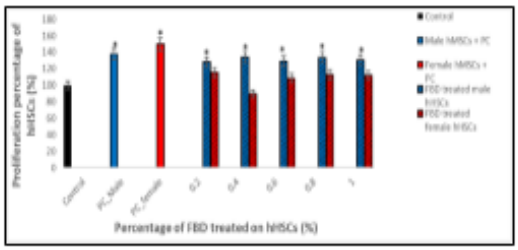
3.2 Effects of botanical preparations on hHSCs

Figure 2 shows the effects of different botanical preparations on male and female hHSCs. As depicted in figure 2A, MLCC shows significant proliferative effects on male and female hHSCs at all tested concentrations, excluding male hHSCs at 0.2 and 0.4 % (P<0.05). Figures 2B and 2C are evident of significant proliferative effects of FBD and VMD on male hHSCs at all tested concentrations, with no significant impact on female hHSCs, but showing significant proliferative effects on female hHSCs at only 0.6% concentration (P<0.05).

2A



2B



2C

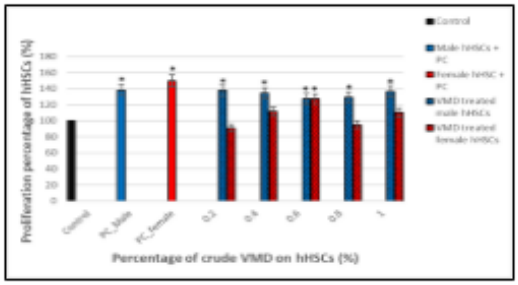


Figure 2. Proliferative stimulatory effects of different botanical preparation on male and female hHSCs. 2A) Effects of MLCC; 2B) Effects of FBD; 2C) Effects of VMD on male and female hHSCs. Significant proliferative percentages are marked with a *(P<0.05).

3.3 Effects of VMD on hFSCs

As shown in figure 3, VMD concentrations of 0.5%, 0.25% and 0.125% with % cellular viability/ proliferation of 242.1%, 304.3% and 273.23%, respectively, exhibited approximately 1.4, 1.7 and 1.5-folds higher % cell proliferation stimulation than the positive control ($p < 0.01$).

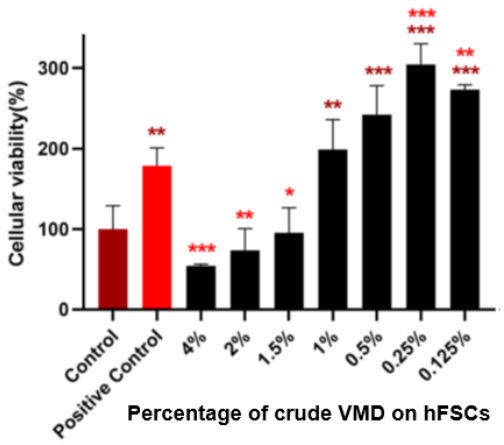


Figure 3. Effect of VMD on hHFC proliferation

Significant proliferative stimulatory activity compared to the control is shown with maroon star marks, while significant inhibitory and proliferative activity compared to the positive control is shown with red star marks

(*p < 0.05, **p < 0.01, ***p < 0.001).

As demonstrated by the above results, the selected botanical preparations demonstrated beneficial significant proliferative stimulatory activities on hMSCs, hHSCs and hFSCs. However, as per the results, the significance of the activity varied depending on the concentrations of botanical extract used, gender of the stem cells, as well as the type of the botanical extract used. These results are on par with previously reports where significant changes of effects of botanical preparations were demonstrated due to the presence of varying concentrations of phytochemicals, and selective genetic effects of genes related to sex chromosomes (Campesi et al., 2019). As an advanced step forward, the stimulants could be tested in organoid cultures directed towards tissue regeneration. It will also be possible to proceed towards clinical trials to stimulate in vivo stem cell populations in patients in need of cell therapy. Therefore, preliminary in vitro studies are a vital requirement to focus on cues from Sri Lankan traditional medicine, directed towards a specific treatment target. As WHO has published guidelines on therapeutic uses of botanical preparations acknowledging their positive effects, the long-term implications of these applications would be improved with the establishment of a global regulatory framework (WHO, 2004). The direct use of animal models to obtain preliminary results on these botanical extracts would have drastically increased the number of animals used, with an additional long duration of experimentation hours, and a tedious workflow (Dandri et al., 2016). Furthermore, some extracts may have manifested different effects during trials due to the differences in the animal species used. However, when using human stem cell platforms, the drawbacks such as expensive equipment and cell culture regents, contamination issues, and unavailability of expert personnel, may restrict researchers diverting from animal model studies to stem cell platforms. Also, an alternative ex vivo cellular platform for screening botanical preparations may not fully reflect the systemic outcomes observed with an in vivo animal model.

4. Conclusion

In-house developed primary human stem cell lines offer a cost-effective and reliable platform for screening botanical preparations that may serve as potential drug leads for stem cell proliferative activity. This approach supports the “replacement” aspect of the 3R principle in animal research. A globally recognized framework for stem cell research platforms and the standardization of botanical preparations would greatly benefit the drug development pipeline by significantly reducing turnaround times.

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