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Article

An *in vitro* comparative analysis of the growth factors present in FBS vs PLAY®

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SUMMARY

Growth factors are natural substances and vital cell culture media components that provide the appropriate environment for cell growth, migration, differentiation, and tissue morphogenesis. Growth factors such as HGF, IGF, TGF-β, FGF, PDGF, VEGF, EGF are present in the growth supplement used for in vitro culturing of animal and human cell-lines. Fetal bovine serum (FBS) is the most widely used growth factor supplement. It contains micro and macronutrients used for clinical and research cell culture such as the culture of Mesenchymal stem cells (MSCs). But it has been observed that MSCs grown in FBS reach senescence by passage. FBS is also becoming more unsuitable due to increasing demand, high price, collection methods, batch to batch variability and immunoreaction in clinical recipients. All these reasons have led the research field to question its suitability in clinical stem cell expansion purposes and find a suitable alternative. In order to address the above issues, we undertook a study to find the growth factor concentrations present in PLAY® which is human-derived, xenogen-free, cost-effective, growth factor rich concentrate. We hypothesized that PLAY® could contain high concentrations of many growth factors required for MSCs to proliferate to a higher passage. Our study found that PLAY® contained higher concentrations of various growth factors such as HGF, VEGF, TGF-β, MMP-2, MMP-9, TIMP-1, and TIMP-2 when compared to FBS. Hence PLAY® could serve as a good substitute for FBS in culturing MSCs for clinical applications. Future studies can be conducted to deduce the mechanism behind PLAY®'s increased proliferative capabilities.

INTRODUCTION

Growth factors are natural compounds and soluble proteins secreted by cells that are capable of regulating different cellular behaviors, such as cell proliferation, migration, and differentiation (1). Cell proliferation is induced by the action of binding to specific transmembrane receptors on target cells (2). Growth factor production is highly regulated in healthy organisms in order to modulate the physiology of the cell (3). Growth factors act on specific cell surface receptors and transmit molecular signals to a cell through a process called signal transduction (4). The growth factors bind to specific transmembrane receptors on the target cell leading to protein phosphorylation, gene expression, protein synthesis, and finally to a biological response (5). The growth factor concentration, the target cell nature, and the presence of co-stimuli, work together to influence proliferation and differentiation of cells. (6). Cells cultured *in vitro* require growth factors to be added to their growth medium in soluble form, as they help in cell survival and cell proliferation. Growth factors also determine cell fate, in order to produce specific phenotypic cells for research or for clinical use (7). The cost of fetal bovine serum (FBS) which is the most widely used cell culture supplement has gone up by more than 300% in the last couple of year. In such a scenario the use of growth factor presents a major production cost for cells for therapeutic use (8).

The culture requirement for stem cells is a million cells per infusion for cancer treatments, therefore the large-scale expansion of multipotent stem cells such as mesenchymal stem cells (MSCs) become of utmost importance. In order facilitate large expansion of MSCs *in vitro* growth factor-rich media is required (9). FBS supplemented with basal media like DMEM or RPMI is the conventional medium used to culture MSCs. However, due to concerns about its methods of collection (namely, the extraction of FBS from a bovine fetus by cardiac puncture without anesthesia) and the safety of using FBS-cultured cells in clinical applications, newer morally acceptable alternatives are being explored (10). It has also been observed in a previous study by Lakkundi *et al.* that MSCs cultured in 10% FBS could grow until passage P10 when compared to MSCs grown in PLAY which could grow until P20 (11). Normally cells undergo replicative senescence (i.e., telomere length shortening) progressively with every passage that can lead to permanent cell cycle arrest (12). This makes it more important to use the right media supplement that can preserve MSCs' regenerative capabilities for larger number of passages.

FBS contains growth factors such as transforming growth factor β (TGF-β), and fibroblast growth factor-2 (FGF-2) which are known to contribute to cell growth and proliferation, although there is batch to batch variability in the concentrations of various growth factors (13, 14). The batch variability depends on easily influenced factors such as seasonal changes and collection/processing methods. These differences are also observed to a higher degree in commercial FBS compared to FBS manufactured for research protocols. This makes FBS unreliable to use in clinical settings and may result in the cellular product being inconsistent (14). However, the biggest disadvantage of FBS lies not with its batch variability but with the immune response that may be provoked in human patients following FBS-cultured MSC injections. Bovine proteins, namely N-glycolylneuraminic acid (Neu5Gc) and bovine apolipoprotein B-100, may be introduced into the body of the patient from MSC injections and result in the production

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of antibodies, which greatly reduces the therapeutic ability of the cultured MSCs due to antibodies to Neu5Gc that target injected MSCs (14).

Alternatives to FBS have been studied extensively, especially over the past decade. These include human platelet lysate (hPL), bovine ocular fluid, sericin protein, and earthworm heat inactivated coelomic fluid (HI-CF) (15, 16). All of the above alternatives can be used for cell expansion; however, they have not been accepted as a complete substitute for FBS. In the case of HI-CF, the fluid lacks cell adherent factors like fibronectin and must be combined with another media to be suitable (16).

Growth factor importance for cell expansion is dependent on their affinity for the cell surface receptors present on the target cell. Essential growth factors in cell culture harbor a high, general affinity for a wide array of cell types, making them suitable to be included in many growth mediums (17). The insulin-like growth factors (IGF-1, 2) play an important role in fetal growth and development. They help in the proliferation, differentiation, and apoptosis of fetal cells *in vitro* (18). TGF-β family signals have an important role to play in maintaining the self-renewal and pluripotency capacity of both human and mouse embryonic stem cells (ESCs) (19). Hepatocyte growth factor (HGF) is a paracrine hormone and plays an important role in epithelial mesenchymal transition (EMT). HGF upregulates mitochondrial-related proteins and contributes to the maintenance of human bone marrow mesenchymal stem cell (hBMMSC) stemness during long-term expansion by preserving mitochondrial function (20). Matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9) are metalloproteases that degrade components of the extracellular matrix and basement membrane. MMPs help in regulating the release and activation of growth factors, chemokines, cytokines, and bioactive molecules, hence contributing to tissue remodeling and maintenance within the body. (21). Tissue inhibitors of matrix metalloproteinases-(TIMP-1, 2) tightly regulates MMPs and controls their action through biomolecules (22). Vascular endothelial growth factor (VEGF) is an angiogenic growth factor that promotes the proliferation of vascular endothelial cells and is also known to have neurotrophic and neuroprotective effects by stimulating the proliferation of neuronal precursor cells (23).

Though there are other alternate growth factor supplements, they cannot be used for cell culture. Usage of FBS for stem cell culture comes with its own set of problems. To circumvent the above-mentioned issues, we have to find a better alternative growth factor supplement. Hence, we embarked on this study to find the concentrations of different growth factors present in PLAY®, growth medium supplement developed and produced by iCREST. PLAY® is intended to replace FBS and to be used with a basal media like DMEM or RPMI. It is a human-derived, pooled (combined from 10 PLAY® samples), xenogen-free, growth factor rich biological concentrate. It is similar to platelet lysate, but the processes incorporated to obtain PLAY® are subject to proprietary standardizations. We hypothesized that PLAY® would contain high concentrations of many of the growth factors required for MSCs to proliferate to high passage numbers and undertook this study to evaluate the quantities of key growth factors in PLAY® versus FBS. We used four batches of PLAY® and one batch of FBS to test for the presence of growth factor concentrations. Our study found that PLAY® contained significantly higher concentrations of various growth factors such as HGF, VEGF, and TGF- β when compared to FBS, though IGF-1 and IGF-2 concentrations were found to be significantly higher in FBS than in PLAY®. Concentrations of MMP-2, MMP-9, TIMP-1, TIMP-2 which are regulators and activators of growth factors were also significantly higher in PLAY® than in FBS. Our results validated Lakkundi *et al.*'s work wherein they used 10% PLAY® as a supplement for stem cell culture, and we suggest that PLAY® may be beneficial for stem cell growth *in vitro*.

RESULTS

Presence of a rich concentration of growth factors in a culture media is essential for *in vitro* cell culture, currently FBS is a major growth factor supplement used for cell culture. Due to some of the disadvantages of FBS, PLAY® an in-house product was considered for stem cell expansion. Our study investigated the presence and concentrations of different growth factors in PLAY® and FBS. The growth factors HGF, VEGF, TGF-β, IGF-1, IGF-2, and the growth factor regulators MMP-2, MMP-9, TIMP-1, TIMP-2 present in PLAY® batches and FBS were determined and quantitatively analyzed. 4 PLAY® batches (each batch comprised of 10 pooled individual PLAY® samples) were studied in duplicates while one batch of FBS served as a comparative setup.

Growth Factor Concentrations of HGF, VEGF, TGF-β1, IGF-1 and IGF-2 in PLAY® and FBS Samples

PLAY® samples contained significantly higher concentrations of HGF (mean of 11835.65 ng/ml ± SD) in comparison to non-existent concentrations in FBS (mean of -0.04 ng/ml ± SD corrected to 0 ng/mL) ($p < 0.001$) **(Figure 1A).** PLAY® samples contained significantly higher concentrations of VEGF (mean of 10.25 ng/mL ± SD) in comparison to FBS (mean of 0.038 ng/mL ± SD) ($p < 0.001$) **(Figure 1B).** PLAY® samples contained significantly higher concentrations of TGF-β (mean of 91.87 ng/mL ± SD) in comparison to FBS (mean of 0.6493 ng/mL ± SD) ($p < 0.001$) **(Figure 1C)**. PLAY® samples contained significantly lower concentrations of IGF-1 (mean of 2.91 ng/mL ± SD) in comparison to FBS (mean of 45.02 ng/mL ± SD) ($p < 0.001$) **(Figure 1D)**. PLAY® samples contained significantly lower concentrations of IGF-2 (mean of 21.1 ng/mL ± SD) in comparison to higher concentrations in FBS (mean of 263.09 ng/mL ± SD) ($p < 0.001$) **(Figure 1E)**.

Growth Factor Concentrations of MMP-2 and MMP-9 in PLAY® and FBS Samples

PLAY® samples contained significantly higher concentrations of MMP-2 (mean of 64.84 ng/mL ± SD) in comparison to non-existent concentration in FBS (mean of -0.068 ng/mL ± SD corrected to 0) ($p < 0.01$) **(Figure 2A).** PLAY® samples contained significantly higher concentrations of MMP-9 (mean of 446.44 ng/mL ± SD) in comparison to FBS (mean of 1.77 ng/mL ± SD) ($p < 0.01$) **(Figure 2B).**

Growth Factor Concentrations of TIMP-1 and TIMP-2 in PLAY® and FBS samples

PLAY® samples contained significantly higher concentrations of TIMP-1 (mean of 398.21 ng/mL ± SD) in comparison to lower concentrations in FBS (mean of 3.57 ng/mL ± SD) ($p < 0.001$) **(Figure 3A)**. PLAY® samples contained significantly higher concentrations of TIMP-2 (mean of

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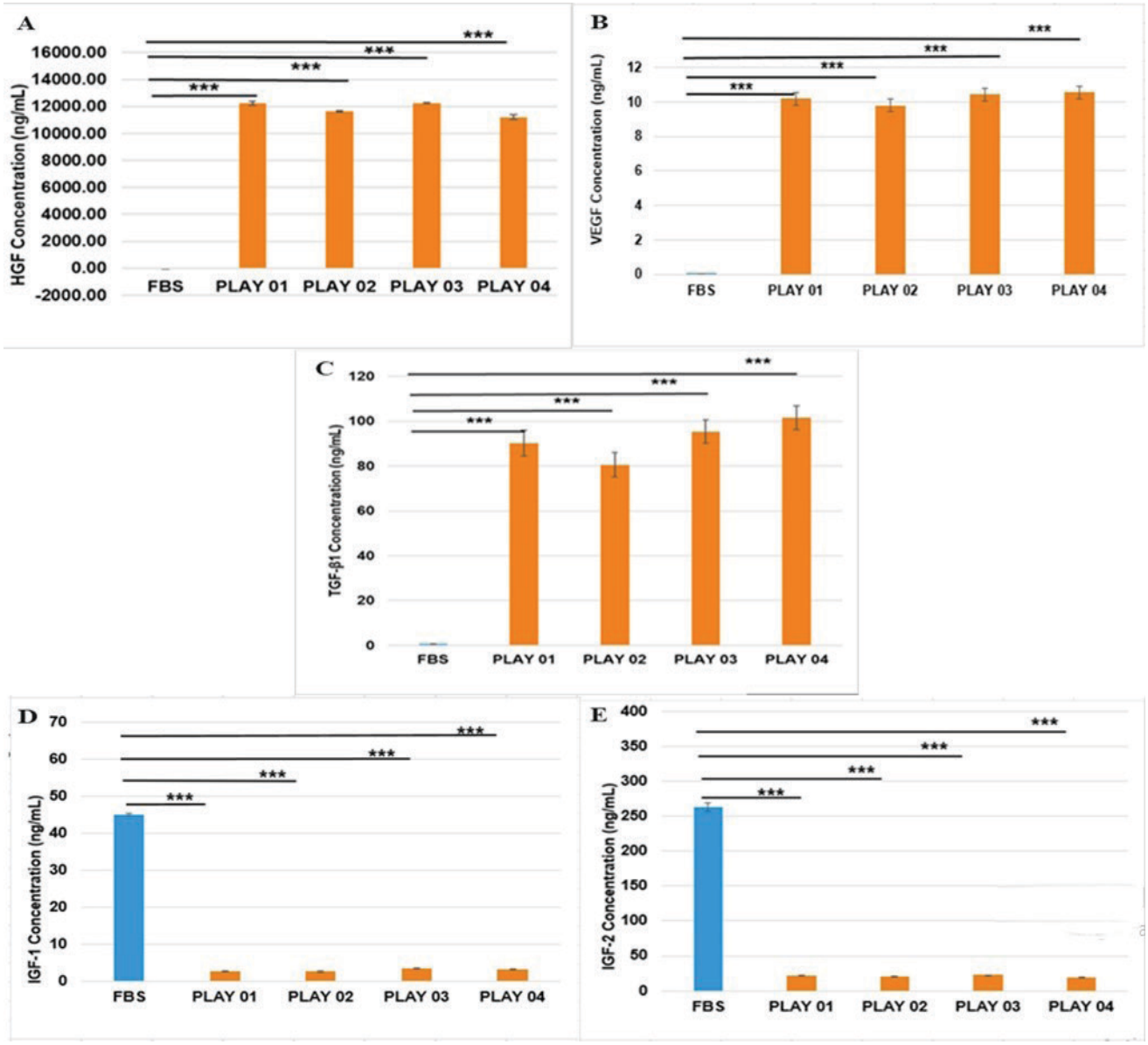

Figure 1: Concentration of growth factors in FBS and PLAY® samples. Graphical representation of growth factor concentrations of (A) HGF, (B) VEGF, (C) TGF-β (D) IGF-1 and (E) IGF-2 in FBS and PLAY samples. Their concentrations were quantified and analyzed through ELISA. (A) HGF concentration was significantly higher in PLAY® samples when compared to FBS. (B) VEGF concentration was significantly higher in PLAY® samples when compared to FBS (C) TGF-β concentration was significantly higher in PLAY® samples when compared to FBS. (D) IGF-1 concentration was significantly higher in FBS when compared to PLAY® samples. (E) IGF-2 concentration was significantly higher in FBS when compared to PLAY® samples. FBS (n=1) and PLAY® samples (n=4). Data values for the above figure **(Figure 1 A, B, C, D, E)** are represented as mean ± SD. Statistical significance was analyzed by ANOVA single factor test. ***p<0.001 is considered to be statistically significant. All samples were run in duplicates. Concentration is represented in ng/mL.

22.41 ng/ml ± SD) (p < 0.001) in comparison to non-existent concentrations in FBS (mean of -0.13 ng/mL ± SD corrected to 0) **(Figure 3B**). But there is significant variability in the levels of TIMP-2 proteins across all the PLAY® batches.

DISCUSSION

PLAY® is a human derived, growth factor rich, blood derivative manufactured at iCREST. Our study tested the presence of growth factor concentrations in 4 batches of PLAY® and 1 batch of FBS. The rationale for such an approach was to validate the efficacy of PLAY® as a growth supplement and as a superior alternative to FBS in terms of growth factor concentrations. The rationale for such an approach was to validate the efficacy of PLAY® as a growth medium and as a superior alternative for FBS. Studies conducted in iCREST lab by Lakkundi et al. have demonstrated the efficacy of PLAY® which supports mesenchymal stem cell growth, while maintaining its pluripotency and differentiation capacity until late passages (passage 20) (11).

A similar study demonstrated that MSCs cultured in hPL have a staggeringly high growth rate triggered by the α-granule stored growth factors, which are released from

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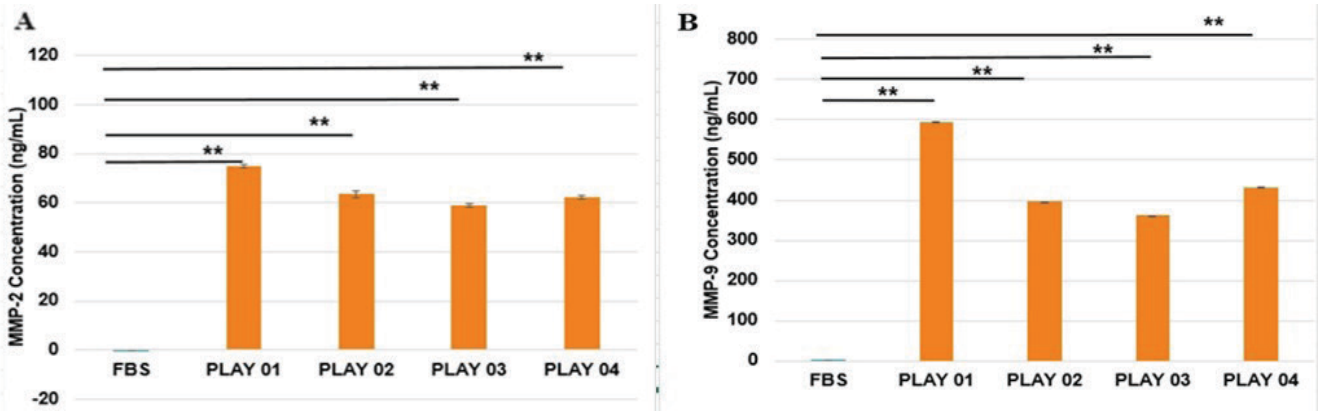

Figure 2: Concentration of MMPs in FBS and PLAY® samples. Graphical representation of MMPs' concentrations in FBS and PLAY samples. Their concentrations were quantified and analyzed through ELISA. MMP-2 concentration was significantly higher in PLAY® samples when compared to FBS (B) MMP-9 concentration was significantly higher in PLAY® samples when compared to FBS. FBS (n =1) and PLAY® samples (n = 4). Data values for the above figure **(Figure 2 A, B)** are represented as mean ± SD. Statistical significance was analyzed by ANOVA single factor test ***p<0.001. All samples were run in duplicates. Concentration is represented in ng/mL.

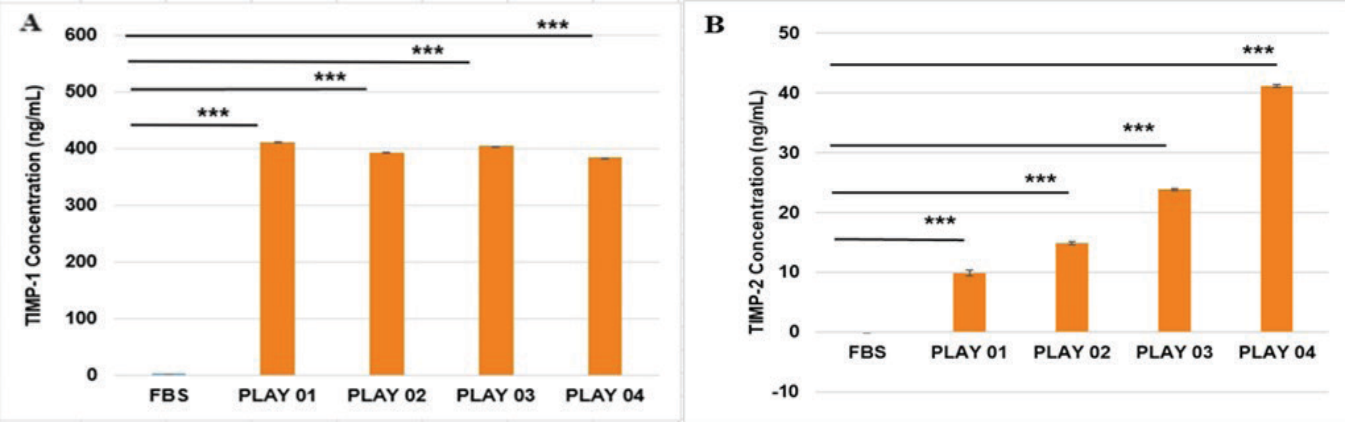

Figure 3: Concentration of TIMPS in FBS and PLAY® samples. Graphical representation of TIMPs' concentrations in FBS and PLAY samples. Their concentrations were quantified and analyzed through ELISA. (A) TIMP-1 concentration was significantly higher in PLAY® samples when compared to FBS (B) TIMP-2 concentration was significantly higher in PLAY® samples when compared to FBS. FBS (n = 1) and PLAY® samples (n = 4). Data values for the above figure **(Figure 3 A, B)** are represented as mean ± SD. Statistical significance was analyzed by ANOVA single factor test ***p<0.001. All samples were run in duplicates. Concentration is represented in ng/mL.

platelets through inducing lysis by freeze-thaw cycles (24). The growth factor concentrations in hPL were analyzed, with hPL exhibiting high concentrations of platelet derived growth factor (PDGF) (208,256 pg/mL) and TGF-β (327,933.33 pg/mL) and slightly lower concentrations of VEGF (665.13 pg/mL) and IGF 1 (1266.43 pg/mL) (15). Our results mirror those of the above studies demonstrating higher concentrations of all the growth factors tested in PLAY®, except for IGF1 and IGF2. However, our work reported lower concentrations of both IGF1 and IGF2 in PLAY® compared to FBS, therefore IGF1 and IGF2 may not be critical for stem cell growth.

Protein phosphorylation is a key step in many signaling cascades, wherein binding of signaling molecules to their cell surface receptors activates cell proliferation (25). There is a specific procedure needed to induce cell growth *in vitro* that includes activation of MAP (mitogen-activated protein) signaling cascades, which can be triggered by high concentrations of signaling molecules like cytokines. Therefore, the MAP cascade is a vital instrument in the overall process of cell growth *in vitro* and *in vivo* as well (26). Growth factors such as HGF, VEGF and TGF-β have been shown to activate the MAP cascades, proving their specific role in cell culture. The cytokines trigger the MAPK (mitogen activated protein kinase) cascade that is regulated by protein phosphorylation, the mechanism being incredibly important in cellular processes such as protein synthesis, cell division, cell growth and cell differentiation (27). As this cascade is dependent on the concentration of the signaling molecule, with higher concentrations of the cytokine activating the at a higher rate and thus enabling culturing of cells to a greater passage number at a quicker rate(15).

MMP-2, MMP-9, TIMP1, TIMP2 proteins have primarily been studied in the context of cancer and wound healing. Therefore, our study may be the first of its kind to determine the presence of MMPs and TIMPS in PLAY®. Our future studies will look into the role of MMPs and TIMPs in stem cells growth and the mechanisms or epigenetic modification of stem cells. The presence of MMP-2, MMP-9, TIMP-1 and TIMP2 along with the other growth factors in PLAY® samples could suggest they influence stem cell growth, maintenance

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in morphology across passages, retention of trilineage differentiation potential, or expression of pluripotency markers in MSCs until passage 20 (11). Since PLAY® is a human blood derivative, it is easy to procure, process, and produce reliably with minimum batch to batch variability. We have shown less than 10% variability in the concentrations of growth factors (except for TIMP2) across all our PLAY® batches. This is an important consideration as FBS, as mentioned before, is prone to batch variability (14).

In our study, we have demonstrated significantly increased concentrations of growth factors such as HGF, VEGF, TGF-B1, matrix metalloproteinases MMP-2 and MMP-9, and tissue inhibitors of matrix metalloproteinases TIMP-1 and TIMP-2 in PLAY® as compared to FBS. We acknowledge that using only one batch of FBS was a limitation in our study. Future studies will involve, multiple batches of FBS, a larger array of growth factors and study in the mechanisms that are involved in maintaining the stemness of MSCs. As we have seen, there are many advantages in using PLAY® as a growth substitute for FBS in *in vitro* culture of MSCs. Due to the high concentrations of growth factors present in PLAY® versus FBS, not having much variability in between the batches, better proliferation rate of MSCs cultured in 10% PLAY when compared to FBS, PLAY® can be a better option for clinical cell expansion purposes(11).

MATERIALS AND METHODS

Study Population

4 PLAY® batches (each batch pooled from 10 individual samples of PLAY®) and 1 batch of FBS (GIBCO cat. no.10270106) were selected for the analysis of growth factors present in them. PLAY® samples were manufactured in a Current Good Manufacturing Practice (CGMP) facility. A CGMP facility is accredited by a national medical regulatory body, and during production the scientists work in close tandem with quality assurances and ethics committees to ensure the safety of their cellular products. Adherence to a standardized protocol for the manufacturing of PLAY® ensured minimal variability between the different PLAY® batches. A thorough quality assurance/quality control (QA/QC) protocol ensured that the PLAY® samples were screened for different diseases such as human immunodeficiency virus (HIV-1,2), Hepatitis C virus (HCV), Hepatitis B virus (HbsAg), Syphilis, Malaria, Cytomegalovirus immunoglobulin G (CMV IgG), and Cytomegalovirus immunoglobulin M (CMV IgM). The QA/QC protocol also ensured that the PLAY® samples were sterile, as checked by Thioglycollate media, and were endotoxin negative, as tested by Limulus amoebocyte lysate (LAL) chromogenic test. These samples were stored in -80°C until further use.

Growth Factors Analysis of PLAY® Batches and FBS Batches by Enzyme-Linked Immunosorbent Assay (ELISA)

The growth factor concentrations of HGF, Catalog No: E-EL-H0084, IGF-1, Catalog No: E-EL-H0086IGF-2, Catalog No: E-EL-H6037, VEGF-A, Catalog No: E-EL-H0111, TGF-β1, Catalog No: E-EL-0162, MMP-2, Catalog No: E-EL-H1445, MMP-9, Catalog No: E-EL-H6075, TIMP-1, Catalog No: E-EL-H0184, TIMP-2, Catalog No: E-EL-H1453, were quantitatively measured in 4 PLAY® batches and 1 FBS batch using commercially available ELISA kits (Elabscience) according to the manufacturer's instructions. The PLAY® batches were diluted with sample diluent to 10,000-fold and no fold dilution for FBS batch in HGF, 100-fold dilution for PLAY® batches and no fold dilution for FBS Batch in TGF-β1 and VEGF. No fold dilutions were included in IGF-1 and IGF-2 for PLAY® and FBS batches. 100-fold dilutions were included for PLAY® batches and no fold for the FBS batch in MMP-2 and MMP-9. 50-fold dilutions were included for PLAY® batches and no fold for the FBS batch in TIMP-1 and TIMP-2. The concentration of each growth factor in ng/mL were estimated from the respective standard curves generated by following the manufactures instructions. The optical densities (OD) of the PLAY® and FBS batches were determined at 450nm were analyzed in the LisaQuant Microplate ELISA reader. The concentration of each cytokine in ng/mL was estimated by plotting the standard and blank values in Microsoft Excel and fitting with the equation $y = mx+c$. We used the sample diluent as a negative control, the standards as the positive control, and the OD values of the blank wells were subtracted from the experimental samples to remove the background signal.

Stastical Analysis

Statistical analyses were performed using an Excel spreadsheet (Excel 2021 for Windows version). FBS (n = 1) and PLAY® batches (n = 4, each batch is made by pooling 10 PLAY samples). Data values were expressed as mean values mean ± SD. Statistical significance was analyzed by ANOVA single factor. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ were considered statistically significant (n = 2, where n = 2 means that the FBS and PLAY® samples were run in duplicates).

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