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Human Patellar Bone Dust has Osteogenic Potential: An *in vitro* Study

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To substantiate the osteogenic differentiation of human Umbilical cord Mesenchymal Stem Cells (hUMSCs) under the influence of Bone Dust (BD) by detecting expression of genes RUNX2 and IBSP in the differentiated cells.
Study Design: *In vitro* study.
Place and Duration of Study: MDRL Cell Culture Lab Ziauddin University and Dr.Zafar H.Zaidi Institute of Proteomics, University of Karachi, between July 2020 and July 2021.
Methodology: We designed an innovative experiment to differentiate hUMSCs into osteoblasts under the influence of BD, without using any conventional differentiating medium. The hUMSCs were isolated from umbilical cord and cultured in a 6-well plate with 3µm inserts, in which BD was lodged, preventing direct contact of BD with hUMSCs. The plates with hUMSCs and BD-filled inserts were incubated at 37°C for 14 days after which RNA extraction, cDNA synthesis and qPCR for two genes was performed. Expression of Runt-related transcription factor-2(RUNX2) and Integrin-binding Sialoprotein (IBSP) were quantified.
Results: Results revealed an increased expression of the late osteogenic marker IBSP(p=0.01)

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signifying that the hUMSCs had undergone differentiation into mature osteoblasts.
Conclusion: This novel experiment identifies BD as a new source for osteogenic differentiation of hUMSCs in *in vitro* experiments.

Keywords: Bone dust; Lineage-specific differentiation; osteogenic differentiation; osteogenic factors.

ABBREVIATIONS

hUMSCs : Human Umbilical Cord Mesenchymal Stem Cells
RUNX2 : Runt-related Transcription Factor-2
IBSP : Integrin-binding Sialoprotein

1. INTRODUCTION

Human Mesenchymal Stem Cells (hUMSCs) have been knowingly differentiated into osteoblasts using various methods. On doing so, these have been witnessed to be affected by a diverse set of factors [1]. These factors include(a) growth in a standard osteogenic medium containing α-MEM 10% FCS, ascorbic acid and β-glycerophosphate, (b)growth on a nanocomposite scaffold of bioactive glass/ gelatin [2], (c) being co-cultured with osteoblasts or osteocytes [3], (d) grown on 3D-scaffolds of poly lactic acid and hydroxyapatite powder [4], and many more.

During lineage-specific differentiation into osteoblasts, these multipotent cells express certain genes examples of which are Runt-related transcription factor-2(RUNX2), Integrin-binding Sialoprotein(IBSP), OSTERIX(Osx), Osteopontin(OPN), and Collagen type 1COLL1 [5]. Of these RUNX2 is a transcription factor that belongs to the Runx family that is first detected in preosteoblasts, indicating early osteoblast differentiation [6] and IBSP is an acidic, noncollagenous glycoprotein that encodes the structural protein of bone matrix and marks later stages of osteoblast differentiation [7].

Several methods of osteoblast differentiation have been employed in the past as discussed above, and many of them identified both the genes during differentiation of osteoblasts. However, these methods are costly as well as time consuming [8].

Bone dust (BD), a powdered bone material produced during orthopaedic surgeries [9,10], has been shown to affect osteoblast proliferation by increased expression of osteogenic factors [11]. Up regulation of RUNX2 and IBSP was observed in studies conducted on osteoblasts under the influence of BD, reinforcing its osteoinductive potential [5]. Nevertheless, no literature is available commenting on the differentiation of hUMSCs into osteoblasts induced by BD. Therefore, the objective of present study was to substantiate the osteogenic differentiation of hUMSCs under the influence of BD by detecting expression of genes RUNX2 and IBSP in the differentiated cells. To the best of our knowledge, the present study would be the first of its kind to identify bone dust as a new source of osteogenic differentiation of hUMSCs *in vitro.*

2. MATERIALS AND METHODS

An in vitro study was designed to compare the gene expressions of RUNX2 and IBSP in UMSCS, cultured alone versus hUMSCs cultured in the presence of BD.

2.1 HUMSCs Collection

Human umbilical cord samples were obtained from babies born to mothers by Caesarian sections, after taking informed consent, in Ziauddin Hospital. hUMSC isolation was performed as per Coulson-Thomas protocol [12]. The cords were rinsed in Phosphate-buffered saline(PBS) and minced tissue pieces were incubated in a medium containing Dulbecco's Modified Eagle Medium(DMEM), L-glutamine, 10% Fetal Bovine Serum(FBS), sodium pyruvate and P/S (M/S Thermofischer Scientific, Life Technologies, USA), in 5% CO2 incubator at 37°C until cells came out of the tissue. When the cell confluency reached 80%, cells were trypsinized and transferred into 15 mL falcon tube to be centrifuged and seeded in sterile tissue culture treated flasks.

Umbilical cord from babies born by spontaneous vaginal delivery to mothers at gestational age less than 37 or more than 42 weeks, or having hypertension, eclampsia, preeclampsia, gestational diabetes, malignancy, were excluded from the study.

2.2 BD Collection and Processing

Samples of BD were taken from patella of male patients, after informed consent, who were undergoing surgery for total knee replacement. BD collection and processing was performed in accordance with the protocol of Gao et al. [11]. All patients underwent surgery according to their routine practice and BD, from patients with known bone disease, autoimmune disease, cancer, endocrine disorders, was excluded from the study.

After collection, BD was washed and rinsed with PBS and lodged in the tissue culture inserts with 3µm pore size at 100mg/insert and submerged under the culture media and suspended over hUMSCs seeded in 6-well plastic tissue culture plates (Fig.1a). This novel experimental design prevented direct contact of BD with the hUMSCs; however, the pores in the insert permitted differentiation factors released from the BD to diffuse into the media to act on the hUMSCs (Fig.1b). Each experiment contained 3-wells of BD and was repeated with three different patient samples. For each experiment, matching control wells were used with equivalent number of cells seeded per well.

2.3 Characterization of hUMSCs and BD through Gene Expression

hUMSCs characterization was performed by extracting RNA using organic extraction method, from the seeded cells. Reverse transcription of 0.5µg of RNA was performed by using Revert Aid First strand cDNA kit (M/S Thermofischer Scientific, Life Technologies, USA), to analyze the hUMSCs markers, i.e. Oct.4, CD90, CD133, CD105 and CD73, by Conventional PCR. BD was characterized using the same protocol for expression of RUNX2 and IBSP and results were noted. The primers are listed in Table 1.

2.4 Gene Expression Analysis of Differentiated Cells through Quantitative POLYMERASE Chain Reaction (q-PCR)

On day 14, total cellular RNA was purified from cells using TRIZOL (M/S Thermofischer Scientific, Life Technologies, USA). Concentration and purity of the isolated RNA was analyzed using Multi Scan Sky spectrophotometer. The reverse transcription was carried out using Revert Aid enzyme with 0.5µg RNA used for each sample. The cDNAs thus obtained was used for q-PCR assays using Maxima SYBR Green/ROX qPCR Master Mix (M/S Thermofischer Scientific, Life Technologies, USA)for up to 40 cycles. GAPDH was used as the house keeping gene. The melting curves were always checked afterward and Ct-values were calculated. Relative gene expression and Fold Changes for the respective genes were calculated using following formulas:

ΔCt = Ct(Gene of Interest) – Ct (GAPDH)
$\Delta\Delta Ct$ = ΔCt(BD-hUMSCs) – ΔCt(hUMSCs)
Fold Change = 2^ – ($\Delta\Delta Ct$)

2.5 Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) version 25.0 (IBM Corp., Armonk, NY) was employed for data analysis. An Independent Sample t-Test was applied for paired data and to compare the difference in fold changes (calculated after getting the Ct values) between the two groups as measured in mean. Results are presented as mean ± SEM/SD and differences among means were considered statistically significant when $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Identification of hUMSCs

Conventional PCR to detect the surface antigens of hUMSCs shows that the hUMSCs positively expressed Oct.4, CD90, CD73, CD105 and CD133 was not expressed (Fig. 2a). HUMSCs, that are multipotent cells with a self- renewing ability, have been useful in regenerative medicine and their characterization has been carried out in the past using expression of hUMSCs-specific markers [13,14]. Thus, by performing their characterization, we have validated the expression of the above mentioned markers in these multipotent, regenerative cells [15,16].

3.2 Characterization of BD

Conventional PCR through Gel Electrophoresis to detect the surface antigens of RUNX2 and IBSP on BD shows that BD positively expressed both RUNX2 and IBSP indicating presence of both early and later-stage osteoblasts (Fig.2b). RUNX2 is an early osteogenic marker whose expression increases in immature osteoblasts [6]. It is a transcription factor and osteoblast inducer [17], which is first detected in preosteoblasts and the expression is upregulated in immature osteoblasts, but downregulated in

mature osteoblasts [6]. Whereas, IBSP is an osteoblast differentiation marker, which produces osteoblastogenesis [18] and is considered to be a late osteoblast marker [7] whose increased expression indicates late stage of differentiation of osteoblasts [19,20]. This indicated that BD contains both early and late bone cells and is viable.

Figure 1a: BD in inserts being lodged in 6-well plate with hUMSCs in DMEM

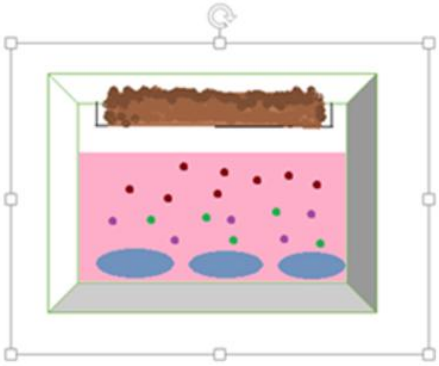

Figure 1b: In vitro methodology. Schematic diagram demonstrating the experimental setup. Primary hUMSCs are seeded in 6-well plates, above which BD is placed in tissue culture inserts with 3µm pore size. The pores in the tissue culture inserts allow factors released from BD to act on hUMSCs

Table 1. Forward and reverse primer sequences

Primer Symbol	Primer direction	Sequences (5′ to 3′)
RUNX2	Forward	GAGGGCACAAGTTCTATCTG
	Reverse	CGCTCCGGCCCACAAATCTC
IBSP	Forward	GGCAGTAGTGACTCATCCGAAG
	Reverse	GAAAGTGTGGTATTCTCAGCCTC
OCT-4	Forward	AAAGACCATCTGCCGCTTTG
	Reverse	GGTTCGCTTTCTCTTTCGGG
CD 73	Forward	GCTCTTCACCAAGGTTCAGC
	Reverse	TCGATCAGTCCTTCCACACC
CD 90	Forward	TTGGATGAGGAGTGGTTGGG
	Reverse	TTGGTTGTGGCTGAGAATGC
CD 105	Forward	TCCATTGTGACCTTCAG
	Reverse	CTTGGATGCCTGGAGAGTCA CCT
CD 133	Forward	TTCTTGACCGACTGAGACCC
	Reverse	CAGGCTAGTTTTCACGCTGG
GAPDH	Forward	GTCTCCTCTGACTTCAACAGCG
	Reverse	ACCACCCTGTTGCTGTAGCCAA

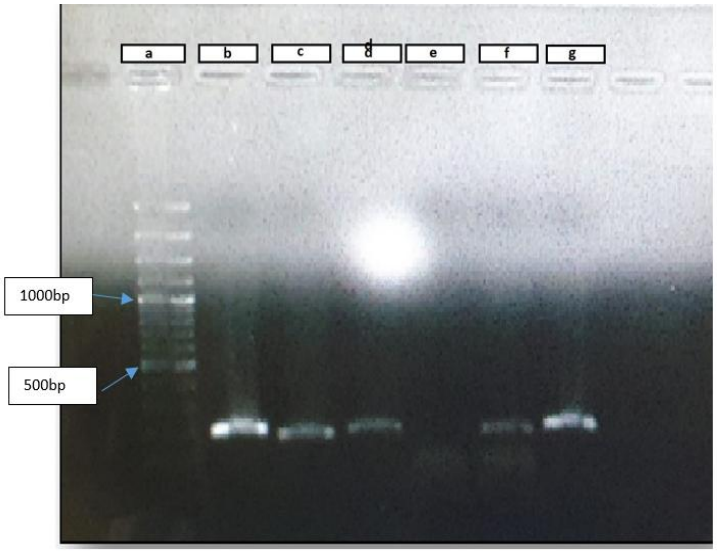

Fig.2a: Identification of hUMSCs using expression of Oct.4, CD90, CD133, CD105, CD73 by Gel Electrophoresis. (a) Ladder used in Gel, (b) GAPDH as the standard for normalization, (c) Oct.4 and (d) CD90 both expressed, (e) CDC133 not expressed, (f)CD105 and (g)CD73 expressed.

Fig.2b: Characterization of BD using expression of RUNX2 and IBSP by Gel Electrophoresis. (a) GAPDH as the standard for normalization, RUNX2(early) and IBSP(late) both expressed.

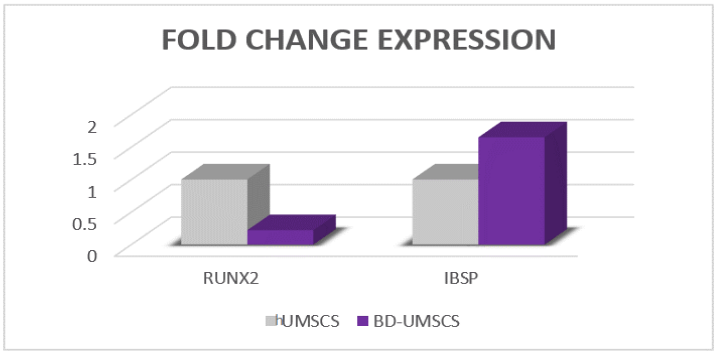

Fig. 3. Mean Fold change expression of RUNX2 and IBSP in cells with BD (BD-UMSCS) and without BD (hUMSCS)

RUNX2 shows decreased expression in BD-UMSCS (P=0.023), whereas IBSP shows increased expression in BD-UMSCs (P=0.010)

3.3 q-PCR of Differentiated hUMSCs

The q-PCR of differentiated hUMSCs under the influence of bone dust revealed significantly enhanced expression of IBSP (*P*= 0.01) and decreased expression of RUNX2 *(P*= 0.023) (Fig.3). This analysis was done on day 14 of the experiment, the results revealing elevated levels of IBSP, indicating later stages of osteoblasts differentiation (Fig.3). The outcome results of q-PCR in our experiment proved to be compatible with the review of related literature, revealing downregulation of RUNX2 in the cells cultured with BD and thus its decreased expression in them.

Osteogenic properties of MSCs have been checked by their release of hematopoietic and non-hematopoietic growth factors [21] chemoattractants, and cytokines [22] along with having immuno-modulatory properties [23], in the presence of osteogenic media [24], as well as when cultured on 3D-scaffolds [4]. However, these strategies are costly and time consuming, warranting the need of a less expensive and convenient method of osteogenic differentiation [8].

BD, which is a powdered bone material that contains small fragments of discarded bone,has been used to check gene expression in osteoblasts [25] and is seen to release osteogenic factors [5]. Osteogenic properties of BD have also been checked on osteoblasts in limited experiments with an increased expression of IBSP [11]. In our study we have used this otherwise discarded material as a differentiating source for hUMSCs.

To the best of our knowledge, no literature has been found that has used BD in ostegenic differentiation of hUMSCs, without using any other differentiating medium. Therefore this unique experiment embarks the discovery of a new source of differentiating hUMSCs into osteoblasts.

4. CONCLUSION

The current study demonstrates that hUMSCs have typical characteristics according to the reported criteria of conventional gene expression. In addition, this experiment reports that BD expresses osteogenic gene markers RUNX2 and IBSP.Finally, this study testifies that BD differentiates hUMSCs into osteoblasts by quantifying the expression of the same genes i.e. RUNX2 and IBSP, without using any osteogenic medium.

DISCLAIMER

The materials used in this research are commonly used in our region and there is no conflict of interest between the authors and product manufacturers. We also declare that this research was not funded by the company providing these materials.

CONSENT

Written and informed consent was taken from the patients prior to sample collection.

ETHICAL APPROVAL

This research was approved by Ethics Review Committee of Ziauddin University (Ref. code: 1830120MAANA).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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