Osteogenic potential of mesenchymal cells derived from canine umbilical cord matrix co-cultured with platelet-rich plasma and demineralized bone matrix

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Canine mesenchymal cells (MSCs) derived from Wharton’s jelly were co-cultured, then supplemented or not supplemented with platelet rich plasma (PRP) and demineralized bone matrix (DBM) to verify osteogenic differentiation. Osteoblastic differentiation followed by mineralized bone matrix production was found to be significantly higher \((p < 0.05)\) when MSCs were associated with PRP/DBM in culture after 14–21-days of induction. Osteopontin and osteocalcin gene expression were significantly superior \((p < 0.05)\) under the same culture conditions after 21 days of observation. In conclusion, addition of PRP to DBM co-cultured with MSCs successfully induced osteogenesis in vitro.

Keywords: bone, mesenchymal stem cells, platelet-rich plasma, umbilical cord
well culture plates, all of which contained DMEM-F12 medium supplemented with B-27 and XenoFree (50×; Gibco, USA). MSCs monolayers were observed at zero (0), seven (7), 14 and 21-days after initial culture. To determine extracellular mineralization, cells were fixed with 4% paraformaldehyde for 15 min, washed five times with ultra-pure water and allowed to dry at room temperature, after which they were stained with Alizarin red. All images were obtained using a digital camera DP73 attached to an IX 70 inverted microscope (Olympus, Japan). Images were analyzed using the cellsSens software (Olympus). Alkaline phosphatase (ALP) activity was measured from culture supernatants in triplicate as previously described [9]. The results were calculated after determining the cutoff point, which was established by the arithmetic mean of the OD values of the negative controls (cells without induction).

Total cellular RNA was extracted from cells with the indicated treatments using Trizol reagent (Invitrogen, USA). cDNA synthesis was conducted using an Enhanced Avian RT First Strand Synthesis Kit (Sigma-Aldrich, USA). Primers for target genes and the internal control were as follows: osteopontin (forward)-5’-CATATGATGGCCAGGTGATAG-3’, (reverse)-5’-CAAGTGATGTGAAAGTCTCCTCTC-3’; osteocalcin (forward)-5’GAGGGCAGCGAGGTGGTGAG-3’, (reverse)-5’TACGCCAGCTGTCACAGTTGG-3’; Runx2 (forward)-5’TGGGAGAGGTACCAGATGGG-3’, (reverse)-5’TCTTGCCTCGTCCACTCCGG-3’. cDNA from the RT reaction was used as the template in quantitative polymerase chain reaction (PCR) with the StepOne real time PCR system (Applied Biosystems, USA). Power SYBR Green PCR master mix was applied to amplify the cDNA (100 ng) according to the manufacturer’s protocols. β-actin was used as a housekeeping gene to normalize the amount of mRNA. Gene expression was measured relative to that of the control. Fold change in gene expression relative to the control was calculated by the 2^ΔΔCt method. All statistical analyses were performed using the SAS 9.1.2 software package (SAS Institute, USA.) and data are presented as the means ± standard deviation based on three replicates for each experiment. One-way analysis of variance (ANOVA) for multiple comparisons or a two tailed student t-test was used to identify significant differences between groups. A p < 0.05 was considered to indicate significance.

The results indicated that PRP and DBM both have strong effects on the differentiation of Wharton’s jelly canine MSCs, which occurs at least partially through induction of ALP and osteocalcin/osteopontin gene expression. We also found that association of co-culture MSCs, PRP and DBM, played a much better role in promotion of osteogenic progenitor proliferation due to its ability to restrain the differentiation at 21 days after induction.

MSCs isolation and expansion showed typical morphology of undifferentiated mesenchymal cells with fusiform fibroblast-like and elongated appearance during all passages (panel A in Fig. 1). Mesenchymal cell phenotyping revealed CD44−, CD105+ and CD71+ (panels B-D in Fig. 1) cells, while they were negative for the hematopoietic cell phenotypic markers CD34− and CD45- (panels B and D in Fig. 1). These findings are similar to those of previous studies [2,4,6,12,14]. The optimum conditions were found to be co-culture of MSCs were with PRP and DBM, which produced the optimum ALP levels (panel A in Fig. 2). Several studies have described mitogenic effects of PRP in stem cells; however, this study was the first to demonstrate these effects in veterinary science [1,8]. In contrast, PRP has been reported to promote a decrease in ALP activity, despite increasing calcium levels [5]. ALP activity is not always directly correlated with the mineralization process [9]; however, in this study, a correlation between these two processes was confirmed (panel A in Fig. 2). No significant differences in Runx2 gene expression were observed during analysis (panel B in Fig. 2). The main role of Runx2 transcription factor is to promote chondrocytes and osteoblast differentiation, which is

Fig. 1. (A) Fibroblast-like morphology of canine MSCs isolated Wharton’s jelly at the 10th passage. Micrographs were taken under phase contrast microscopy. 40× magnification. MSCs phenotyping by flow cytometer analysis: (B) CD34−/CD44+, (C) CD45−/CD271+, (D) CD45−/CD105+. Data from surface markers were obtained by histograms generated using an Attune acoustic focusing cytometer, and auto-fluorescence was excluded as a global compensation tool (cutoff > 10^4). Y-axis corresponds to the number of positive labeled cells detected by BL1A filter (488 nm).
associated with bone formation [2,5]. Interestingly, when canine Wharton’s jelly MSCs were co-cultured with PRP and DBM, osteocalcin and osteopontin genes were expressed in higher levels when compared to other combinations (panel B in Fig. 2). Osteocalcin is considered a late marker of osteogenesis, while osteopontin has been implicated as an important factor in bone remodeling [3,11]. These findings suggest that an association of PRP and DBM could improve MSCs differentiation into osteoblast cells and facilitate bone formation, as partially demonstrated here.

To expand the population of osteogenic progenitors would be advantageous for clinical application of MSCs to the reconstruction of massive bone defects. Mesenchymal cell expansion is currently conducted by adding fetal bovine serum or growth factors, which could lead to sanitary problems, especially infectious pathogen transmission [2]. Moreover, the PRP applied in this study are safer to use than homologous recombinant growth factors and is free from concerns regarding disease transmission [2].

Overall, the results of this study strongly suggest that PRP and DBM co-cultured with MSCs have the potential for use in an osteo-induction in vitro model with future clinical applications to avoid the risk of graft versus host disease, leading to better acceptance by patients. Further studies are ongoing to explore use of this in vitro model during orthopedic surgeries.

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Conflict of Interest

There is no conflict of interest.

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