The Effect of Osteogenic Induction Medium on Mineralization between Human Jaw Periosteum Cells and Dental Pulp Cells

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Abstract

The most common birth defect in craniofacial area is a cleft defect with an incidence of 1.7:1000 live births. The current treatments involve many steps of surgical procedures and cause morbidity at the donor site when harvesting bone for filling the gap defect. It may be possible to treat cleft palate defects by bone regeneration strategies using osteoprogenitor cells to form the hard tissue. The aims of this project are to select a suitable cell source between human jaw periosteum (HJPs) and dental pulp cells (DPCs) for repairing the bone part of a cleft palate.

Donor who attended a wisdom tooth surgical removal was collected both HJPs and DPCs for the same time at Dental Hospital, Naresuan University. In this study, 3 donors were used with ethical approved by Human research committees, Naresuan University. Both HJPs and DPCs were isolated and cultured in the osteogenic induction medium which supplemented with Dexamethasone (Dex). A cell proliferation was measured by using Hoechst 33258. An osteogenic differentiation potential was measured by alkaline phosphatase (ALP) activity on day 7, 14, 21, and 28 days and calcium mineralization by using alizarin red staining on day 28.

The HJPs and DPCs showed increased in the cell proliferation overtime for 28 days of culture with no difference between cell types. HJPs showed the osteogenic potential by increasing ALP activity over 21 days and decreasing on day 28 of experiment. However, DPCs showed an increasing ALP activity over 14 days and then decreasing on day 21 and 28. The calcium mineralization on monolayer culture showed no difference between HJPs and DPCs.

Both HJPs and DPCs have the osteogenic potential under Dex supplementation. Bone regeneration strategies by using both HJPs and DPCs could benefit in cleft palate treatment compared to the current treatments (autologous bone graft from iliac crest) to promote bone formation at the defect area and enhance development of facial structure in the future.

Keywords: Cleft palate, bone tissue engineering, osteoprogenitor cells

Introduction

A common oral and facial malformation birth defect is a cleft lip and/or cleft palate with an approximately 1.7 per 1000 live births in worldwide (Mossey, Little, Munger, Dixon, & Shaw, 2009). The current treatment necessary composes of multiple operations over several years and involves bone harvesting from the hip bone (iliac crest) or chin, resulting in morbidity at the donor site. The alveolar ridge area is usually repaired with an autologous bone graft to enhance and support a permanent tooth eruption. An alternative bone graft was suggested and investigated by many researchers on their potential as a for bone reconstruction of the cleft palate (Lohberger et al., 2013). Moy, Lundgren, and Holmes (1993) reported that the result of bone graft substitute (BGS) materials implanted alone instead of autologous bone graft presented a lower success rate compared to the combination of cells and BGS. This suggests that bone regeneration strategies using suitable cells combined with a supportive biomaterial scaffold may be a more effective treatment for cleft palate repair than biomaterials alone (Behnia, Khojasteh, Soleimani, Tehranchi, & Atashi, 2012; Khojasteh, Eslaminejad, & Nazarian, 2008).

Osteoprogenitor cells are the mesenchyme precursor cells have been widely used for bone regeneration due

to their ability to self-renew and committed into the bone lineage. These cells could capable differentiate into osteoblast-like cells, using specific culture media. Harvesting bone marrow osteoprogenitor cells (OPGs) from the iliac crest presented a lower risk of infection, fewer arterial and nerve injuries, and less pain at the operation site compared to the traditional harvesting of iliac crest bone grafts (Samee et al., 2008). Bone regeneration strategies using OPGs from patients have shown promise for a cleft defect reconstruction at the alveolar ridge region; resulting in a successful bone repair and support of permanent tooth eruption over the post following two years (Hibi, Yamada, Ueda, & Endo, 2006). Another interesting cell source for bone repair is human jaw periosteal cells (HJPs) and dental pulp cells (DPCs). HJPs reside in the inner layer of connective tissue membrane that covers the outer surface of all bones, except at the joints of long bones, they can be extracted from human jaw periosteum (HJP) and appear to contain a sub-population of OPGs. Studies have shown that HJP derived cells show promise for bone regeneration due to reduced donor site morbidity and time of operation compared to the traditional autologous bone graft from iliac crest (Trautvetter et al., 2011). HJP tissue is easy to harvest during wisdom tooth removal or other routine maxillofacial surgical procedures. For patients with cleft palate, HJP tissue could be harvested from the palate at the time of palatal closure which typically takes place at 9-12 months of age (Pradubwong, 2007). The other interesting cell source is DPCs which were reported by many studied showed an osteogenic potential (Estrela, Alencar, Kitten, Vencio, & Gava, 2011; Rodríguez-Lozano et al., 2011). Dental pulp tissue could harvest during a wisdom tooth removal or primary tooth exfoliation. The objectives of this study were to understand how to regenerate bone tissue by selected cell sources for developing cleft palate repair.

Methods and Materials

All chemicals and culture consumables were obtained from Sigma-Aldrich (Singapore) unless otherwise stated and used as supplied.

1. Cell Culture

Two different cell types from the same donor were used in this study 1) human jaw periosteal cells (HJPs) isolated from the periosteum tissue and 2) dental pulp cells isolated from the dental pulp tissue of a non-functional tooth from the same patient who undergoing maxillofacial surgery at the Dental Hospital, Naresuan university (with written informed consent). These tissues collection were conducted under ethical approval 507/59 from the Naresuan university ethical committee, Phitsanulok, Thailand.

All cell types were cultured in a basal culture medium (BCM) which consisted of α-MEM culture medium (Lonza®, Basel, Switzerland), supplemented with 10% fetal bovine serum (FBS; v/v) (Capricorn, Ebsdorfergrund, Germany), 2 mM L-glutamine (Hyclone, Massachusetts, USA), 100 mg/ml penicillin and streptomycin (P/S) (Caisson labs, Utah, USA).

HJPs and DPCs were cultured in the 75 cm^2 tissue-culture flasks (Nunc, Massachusetts, USA). For osteogenesis induction media, a basal culture medium was supplemented with 50 µg/ml ascorbic acid-2-phosphate (AA) and 5 mM beta-glycerophosphate (β-GP), and the addition of 10nM of dexamethasone (Dex). 10 nM Dex was added to HJPs and DPCs cultures based on previous experiments in our laboratory to establish the best Dex concentration for osteogenic induction (Puwanun, 2014). All cells were cultured at 37ºC in 5% CO_2 in a humidified atmosphere. Media was changed every 2-3 days. HJPs and DPCs were used between passages 2-5, as suggested by De Bari and colleaques (De Bari et al., 2006).

2. Cell isolation

Three donors were included in this study with age between 20-23 years with healthy and non-smoking. One donor could provide both HJPs and DPCs to reduce subject variation. Briefly, removed tissue of approximately 5x5 mm^2 was rinsed with phosphate buffered saline (PBS; DulbeccoA, England) containing 100 mg/ml P/S, cut into smaller pieces and added to 0.25% collagenase type II in BCM without FCS and incubated at 37ºC for 3 h (Samee et al., 2008). DPCs were isolated from dental pulp tissue. Briefly, remove tissue from pulp chamber was removed by using a sterile dental diamond bur and followed by chisel and hammer to split the tooth into 2 parts (Phumpatrakom & Srisuwan, 2014). The tissue was rinsed with phosphate buffered saline containing 100 mg/ml P/S, cut into smaller pieces and added to a solution of 3 mg/ml collagenase (Gibco, Massachusetts, USA) and 4 mg/ml dispase (Gibco, Massachusetts, USA) for 1 hour until the tissue digested (Vishwanath et al., 2013). After this time, the cells were centrifuged, the supernatant was removed and the cells were placed into a 25 cm^2 tissue-culture flask (Nunc, Massachusetts, USA) in 5 ml of fresh BCM. Fresh media was added to the flask every 2-3 days for 7 days. After 7 days the culture media and non-adherent cells were removed and the adherent population was termed passage 0 (Chao et al., 2012; Dominici et al., 2006).

3. DNA measurement and alkaline phosphatase activity

DNA quantification was assessed using fluorescent bisbenzimide (Hoechst 33258) is a selective binding for double strand DNA (dsDNA). An increase in fluorescence was measured using fluorescence spectrophotometer (FL600, BioTek, USA) with a 96-well black microplate (Nunc, ThermoFisher Science, USA) as per the manufacturer's instructions. Briefly, 500 µL of cell lysate buffer was added to the cells for 20 min at 37°C before scraping to remove cell lysate. Cell lysates were freeze-thawed three times, vortexed and centrifuged. Cell lysate and 1µg/ml Hoechst working solution were mixed and the fluorescence intensity measured with an excitation wavelength of 360 nm and emission wavelength of 460 nm (Oliveira et al., 2006). The total DNA was calculated from the fluorescence emission to nano-gram per ml using a standard curve. To determine alkaline phosphatase (ALP) activity, cell lysate was mixed with an 'Alkaline Phosphatase Yellow Liquid Substrate' (Sigma-Aldrich, USA) based on p-nitrophenol phosphate and the absorbance was measured using a spectrophotometer (Bio-Rad, USA) at 405 nm every minute for 30 min. The enzyme activity was calculated as nano-mol of para-nitrophenol per min (nmol pNP/min) divided by DNA (µg/ml).

4. Calcium mineralization staining

Calcium mineralization staining was performed on day 28 for monolayer culture. Total calcium mineralization was measured by staining with 1 mg/ml alizarin red in distilled water (dH_2O), adjusted to pH 4.1 with ammonium hydroxide for 20 min at room temperature. All unstained dye was removed with dH_2O and left to air-dry. The stained samples were dissolved by adding 500 µl cetylpyridinium in 10mM sodium phosphate (10% v/v) for 30 min at room temperature and the absorbance of the eluted stain was measured at 570 nm using a spectrophotometer (Gregory, Gunn, Peister, & Prockop, 2004).

5. Statistics

Data are expressed as mean values ± standard error of mean (SEM). Numbers of replicates are stated in the figure legend. 'N' represents a biological repeat (separate experiment) and 'n' represents a technical repeat (different samples within one experiment). Statistical analysis was performed using SPSS (IBM SPSS statistics 21). DNA quantification, ALP activity, and calcium mineralization were analyzed using an independent (unpaired) *t*-test. The differences were

considered to be statistically significant when the p-value was less than or equal to 0.05 ($p \leq 0.05$).

Results

After 7 days, both of DPCs and HJPs cell morphologies showed change under the osteogenic induction medium from the spindle cell shapes which were similar to the fibroblastic-like cells to a cobblestone-shape which is similar to a typical osteoblastic cell shape (Figure 1). DNA quantifications of both HJPs and DPCs were assessed using a fluorescent bisbenzimide (Hoechst 33258) dye test after day 7, 14, 21, and 28. DNA increased in all cell types (HJPs and DPCs) over 28 days of experiment (Figure 2). Total DNA of DPCs was higher than HJPs with no significantly difference. The highest ALP activity normalized to DNA of the DPCs was on day 14, however, the HJPs was on day 21. Then the ALP activities of both cell types were decreased after the peaks (Figure 3). Total calcium mineralization of DPCs and HJPs were measured by using alizarin red staining after 28 days. They showed no significantly different between DPCs and HJPs (Figure 4A). The photographs of whole culture well alizarin red staining showed that they were contained the dark staining after 28 days in the cell culture (Figure 4B for DPCs and 4C for HJPs).

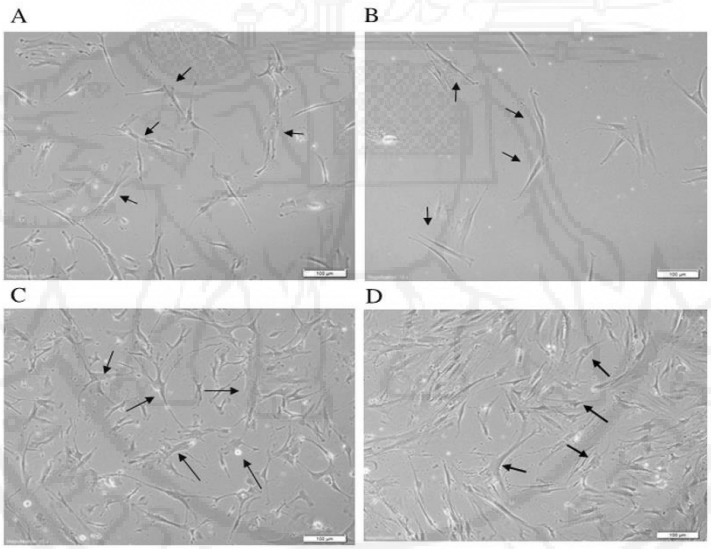

Figure1 Phase contrast images of the DPCs (A) and the HJPs (B) were cultured on the basic culture media for 7 days, the black arrows showed the spindle cell shape similar to fibroblastic-like cells. The cells were cultured on the osteogenic induction medium for 7 days (C for DPCs, D for HJPs: the black arrows showed the cobblestone-shape). 100 X magnification, Scale bar=100μm

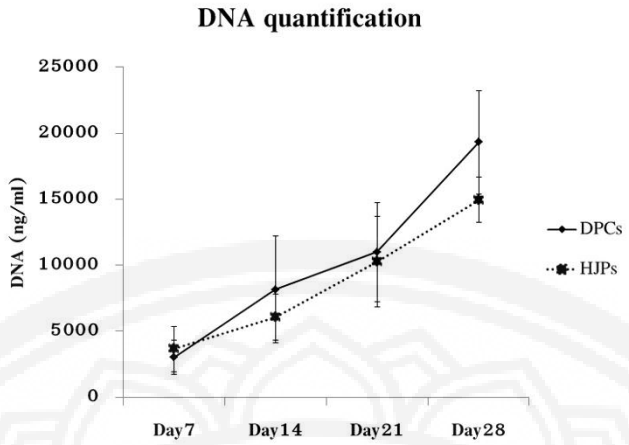

Figure 2 DNA quantification measured by Hoechst on the DPCs (dark line) and HJPs (dot line) on the osteogenic induction medium. Data presented as mean ± SEM, (N=3, n=3), an independent (unpaired) *t*-test.

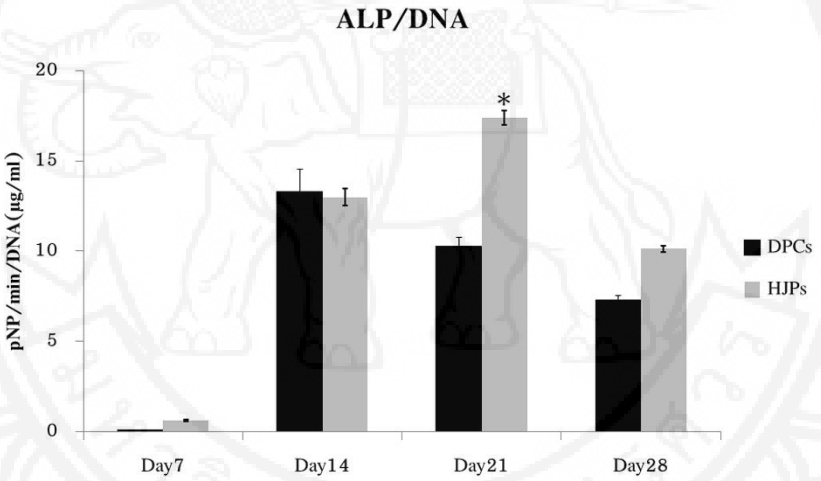

Figure 3 ALP activity normalized to DNA (μg/ml) on the DPCs (black column) and HJPs (grey column) on the osteogenic induction medium. Data presented as mean ± SEM, (N=3, n=3), * = $p<0.05$ comparison between DPCs and HJPs, an independent (unpaired) *t*-test.

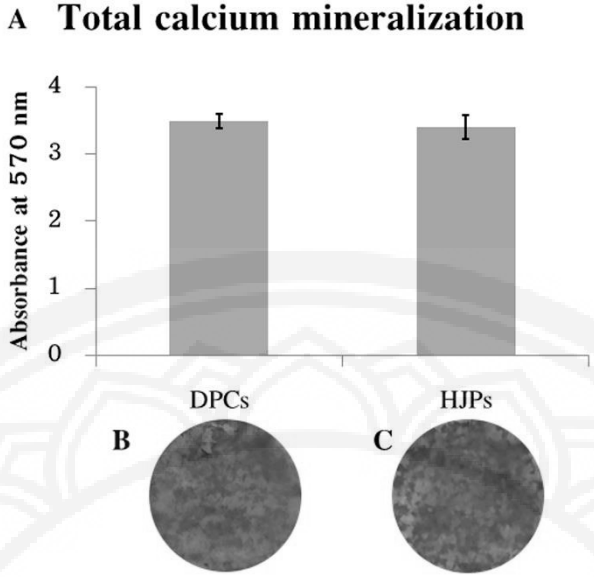

Figure 4 Total calcium mineralization of the DPCs and HJPs (A) using alizarin red staining. The photoimages show a representative set of alizarin red staining of the DPCs (B) and HJPs (C). Data is presented as mean ± SEM, (N=3, n=3), an independent (unpaired) *t*-test.

Discussion

Bone tissue regeneration may become an option for cleft palate reconstruction. One important part of this strategy is the osteoprogenitor cell source used for bone formation. The main aims of this study were to evaluate suitable cell sources for proliferation and osteogenic differentiation of both DPC and HJP cells in monolayer culture. This study used 3 donors and performed in triplicate. In this study we used α-MEM media based on the previous report that α-MEM media is suitable to use for isolation and culturing bone marrow cells (Chen et al., 2009; Coelho & Fernandes, 2000). The α-MEM media compose of Vitamin B12, AA and non-essential amino acid in addition to the components of DMEM media. This medium may facilitate HJPs and DPC cell proliferation and enable these to differentiate into the osteoblastic linage in a similar way as bone marrow cells. Several studies used α-MEM to culture these types of cells. However, Nakamura et al. (2008) found that human MSCs cultured on both DMEM media and specific MSC basal medium were not different in their cell behavior including growth rate and an osteogenic differentiation. It is known that a combination of Dex, AA and β-GP can be used to induce calcium mineralization by MSCs (Langenbach & Handschel, 2013). Both DPCs and HJP cells used in these experiments could differentiate into osteoblastic-like cells. However, there is limited literature on the suitable cell source that enable osteoblastic differentiation and mineralization. Therefore these cells were measured to investigate whether they response to osteogenic induction medium in the same way as mature MSCs. Our study found that the DNA of both cell types increased overtime for 28 days indicating that cells proliferated. Osteogenic induction medium which supplemented with Dex promoted ALP activity (an early maker for osteogenic differentiation) and change cell phenotype to osteoblastic-like cells (Cheng et al., 1994; Kaveh et al., 2011). The highest ALP activity of DPCs was the highest on day 14, however, HJPs was the highest on day 21. A potential reason is that ALP activity normally decreases before the beginning of mineralization (before day 21) where the calcium mineralization has been accelerated (Lian & Stein,

1995). Total calcium mineralization plays a role as a late marker of osteogenic differentiation. There showed no difference between the DPCs and HJPs. Comparison two different osteoprogenitor cells indicated that both of DPCs and HJPs showed the similar cell proliferation and osteogenic potential. It might be due to the similar embryological origin of these cells from neural crest (Ichikawa et al., 2015; Tatullo, Marrelli, Shakesheff, & White, 2015).

Conclusion

The main finding of this study showed that both of DPCs and HJPs have the similar osteogenic potential and could be differentiate into osteoblastic-like cells and deposit calcium mineralization. Isolated DPCs and HJPs could be the autologous cell source for craniofacial bone regeneration. It should be a new optional treatment to reduce donor site morbidity and time of surgical treatment. The use of both DPCs and HJPs are promising cell sources for bone tissue regeneration for small defects such as periodontal disease and cleft palate.

Suggestion

Our study should investigate other markers of osteogenic differentiation of the cells such as surface antigens, cytokines, and the expression of genes involved in the initial stages of differentiation e.g. Runx2, osterix (OSX), activating transcription factor 4 (ATF4), collagen type I, and BMP-2 (Malgorzata, 2012). For the future work, it should investigate multiple donors of osteoprogenitor cells to better understand the variability from different individuals. Moreover, the next study should be used in clinic and compared the bone regeneration between iliac bone graft and bone tissue engineering graft derived from HJPs and/or DPCs.

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