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Higher Concentration of Ascorbic Acid as a Sole Induction Factor for Osteogenesis on MC3T3-E1 Cell Model

(Kepekatan Tinggi Asid Askorbik sebagai Faktor Aruhan Tunggal untuk Osteogenesis pada Model Sel MC3T3-E1)

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ABSTRACT

This research aimed to investigate the potential of ascorbic acid (Asc) to act independently as an osteogenic induction factor in a murine pre-osteoblast (MC3T3-E1) model. MC3T3-E1 cells were seeded in culture wells and hydroxyapatite scaffold for two-dimensional and three-dimensional analyses respectively. Cell morphology, viability, osteoblast differentiation, and mineralisation potentials of MC3T3-E1 cells were compared between induction of standard (50 µg/mL) and doubled (100 µg/mL) Asc concentrations in growth media. Cells with fibroblast-like morphology became confluent earlier on day 6 in the standard group compared to the doubled group on day 9. Cell viability and differentiation potential were significantly increased in the doubled group ($p < 0.01$). Mineralisation occurred in the doubled group after 15 days of seeding but no mineralisation was seen in the standard group. Findings were similar in 3D analysis whereby mineralized nodules were seen only in the doubled group. The relative expression of collagen 1(α) and osteocalcin genes were increased in the doubled group than the standard group. Doubling Asc concentration in a growth medium to 100 µg/mL can induce viability, differentiation, and mineralisation of MC3T3-E1 cells. Thus, higher concentration of ascorbic acid can potentially be used as the sole induction factor in osteogenic medium.

Keywords: Ascorbic acid; growth media; MC3T3-E1 cells; osteogenic differentiation; sole induction

ABSTRAK

Penyelidikan ini bertujuan untuk mengkaji potensi asid askorbik (Asc) bagi tindak balas secara bebas sebagai faktor induksi osteogenik dalam model pra-osteoblas murine (MC3T3-E1). Sel MC3T3-E1 dibenihkan dalam telaga kultur dan perancah hidroksi apatit untuk analisis dua dimensi dan tiga dimensi. Morfologi sel, kebolehidupan sel, pembezaan osteoblas dan potensi pemineralan sel MC3T3-E1 dibandingkan antara induksi kepekatan piawai (50 µg/mL) dan kumpulan dua kali ganda (100 µg/mL) kepekatan asid askorbik di dalam media pertumbuhan. Sel dengan morfologi seperti fibroblas menjadi konfluensi pada hari ke 6 dalam kumpulan piawai berbanding kumpulan dua kali ganda pada hari ke 9. Kebolehidupan sel dan potensi pembezaan meningkat dengan ketara pada kumpulan dua kali ganda ($p < 0.01$). Pemineralan berlaku pada kumpulan yang berlipat ganda selepas 15 hari pengkulturan tetapi tiada pemineralan dilihat pada kumpulan piawai. Penemuan yang sama dilihat dalam analisis 3D manakala nodul mineral hanya dilihat pada kumpulan dua kali ganda. Ekspresi relatif gen kolagen 1(α) dan osteokalsin meningkat pada kumpulan dua kali ganda berbanding kumpulan piawai. Menggandakan kepekatan asid askorbik dalam medium pertumbuhan hingga 100 µg/mL boleh mengaruh keviabelan, pembezaan dan pemineralan sel MC3T3-E1. Oleh itu, kepekatan asid askorbik yang lebih tinggi berpotensi digunakan sebagai faktor induksi tunggal dalam medium osteogenik.

Kata kunci: Aruhan tunggal; asid askorbik; media pertumbuhan; pembezaan osteogenik; sel MC3T3-E1

Introduction

Bone grafting in dentistry is largely applied for implant and periodontal procedures in adults, and alveolar cleft repair in children (Guo et al. 2011; Jimi et al. 2012; Kumar et al. 2013). Autogenous bone is the gold standard because of its excellent immunohistocompatibility and high osteogenic cells content (Roberts et al. 2012). However, the site of harvest presents an additional area of pain and infection risk which are undesirable post-operative morbidities (Kumar et al. 2013). In addition, the volume of bone material which can be harvested may be insufficient, particularly in the paediatric population (Roberts et al. 2012). Therefore, tissue engineering-enhanced graft materials have become a popular alternative to overcome the limitations of autogenous graft (Hammoudeh et al. 2017; Mac Isaac et al. 2012). Bone regeneration via this method is achieved by inducing functional osteoblasts on a three-dimensional scaffold with biological factors (Hayrapetyan et al. 2015). The standard induction factors for osteogenic differentiation comprises a combination of dexamethasone, β- glycerophosphate (β-GP) and L-ascorbic acid (Asc) in the concentration 100 Nm, 10 Mm, and 50 µg/mL, respectively. However, osteogenesis has been shown to occur with the addition of only β-GP and Asc. β-GP acts as a source of inorganic phosphate to activate signalling pathways responsible for osteogenic gene expression (Langenbach & Handschel 2013). This is necessary for bone mineralisation (Vater et al. 2011). Nonetheless, β-GP induces matrix mineralisation at the expense of reduced cell proliferation (Ariffin et al. 2017). At a concentration above 2 mM, it can also lead to dystrophic mineralisation which causes increased false-positive results (Langenbach & Handschel 2013). Therefore, there is a need to determine if β-GP can be eliminated from an osteogenic medium without adverse effects on bone formation.

The role of Asc in osteogenic differentiation is primarily related to collagen secretion into the extracellular matrix (ECM) and its subsequent maturation. Asc stimulates pro-collagen type I messenger ribonucleic acid which is responsible for collagen synthesis and acts as a cofactor for hydroxylases for collagen maturation (Finck et al. 2014). Osteoblast differentiation requires cells to be in contact with the collagen-rich ECM before activation of downstream signalling cascade can occur (Langenbach & Handschel 2013). Specifically, the interaction of bound α2β1 integrin ligands with collagen activates the extracellular related kinase (ERK1/2) in the mitogen-activated protein kinase (MAPK) signalling pathway. This leads to phosphorylation of Runx2 (osteoblast master transcriptional regulator) and accumulation of P-ERK in the nucleus which upregulate production of osteoblast marker genes like osteocalcin (OCN) and osterix (OSX) (Aghajanian et al. 2015; Langenbach & Handschel 2013). On the other hand, Asc has not been shown to induce mineralisation of MC3T3-E1 cells on its own. Two MC3T3-E1 cell studies that reported negative findings had utilized concentrations at or below 50 µg/mL Asc (Marsh et al. 2009; Quarles et al. 1992). The effects of using higher Asc concentrations towards osteogenesis is scarcely researched. Human mononuclear cells were used in one study while G292 osteosarcoma cell line was used in another (Fernandes et al. 2017; Hadzir et al. 2014). Both studies were conducted under two-dimensional culture conditions. The use of human primary cells may introduce inconsistencies as the osteoblast phenotype may differ according to location where cells were isolated. Furthermore, the proliferation profile can be affected by donor-related factors such as age. On the other hand, cell proliferation rate of G292 cells may not represent normal bone physiology due to the loss of contact inhibition property that is characteristic of malignant cell lines (Czekanska et al. 2012). In addition, the actual concentration of Asc in the experiment set up was ambiguous in the first study as the authors did not detail the type of growth medium used (Hadzir et al. 2014).

MC3T3-E1 cells are an osteogenic progenitor cell line derived from the calvaria of newborn mice (Kodama et al. 1981). They undergo the sequential processes of osteogenesis (adhesion, proliferation, differentiation, and mineralisation) when cultured in the correct osteogenic medium (Sudo et al. 1983). Unlike other malignant cell lines such as SaOs-2 and MG-63, they also demonstrate replicative senescence which is similar to human cells (Czekanska et al. 2012). As such, MC3T3-E1 cells are good candidates to study *in vitro* osteogenesis.

Hence, the aim of this study was to determine the effects of increased Asc concentrations on the mineralisation of a well-established phenotypically stable MC3T3-E1 pre-osteoblast cell line in the absence of β-GP.

Materials and Methods

Culture media preparation

Culture medium for this research consisted of alpha-minimal essential medium (α-MEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS)

(Thermo Fischer, USA), 1% penicillin-streptomycin solution (Gibco, USA) and 1 mM sodium pyruvate (Sigma, USA). The α-MEM used in this study contained a pre-existing 50 µg/mL Asc (catalogue number 12561-056), which is the concentration in a standard osteogenic medium (Vater et al. 2011). This culture medium was hereafter referred to as standard medium. The test medium had double the ascorbic acid concentration by addition of 50 µg/mL Asc A4403 (Sigma, USA) into the standard medium.

GRANUMAS® SCAFFOLD PREPARATION

GranuMas® (GranuLab, Malaysia) is a synthetic graft material made of calcium phosphate hydroxyapatite which is sourced from limestone. It is sold in many shapes including spherical, cuboids, pins, and chips. The hydroxyapatite in this scaffold resembles the mineral content of bone, hence its use in bone repair studies (Nawi et al. 2013; Zakaria et al. 2016; Zulkifly et al. 2008). Spherical granulated scaffolds with a particle size between 0.2 and 1.0 mm in diameter were used in this study. The gamma-ray irradiated granules were aliquoted into 24-well plates to cover the bottom of the well-plates as a dense monolayer (Seebach et al. 2010). The scaffolds were soaked in 200 µL standard medium for 24 h in 5% carbon dioxide incubator prior to cell seeding. This was done to minimize ion release from the particles (Cordonnier et al. 2014).

CELL CHARACTERIZATION IN TWO-DIMENSIONAL MODEL

A two-dimensional cell culture was first performed to establish basic cell characters without interference from scaffold material.

MC3T3-E1 CELL CULTURE

MC3T3-E1 subclone 14 cells (ATCC No: CRL -2594™) were chosen as they are a well-established osteogenesis model which reflects the full phenotypic differences from pre-osteoblasts to mature osteoblasts. MC3T3-E1 cells were grown in T75 flasks containing 3 mL standard medium. Once the cells reached confluence, they were detached with trypsin/EDTA and passage into experiment well plates at 2×10^4 cells/cm^2 seeding density. Standard medium was used as the control and medium with doubled Asc concentration was used in the test group. Culture media were refreshed every 3 days. Three biological replicates (n = 3) were used according to the sample size in published studies (Fu et al. 2017; Yazid et al. 2010). Day 0 of analysis was defined as 24 h after cell seeding onto experimental plates.

CELL MORPHOLOGY VIA LIGHT MICROSCOPY

Cell morphology was examined under phase contrast inverted microscope. Cells were seeded in 6-well plates at a seeding density of 2×10^4 cells/cm^2 and incubated at 5% CO_2 and 37 °C conditions. Cell images were captured every three days up to 21 days using CellB imaging software (Olympus, USA). The media were removed and replaced with phosphate-buffered saline (PBS) solution (Gibco, USA) prior to viewing each time because Asc is photosensitive.

CELL VIABILITY VIA MTT ASSAY

The number of viable MC3T3-E1 cells in each group was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, USA). Cells were seeded in 96-well plates with the same seeding density for analysis on day 0, 3, 6, 9, 12, 15, 18, and 21. On each day of analysis, old medium was removed while 10 µL of MTT solution and 90 µL fresh complete medium were added into each well. The cells were incubated for 4 hours at 37 °C, after which the medium was discarded. Subsequently, 110 µL dimethylsulphoxide (DMSO) (Merck, Germany) in glycine buffer pH 7.4 (Sigma, USA) was added to dissolve the purple Formozan salts. Optical density (OD) of the salts was read at 570 nm. A standard curve with known seeding densities was plotted to obtain the number of viable MC3T3-E1 cells from the OD values.

CELL DIFFERENTIATION VIA ALP ACTIVITY

Cell differentiation was measured by level of alkaline phosphatase (ALP) enzyme. Seeding density of 2×10^4 cells/cm^2 was used. ALP assay was carried out every three days, similar to MTT assay. Cells were lysed and harvested per SensoLyte® (AnaSpec, USA) kit protocol. Resulting cell suspension was agitated and then centrifuged at 2500 G for 15 min in 4 °C. 50 µL supernatant was placed in a 96-well followed by addition of 50 µL p-nitrophenyl phosphate (pNPP) substrate solution. The reagents were incubated at 37 °C for 60 min. Absorbance was read at 405 nm using spectrophotometer. This represented the hydrolysis of pNPP by ALP enzyme at 37 °C.

CELL MINERALISATION VIA VON KOSSA STAINING

Cell mineralisation was detected via von Kossa staining which stained mineral deposits. Seeding density and observation interval were same as MTT and ALP assays. The old medium was removed on each day and the cells were washed with 100 µL PBS. The cells were then fixed with 10% (v/v) formalin for 30 min and rinsed with deionized water thrice. Subsequently, the formalin was removed, and the well plate left uncovered overnight to dry out any residues. On the next day, the cells were washed with cold deionized water once, stained with freshly prepared 5% (v/v) silver nitrate solution for 30 min and then rinsed well with deionized water for three times. Following this, the cells were developed with fresh 5% (v/v) sodium carbonate in 25% (v/v) formalin for 5 min for mineral and matrix staining. After three washes with deionized water, the cells were finally fixed with 5% (v/v) sodium thiosulfate for 2 min to remove any unreacted silver nitrate. Finally, the cells were washed well with deionized water three times and air-dried. The sample was viewed under microscope to determine presence of brown mineral deposits. All solutions used for von Kossa staining were from Sigma-Aldrich, USA.

CELL CHARACTERIZATION IN THREE-DIMENSIONAL MODEL

Following the establishment of cell characteristics in 2D cultures, the experiment was repeated in a 3D model to better represent cell behaviour in *in vivo* environment (Matthews et al. 2014). Observation interval was increased to seven days based on the results from 2D analyses. This uniform interval was chosen as it encompassed cellular events of interest.

MC3T3-E1 CELL SEEDING ON GRANUMAS® SCAFFOLD

MC3T3-E1 cells were loaded as a 200 µL suspension in standard medium onto GranuMas® scaffold at a seeding density of 5×10^4 cells/cm^2 in 24-well plates. The construct was incubated in 5% carbon dioxide at 37 °C for 1 h to ensure cell attachment before addition of another 300 µL of standard medium. After 24 h, the old medium was removed and replaced with a new 500 µL standard or test medium. This marked day 0 of analysis. The culture medium was changed every three days for both groups.

CELL MORPHOLOGY USING SCANNING ELECTRON MICROSCOPY (SEM)

Cell morphology and adherence on the scaffold was done by scanning electron microscopy (SEM) analysis on day 7 and 21. Cells were fixed with 2.5% cold glutaraldehyde (Merck, Germany) overnight and then serially dehydrated with undenatured ethanol (Merck, Germany) by incubation with a 6-step alcohol gradient (30, 50, 70, 80, 90, and 100%) for 10 min in each step, and additional 20 min during the last step. Once dehydrated, the cell-scaffold construct was transferred for critical point drying (Leica, USA) for 2 h followed by sputter-coating with gold (Quorum Tech, UK). Images were viewed digitally with scanning electron microscope (Zeiss, UK).

CELL VIABILITY VIA MTT ASSAY

Measurement of viable MC3T3-E1 cells on the scaffold was carried out on day 0, 7, 14, and 21. A seeding density of 5×10^4 cells/cm^2 was used in 24-well plates. On each day, the old medium was removed, and the cell-scaffold construct was transferred to the bottom of a 96-well plate in a monolayer before addition of MTT solution. This prevented false positive results by eliminating the probability of counting cells that have attached to the bottom of the 24-well plate (Kumar et al. 2013). The assay was conducted per the method in two-dimensional characterisation of MC3T3-E1 cells. Similarly, an MTT standard curve for 3D culture was plotted with known seeding densities to normalize the optical density readings to actual number of cells.

CELL DIFFERENTIATION VIA ALP ACTIVITY

The activity of cell differentiation was measured by level of alkaline phosphatase (ALP) enzyme. ALP assay was carried out on day 7, 14, and 21. The same experiment protocol was undertaken per two-dimensional culture, with initial seeding density of 5×10^4 cells/cm^2 in 24-well plates.

CELL MINERALISATION VIA QUANTITATIVE POLYMERASE CHAIN REACTION

Mineralisation of MC3T3-E1 cells in a three-dimensional model was assessed via quantitative polymerase chain reaction (qPCR). Cells were cultured for day 0 and day 14 analyses. Old medium was removed on each day of analysis. Cells were lysed using the TRIzol® (Invitrogen, USA) protocol and their ribonucleic acid (RNA) content was extracted with innuPREP RNA Mini Kit (Analytik Jena). Briefly, 1 mL of TRIzol® reagent (Invitrogen, USA) was added into each well and incubated for 5 min in room temperature. The lysate was homogenized by

use of pipette. Subsequently, the lysate was transferred into an Eppendorf tube and 0.2 mL chloroform was added. The solution was incubated for another 2 min at room temperature before being centrifuged at 12000 G for 15 min at 4 °C. After this point, the mixture would be separated into three phases containing RNA, DNA, and protein, respectively. Only the aqueous phase which contained RNA were transferred into a new receiver tube. RNA purification was done by a series of alcohol washes and centrifugation.

Extracted RNA was directly transcribed into complementary deoxyribonucleic acid (cDNA) using the SensiFAST™ cDNA Synthesis Kit (Bioline, United Kingdom). Collagen Type 1α (Col1α) and osteocalcin (OCN) genes were selected for qPCR analysis as they represented extracellular matrix formation and mineralization, respectively (Motamedian et al. 2016; Wahab et al. 2020). The primer sequence for these target genes as well as the housekeeping gene GAPDH were adapted from previous studies on MC3T3-E1 cells and were listed in Table 1 (Fu et al. 2017; Wahab et al. 2020). Quantitative polymerase chain reaction was carried out using SensiFAST™ SYBR® No-ROX Kit (Bioline, United Kingdom). The results were quantified using the 2^-ΔΔCt method (Livak & Schmittgen 2001); where Ct values of target genes were normalized to that of the housekeeping gene, and relative expression between treatment and control groups was determined. Each analysis was repeated thrice.

TABLE 1. List of osteoblast markers and housekeeping gene with associated nucleotide sequence

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')	Size (bp)
Osteocalcin (OCN) (NM_001305449)	AGGGCAATAAGGTAGTGAA	CGTAGATGCGTCTGTAGGC	159
Collagen (Col1α) (NM_007742)	CTTCACCTACAGCACCCTTGT	AAGGGAGCCACATCGATGAT	120
Housekeeping gene (GAPDH) (NM_001289726)	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA	223

STATISTICAL ANALYSIS

Data was analysed using Statistical Package for Social Sciences (IBM® SPSS) Version 22.0 (Chicago, Illinois). From the Shapiro-Wilk normality test, all the data were normally distributed. Therefore, differences in cell characteristics between mediums with standard and doubled Asc concentration were tested using the independent t-test. Changes in cell characteristics within each experimental group were tested with one-way repeated measures ANOVA. Statistical significance was set at $p < 0.05$.

RESULTS

CELL CHARACTERISATION IN TWO-DIMENSIONAL MODEL CELL MORPHOLOGY

The initial morphology was spherical as MC3T3-E1 cells were still floating within the culture medium immediately after seeding (Figure 1(a), 1(e)). After one day, the cells attained a fibroblastic shape and maintained this morphology throughout the study (Figure 1(b), 1(f)). This corresponded to the adherent behaviour of MC3T3-E1 cells. Cell confluence was noted on day 6 in the standard group, and on day 9 in the doubled group. On longer incubation, there was overlapping of cell layers in both culture conditions. Cells became more elongated as their cytoplasm extended. White opaque materials present in the culture in later stages were likely metabolic wastes.

CELL VIABILITY

Number of viable cells was significantly higher in the presence of doubled Asc concentration on all days except day 0 ($p < 0.05$) (Figure 2). After correction to the initial number of cells on day 0 in each group, cells number increased at a significantly faster rate in the doubled group on all days of analysis compared to that in the standard group (Table 2). Having said that,

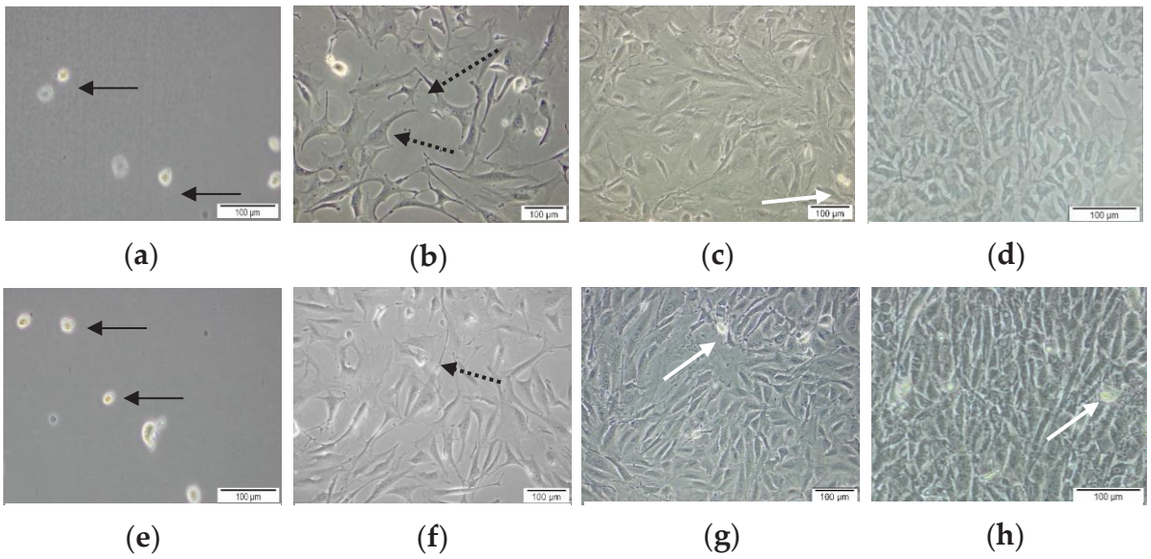


FIGURE 1. Morphology of MC3T3-E1 cells in 2-dimensional culture. (a-d) Cells cultured in 50 µg/mL Asc; (e-h) Cells cultured in 100 µg/mL Asc. Cells were spherical shaped immediately after seeding (a, e; black arrows) and attained fibroblastic features after one day (day 0) (b, f; black broken arrows showing long cytoplasmic extension, resulting in spindle-shaped cells). Cells in 50 µg/mL Asc became confluent on day 6 (c) while cells in 100 µg/mL Asc were confluent on day 9 (g). On longer incubation, cells in both groups became densely packed and lost their monolayer characteristics (d, h). White arrows showing white opaque materials which were likely metabolic wastes. Magnification ×10

cell proliferation showed a peak activity in doubled group on day 15 after which the rate began to reduce significantly. Cells in the standard group maintained a positive increment in cell viability up to day 18 and then plateaued. At the end of the study observation, there was an approximately 4-fold increase in number of viable cells in the doubled group compared to only a 3-fold increase in the standard group.

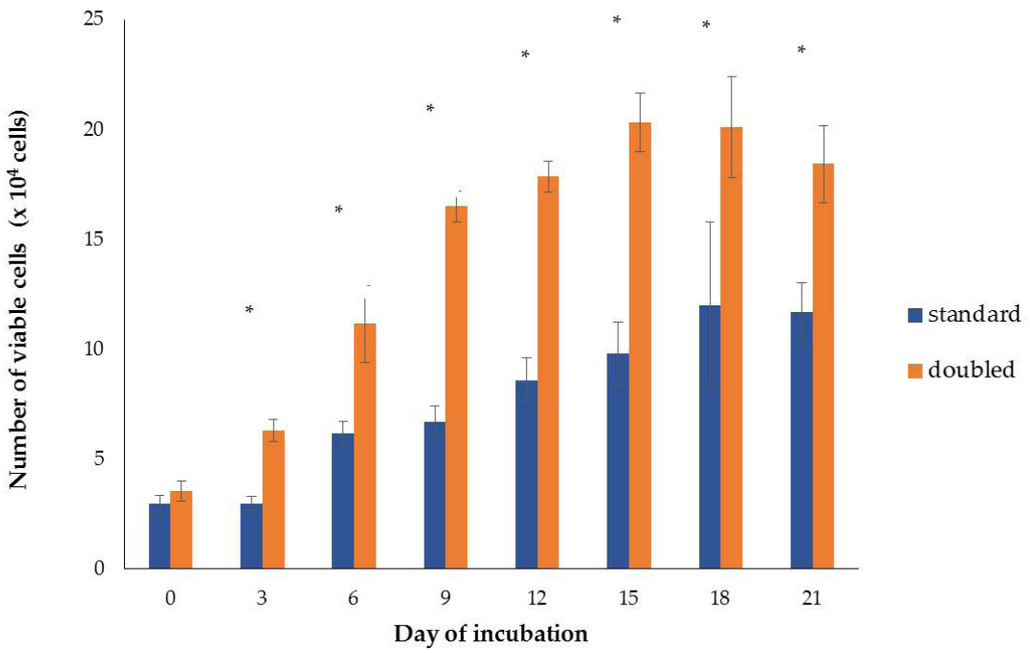


FIGURE 2. Viability of MC3T3-E1 cells in two different Asc concentrations Standard = 50 µg/mL Asc; doubled = 100 µg/mL Asc. Values expressed in mean ± SD. n = 3 (* = $p < 0.05$)

TABLE 2. Comparison of cell proliferation between two Asc concentrations in 2D culture. Cell proliferation was measured from the change in number of viable cells relative to day 0 of each group. Values were analysed with one-way repeated measures ANOVA and expressed in mean difference ± standard error. n = 3 (* = $p < 0.01$ for intragroup differences; # = $p < 0.01$ for intergroup differences)

Day, d	Change in number of viable cells relative to day 0, ΔN (× 10^4 cells)			
	Standard ascorbic acid concentration		Doubled ascorbic acid concentration	
	Mean difference ± S.E, Δn (× 10^4 cells)	Fold change, $\Delta n/n_0$	Mean difference ± S.E, Δn (× 10^4 cells)	Fold change, $\Delta n/n_0$
3	0.036 ± 0.163	0.01×	2.476 ± 0.294	0.77× *,#
6	3.205 ± 0.230	1.06× *	7.599 ± 0.414	2.14× *,#
9	3.727 ± 0.288	1.26× *	12.947 ± 0.291	3.65× *,#
12	5.636 ± 0.391	1.90× *	14.306 ± 0.456	4.03× *,#
15	6.841 ± 0.532	2.31× *	16.771 ± 0.657	4.72× *,#
18	8.911 ± 1.225	3.01× *	16.549 ± 0.640	4.66× *,#
21	8.740 ± 0.466	2.95× *	14.872 ± 0.523	4.19× *,#

CELL DIFFERENTIATION

ALP activity level was used as an early marker of osteoblast differentiation. The trend of cell differentiation followed closely that of cell viability (Figure 3). This proved that ALP enzyme was a good marker of viable cell activity. When analysed for intragroup changes in ALP activity, cells in the standard group produced higher ALP level than cells in the doubled group during the first three days. However, the difference was not significant (Table 3). Doubling of the Asc concentration resulted in significantly higher ALP activity on all days from day 9 onwards ($p < 0.01$). The maximum difference was seen on day 15 where ALP level in the doubled group was more than twice as high as that in the standard group. ALP level in the doubled group reduced after day 15 while that in standard group plateaued. Nevertheless, the amount of ALP was still significantly higher in the former group.

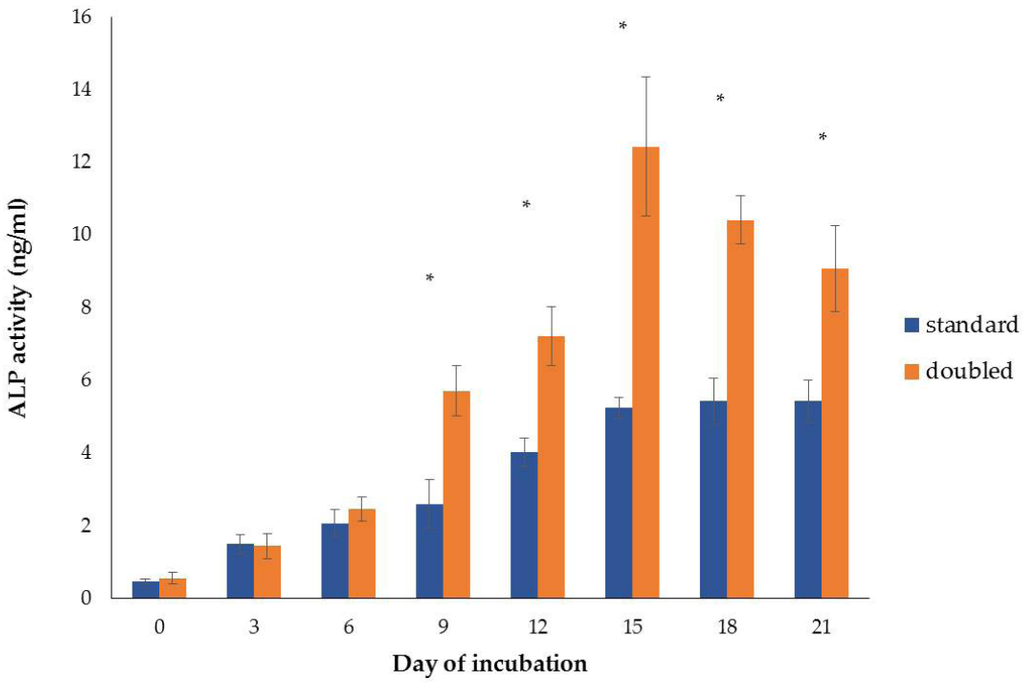


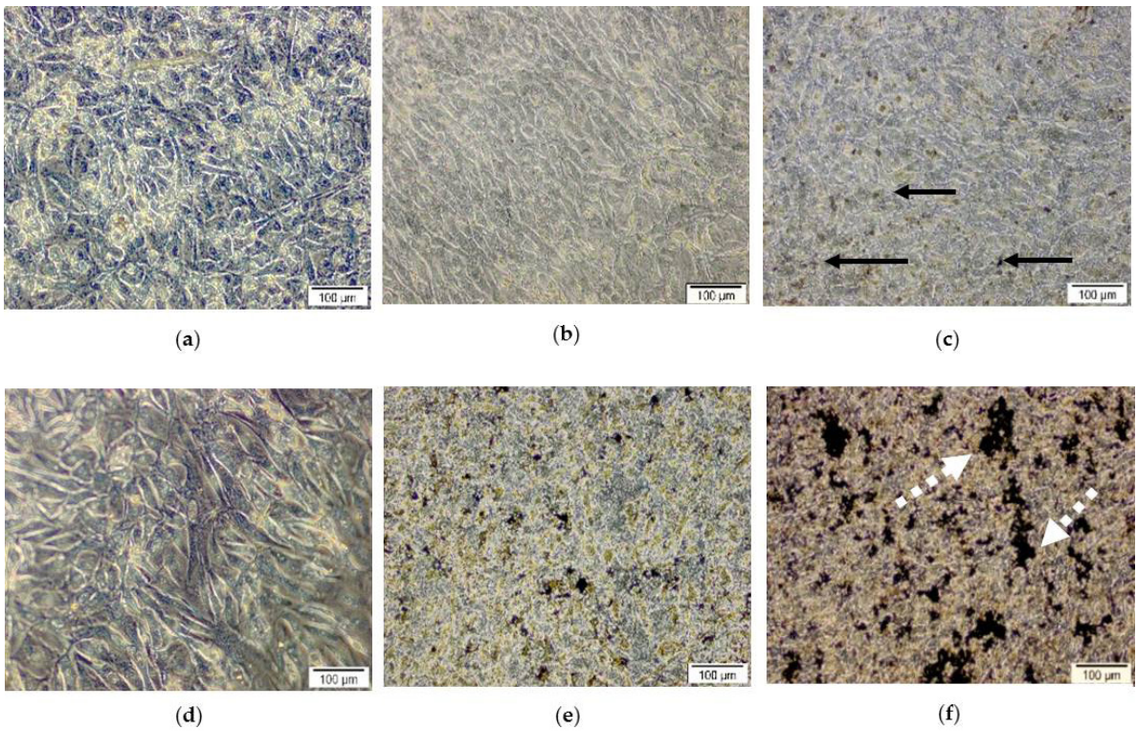
FIGURE 3. ALP activity of MC3T3-E1 cells in two different Asc concentrations. Standard = 50 µg/mL Asc; doubled = 100 µg/mL Asc. Values are expressed in mean ± SD. n= 3 (* = p <0.05)

TABLE 3. Comparison of changes in ALP activity between two Asc concentrations in 2D culture. Change in ALP activity was measured relative to day 0 of each group. Values were analysed with one-way repeated measures ANOVA and expressed in mean difference ± standard error. n = 3 (* = $p < 0.01$ for intragroup differences; # = $p < 0.01$ for intergroup differences)

Day, d	Change in ALP activity relative to day 0 (ng/mL)			
	Standard ascorbic acid concentration		Doubled ascorbic acid concentration	
	Mean difference ± S.E, Δx (ng/mL)	Fold change, $\Delta x/x_0$	Mean difference ± S.E, Δx (ng/mL)	Fold change, $\Delta x/x_0$
3	1.038 ± 0.118	2.3× *	0.894 ± 0.382	1.6×
6	1.609 ± 0.109	3.5× *	1.914 ± 0.307	3.5× *
9	2.141 ± 0.232	4.6× *	5.155 ± 0.423	9.3× *, #
12	3.565 ± 0.209	7.7× *	6.655 ± 0.348	12.0× *, #
15	4.784 ± 0.152	10.4× *	11.879 ± 0.541	21.4× *, #
18	4.963 ± 0.175	10.7× *	9.861 ± 0.204	17.8× *, #
21	4.969 ± 0.344	10.8× *	8.519 ± 0.396	15.4× *, #

CELL MINERALIZATION

Mineralised cells can be differentiated from non-mineralised cells via von Kossa staining. A non-stained cell is undifferentiated, a lightly brown stained cell is semi-differentiated, and a dark brown stained cell is fully mineralised or mature. Cells in this study were unmineralized by day 12 in both groups (Figure 4). Mineralisation was evident on day 15 in the doubled



(**a**) (**b**) (**c**)

(**d**) (**e**) (**f**)

FIGURE 4. Cell mineralisation assessment by von Kossa staining. Magnification ×10. (a-c) MC3T3-E1 cell cultured in standard group (50 µg/mL Asc); (d-f) MC3T3-E1 cell cultured in doubled group (100 µg/mL Asc). Cells were confluent in both groups by day 12 (a, d). Obvious mineralisation began on day 15 in the doubled group with multiple dark brown deposits (white block arrows) interspersed between light brown stains (white line arrows) (e). No brown stains were noted in standard group (b). By day 21, more mineralisation had occurred in the doubled group with large surface area covered by dark brown deposits (white broken arrows) (f). Comparatively, only very faint light brown stains were present in standard group (black arrows) (c)

group. The surface area of darkly stained cells increased on day 21, which indicated continuous and increased mineralisation in the doubled group. In contrast, no dark brown stains were appreciated in the standard group on any observation day, proving that mineralisation did not occur during the experiment period. Light brown stained cells seen on day 21 represented semi-differentiated cells.

CELL CHARACTERISATION IN THREE-DIMENSIONAL MODEL CELL MORPHOLOGY

Following 7 days of culture, cells were able to anchor to the hydroxyapatite scaffold and propagate between granules. They appeared stretched with filamentous pseudopodia (Figure 5(a), 5(b)). There was no difference in cell morphology or cell-scaffold adhesion noted between the standard and doubled groups. Thick accumulation of extracellular matrix was seen in the test group on day 21, with white opaque particles appearing on top of the matrix (Figure 5(c)). In contrast, no such particles were seen on the cell in the control group. The extracellular matrix was also thinner as the scaffold granule beneath the cell were still visible (Figure 5(d)).

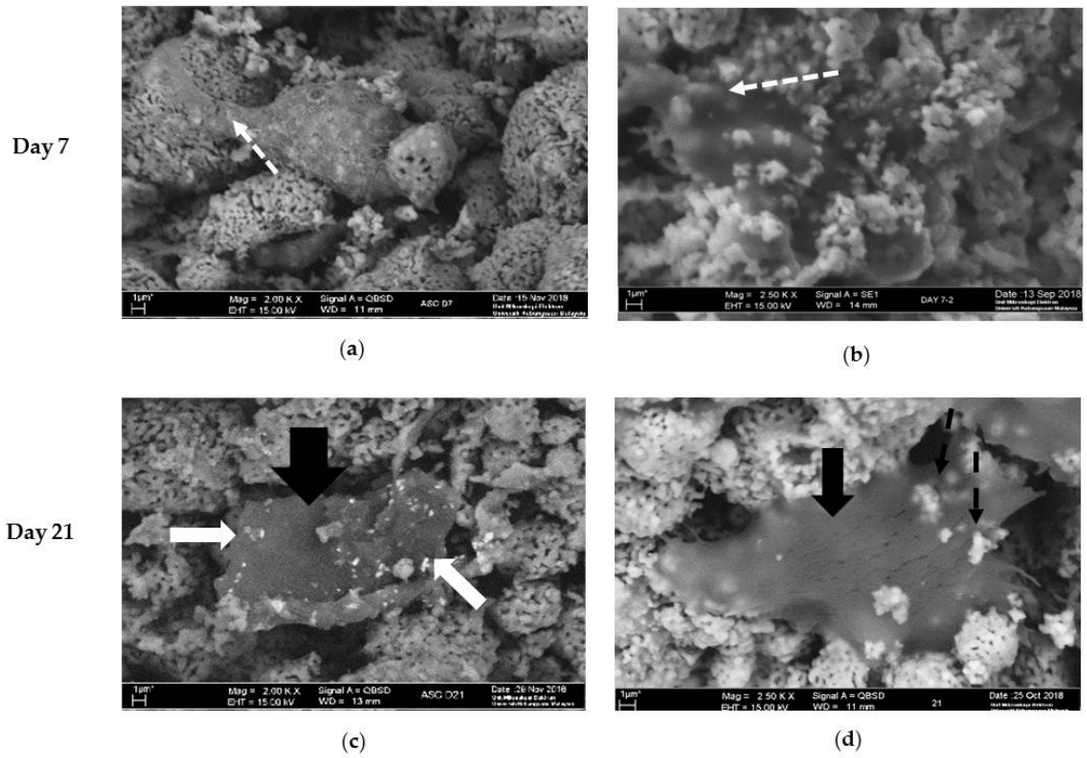


FIGURE 5. Cell morphology viewed under scanning electron microscopy. (a,c) Cells cultured in 100 µg/mL Asc; (b,d) Cells cultured in 50 µg/mL Asc. Cells were spindle-shaped with pseudopodia (white arrows, broken line) extending between granules on day 7. Mineralized particles (white arrows) were seen in the doubled group on day 21, with thick accumulation of extracellular matrix (black arrow, thick) (c). No mineralized particles were found in the standard group; and the extracellular matrix appeared thin (black arrow, thin) as scaffold material beneath the cell was still visible (black arrows, broken line) (d). Magnification ×2000 – ×3000

CELL VIABILITY

The number of viable cells showed an overall increase from day 0 in both groups, which indicated that the scaffold was non-toxic for MC3T3-E1 cells (Figure 6). In the doubled group, maximum cell number increase was reached on day 14 after which it plateaued as evident from the fold change (Table 4). In contrast, cell number continued to rise to day 21 in the standard group. Despite the intragroup differences, increased ascorbic acid concentration significantly increased number of viable cells on all days of experiment at $p < 0.05$ and $p < 0.01$.

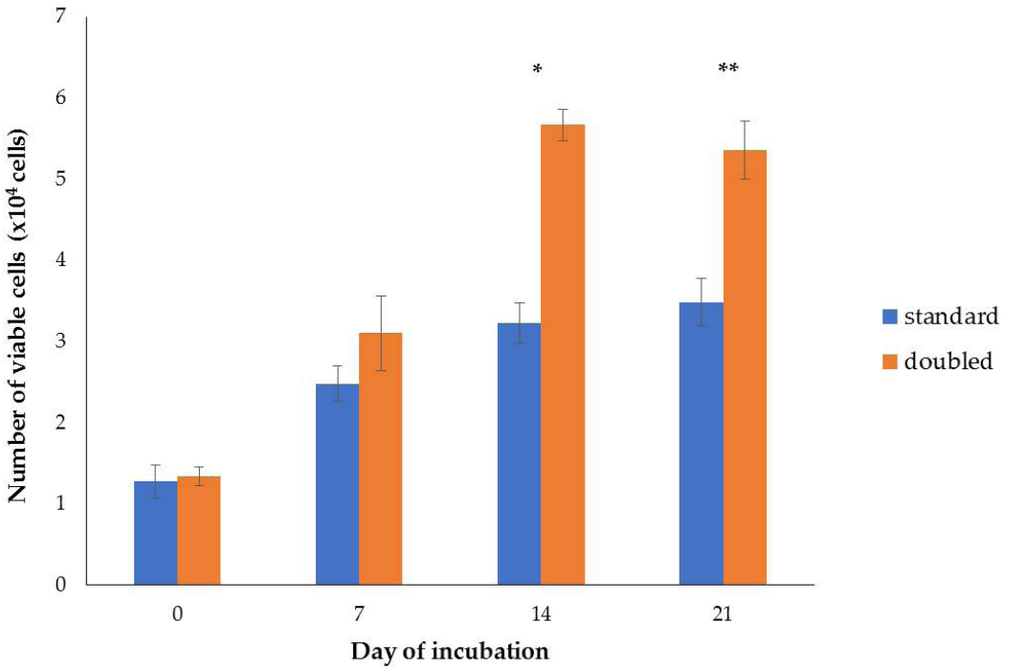


FIGURE 6. Comparison of number of viable cells between treatment groups in three-dimensional cultures. Standard = 50 µg/mL Asc; doubled = 100 µg/mL Asc. Values expressed in mean ± SD. n = 3. (* = $p < 0.01$; ** = $p < 0.001$)

TABLE 4. Comparison of cell proliferation between two Asc concentrations in 3D culture. Cell proliferation was measured from the change in number of viable cells relative to day 0 of each group. Values were analysed with one-way repeated measures ANOVA and expressed in mean difference ± standard error. n = 3 (* = $p < 0.01$ for intragroup differences; $ = $p < 0.05$ for intergroup differences; # = $p < 0.01$ for intergroup differences)

Day, d	Change in number of viable cells relative to day 0, ΔN (× 10^4 cells)			
	Standard ascorbic acid concentration		Doubled ascorbic acid concentration	
	Mean difference ± S.E, Δn (× 10^4 cells)	Fold change, $\Delta n/n_0$	Mean difference ± S.E, Δn (× 10^4 cells)	Fold change, $\Delta n/n_0$
7	1.208 ± 0.092	0.95× *	1.762 ± 0.196	1.31× *, $
14	1.956 ± 0.614	1.54×	4.328 ± 0.172	3.23× *, #
21	2.213 ± 0.127	1.74× *	4.013 + 0.160	2.99× *, #

CELL DIFFERENTIATION

The ALP activity of MC3T3-E1 cells in three-dimensional model was shown in Figure 7. Detection of ALP enzyme level was carried out from day 7 onwards as the results from 2D analysis showed unremarkable difference between groups prior to day 6. An upward trend was noted in both groups from day 7 to day 21. However, ALP activity was raised to three times the initial value when 100 µg/mL Asc was used in comparison to only 1.4 times in 50 µg/mL Asc (Table 5).

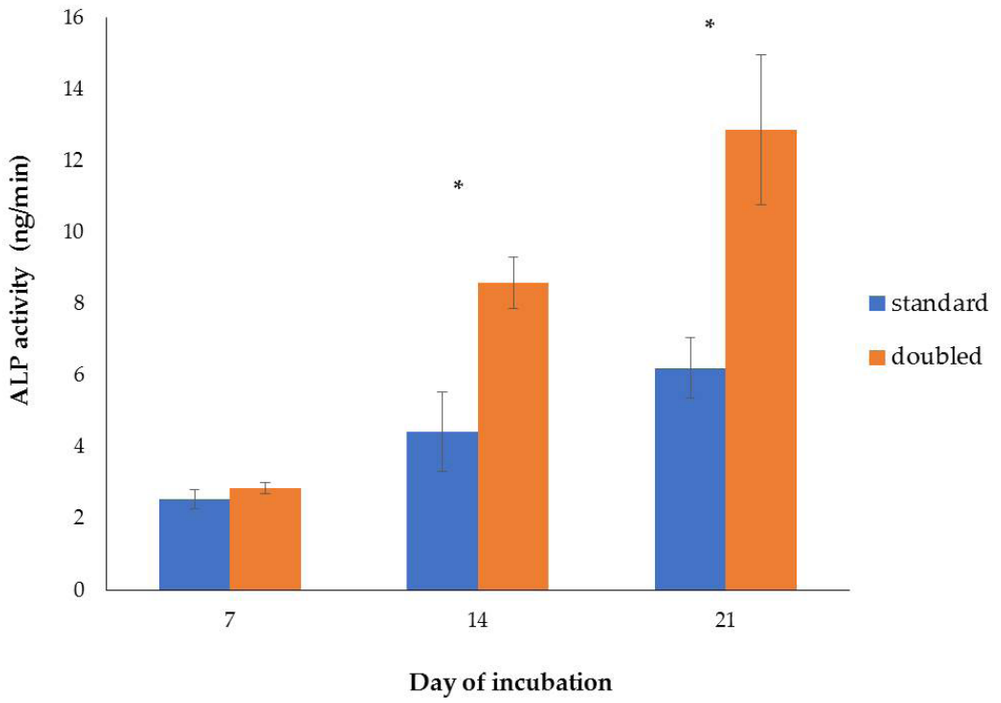


FIGURE 7. Comparison of ALP activity between groups in three-dimensional cultures Standard = 50 µg/mL Asc; doubled = 100 µg/mL Asc. Values are expressed in mean ± SD. n = 3 (*= $p < 0.001$)

TABLE 5. Comparison of changes in ALP activity between two Asc concentrations in 3D culture. Change in ALP activity was measured relative to day 7 of each group. Values were analysed with one-way repeated measures ANOVA and expressed in mean difference ± standard error. n = 3 (* = $p < 0.01$ for intragroup differences; # = $p < 0.01$ for intergroup differences)

Day, d	Change in ALP activity relative to day 7 (ng/mL)			
	Standard ascorbic acid concentration		Doubled ascorbic acid concentration	
	Mean difference ± S.E, Δx (ng/mL)	Fold change, $\Delta x/x_7$	Mean difference ± S.E, Δx (ng/mL)	Fold change, $\Delta x/x_7$
14	1.889 ± 0.447	0.74× *	5.739 ± 0.398	2.0× *,#
21	3.669 ± 0.445	1.44× *	10.008 ± 0.777	3.5× *,#

CELL MINERALISATION

Due to the calcium content in the hydroxyapatite scaffold used in this study, von Kossa staining would not be able to differentiate between mineralised cells and scaffold mineral. Gene expression analysis was used instead to confirm the process of MC3T3-E1 cell mineralisation. Type 1 alpha collagen (Col1α) and osteocalcin (OCN) gene expressions were illustrated in Figure 8. Both gene expressions were upregulated from day 0 to day 14, with the results significantly higher at double Asc concentration ($p < 0.05$). The expression of Col1α gene was upregulated by approximately 20 folds in the doubled group from 1.00 ± 0.07 to 19.38 ± 1.95. In contrast, Col1α in the standard group was only upregulated by approximately two folds from 1.00 (0.03) to 1.98 (0.30). The OCN gene was upregulated by six folds in the doubled group and only two folds in the standard group.

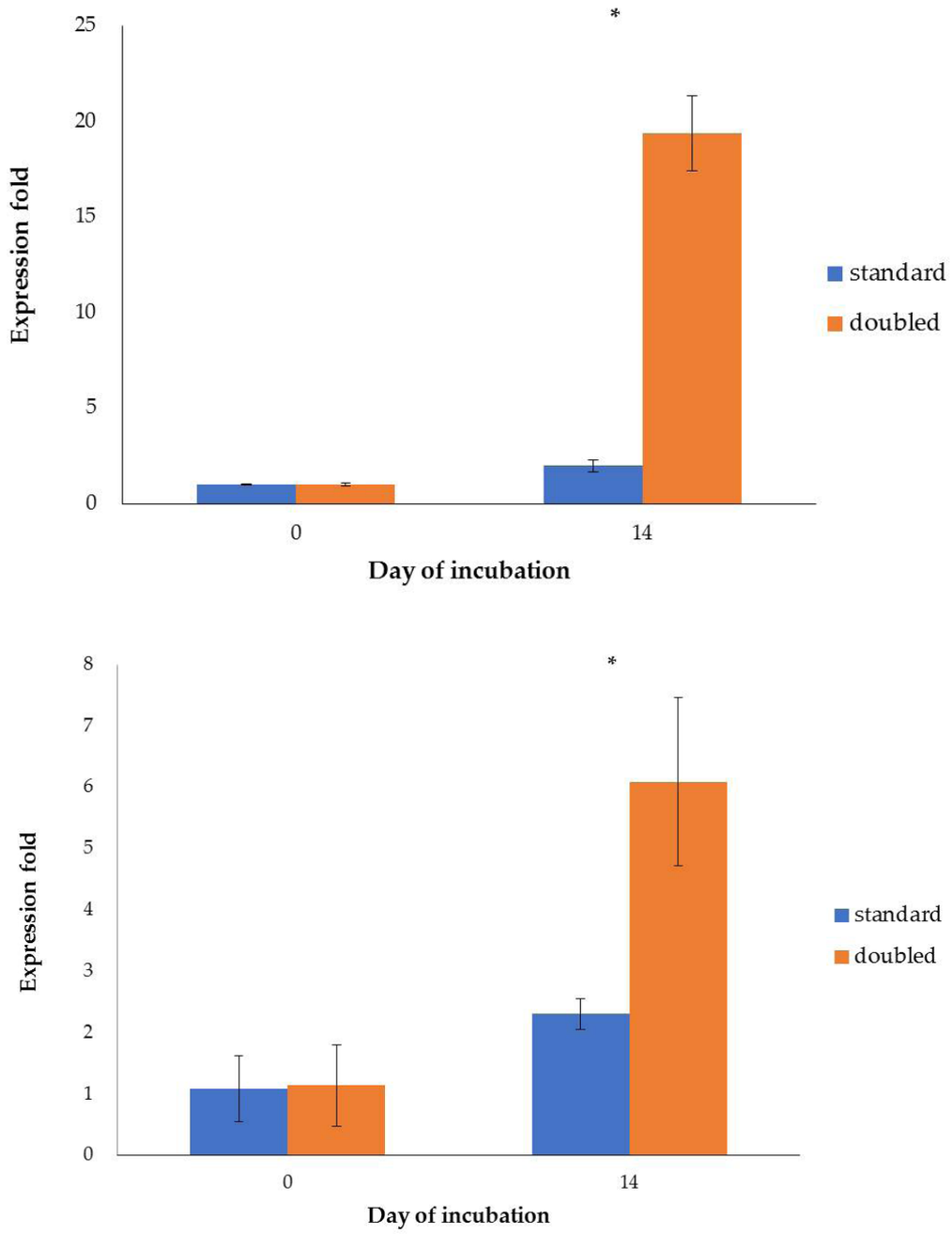


FIGURE 8. Comparison of relative expression of (a) Col1α and (b) OCN genes between groups. Standard = 50 µg/mL Asc; doubled = 100 µg/mL Asc. Values are expressed as mean ± SD. n=3 (*= $p < 0.05$)

DISCUSSION

The aim of this study was to determine the effect of increased Asc concentration on mineralisation potential of MC3T3-E1 cells when β-GP is absent. The total amount of Asc experimented were 50 µg/mL and 100 µg/mL to represent the recommended and doubled concentrations in a standard osteogenic medium, respectively. Maximum collagen production

and osteoblast differentiation had been shown with Asc concentration of 50 µg/mL; however, mineralised nodules were minimally detected (Franceschi & Iyer 1992). Addition of a further 50 µg/mL Asc fell within the optimal range in which Hadzir et al. (2014) found that human suspension mononuclear cells were able to mineralise in the absence of β-GP. Admittedly, further studies are needed to determine the optimal Asc concentration to obtain mineralisation for MC3T3-E1 cells. Inclusion of a positive control group containing β-GP at this point of study would be premature as quantitative and qualitative comparisons could not be made against an optimal Asc concentration. On the flip side, a negative group without Asc was also deemed unnecessary as an Asc-deficient MC3T3-E1 cell system is not able to convert procollagen to mature collagen or to stimulate mineralisation (Franceschi et al. 1994).

The sequential change of cell morphology as observed from initial seeding to post-confluent stage was similar in both groups and in accordance to the findings of previous studies (Hong et al. 2010; Sudo et al. 1983; Wan et al. 2018). However, we were not able to detect a mosaic appearance during confluency as reported by Sudo et al. (1983). This difference may be due to dissimilarity in defining the stage of confluence. In present study, cells were labelled as confluent when they covered 80% of the bottom of culture plates (Yan et al. 2014). Nevertheless, the mosaic appearance was observed as cells multiplied and overlapped each other. Also, from the initial seeding, the doubled concentration group took longer to attain 80% confluence (9 Vs 6 days in standard group). This could be due to the earlier differentiation induction in the presence of more concentrated Asc. The earlier initiation of differentiation-inducing transcription factors may have altered the expression of cell cycle regulators (Ruijtenberg et al. 2016).

Cell viability refers to the ratio of live and dead cells in a population, which should parallel cell proliferation in a non-cytotoxic culture system. Cells in both groups experienced slower albeit still-positive growth rate immediately after confluence. This could be explained by reduced cell metabolism as a result of contact inhibition and the low proliferative capacity of differentiated cells (Gerard & Goldbeter 2014; Ruijtenberg et al. 2016). At all time-points, cell viability was higher in the doubled group than in standard group. Results from light microscopy and MTT assay reaffirmed the role of Asc to stimulate cell proliferation and proved that the 100 µg/mL concentration was not cytotoxic in this cell system. It was shown in the literature that collagen participates in promoting cell viability (Fujisawa et al. 2018). Asc is known to be an important cofactor of the hydroxylase that participates in the post-translational modification of the collagen molecules and thus increases the availability of functional collagen (Chan et al. 1990). The occurrence of mitochondrial activation is also crucial to maintain cell viability. In the osteogenic cells, Asc increases HIF1α hydroxylase activity, suppresses the HIF1a transcription reaction, leading to mitochondrial activation (Fujisawa et al. 2018).

Alkaline phosphatase enzyme (ALP) is an established early marker of osteoblast differentiation (Farinawati et al. 2018; Hadzir et al. 2014). The regulatory function of Asc on osteoblast differentiation can be seen from the detection of low-level ALP activity during early proliferative stage, to a significantly increased level after confluence. There was a peak level on day 15 in the doubled group, after which the ALP decreased. This corresponded with the period of bone matrix maturation as described by Choi et al. (1996). As basal level of ALP secretion increases, the enzyme utilizes phosphate as a substrate and releases free inorganic phosphates which induces mineralisation (Farinawati et al. 2018; Hadzir et al. 2014). Hence, mineralized cells could be visualized with von Kossa staining from day 15 in the doubled group. No mineralisation was seen in the standard group up to day 21. This is because mineralisation begins only after maturation of extracellular matrix (Quarles et al. 1992). Failure of the extracellular matrix to mature will result in failure of mineralisation despite presence of viable osteoblasts and endogenous inorganic phosphate source (Marsh et al. 2009). The pooled findings from ALP assay and von Kossa staining indicated that 50 µg/mL Asc can induce osteoblast differentiation and bone matrix formation; but an increased concentration is necessary for bone matrix maturation. Our finding also proved that mineralisation can occur in the absence of β-GP; a result which corroborated with another study (Hadzir et al. 2014). In recent years, it is known that enzymes belonging to the 2-Oxoglutarate/Fe^{2+}-dependent dioxygenases (2-OGDD) family are involved in collagen maturation (Kuiper & Vissers 2014). Increased availability of Asc, which is a specific cofactor for this group of enzymes, may help in the enhancement of collagen matrix maturation. Col1α was expressed in both Asc concentrations in the 3-dimensional culture medium. This was expected as ascorbic acid functions as a cofactor for collagen production. However, the expression was significantly higher in the doubled concentration as collagen

production increased (Harada et al. 1991; Wahab et al. 2020; Yazid et al. 2019). As a later marker for bone formation, osteocalcin (OCN) level was raised on day 16 in the literatures of two-dimensional cultures (Choi et al. 1996; Quarles et al. 1992). OCN level in the 3-dimensional culture of this study was detected in the test group on day 14. The timing correlated with the findings from other studies (Motamedian et al. 2016; Wahab et el. 2020; Wan et al. 2018). OCN was also detected in the control group on day 14; albeit at a low level. This was similarly reported by a group who assessed dental pulp stem cells behaviour on three different types of scaffolds (Motamedian et al. 2016). Nevertheless, mineralised nodules were only seen in the test group on day 21. We reasoned that at low level of ascorbic acid concentration, the expression of OCN was insignificant to result in clinical evidence of mineralisation.

In our study, the number of viable cells in 3D cultures was much lower compared to 2D cultures. This result echoed studies which used an identical scaffold or other granulated biomaterials (Motamedian et al. 2016; Nawi et al. 2013). While all efforts have been made to prevent seeding of cells onto bottom of well plates instead of scaffold material, there was inadvertent loss of cell population during the multiple transfers of scaffold during analysis. Studies which utilized scaffold in tablet formulation would report higher cell proliferation and/or viability in 3D models as the surface area of the tablet for initial cell adhesion is larger (Matthews et al. 2014; Shamsuddin et al. 2017). Nonetheless, the comparison of cell behaviour between two different Asc concentrations were similar to that of two-dimensional model. Cell morphology, viability, differentiation and mineralisation potential were better in 100 µg/mL compared to 50 µg/mL concentration.

The main limitation of this study is the use of MC3T3-E1 cells which are different in terms of cell type and species from human primary cells. However, this cell type was selected as it is a well-established and well-characterised osteogenic model and possible confounding factors related to host differences could be avoided. Secondly, the concentration of Asc used in this study was only limited to 50 and 100 µg/mL.

Since Asc is a non-toxic and readily available substance, increased concentrations of Asc may be a promising inducing factor to be used to create a localized microevironment for the enhancement of the mineralisation potential of various tissue-engineered bone grafts, for example, alveolar bone grafts in dentistry. Futher *in-vitro* and *in-vivo* bone defect studies could be conducted to determine the minimal and maximum concentrations of Asc that can be added into an osteogenic medium without adverse effects.

Conclusions

In conclusion, this study established that higher concentration of Asc at 100 µg/mL in the absence of β-GP can induce complete bone formation. The potential of more concentrated Asc to be used as the sole induction factor in an osteogenic medium was proven.

Acknowledgements

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