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Effect of essential and nonessential amino acid compositions on the *in vitro* behavior of human mesenchymal stem cells

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Abstract−Mesenchymal stem cells (MSCs) from bone marrow appear to be an attractive tool for use in tissue engineering and cell-based therapies due to their multipotent capacity. The majority of studies on MSCs have been restricted to the roles of growth factors, cytokines, and hormones. Based on previous reports demonstrating the important roles of amino acids, we sought to evaluate the effect of essential amino acids (EAs) and nonessential amino acids (NEAs) on the proliferation and differentiation of MSCs. The results showed that the EA/NEA compositions during culture could significantly modulate MSC proliferation and differentiation and, especially, that EAs served as a potent positive modulator in the proliferation of MSCs without causing a deficit in the differentiation capacity of the cells. These results will be very useful in the production of MSC-based cell therapy products for use in the field of tissue engineering and regenerative medicine.

Key words: Amino Acid, Proliferation, Differentiation, Mesenchymal Stem Cell, Bone Marrow

INTRODUCTION

Mesenchymal stem cells (MSCs) can be derived from specific organs, such as the gut, lung, liver, and bone marrow. MSCs isolated from bone marrow have been shown to have multilineage potential and have been used experimentally in cell-based therapies. MSCs are capable of giving rise to multiple mesenchymal cell lineages, such as fibroblasts, osteoblasts, chondrocytes, and adipocytes, under specific culture conditions. In contrast to other adult cells, such as ligament cells, chondrocytes, and osteoblasts, MSCs are not rejected and can be easily obtained after bone marrow aspiration and subsequent *in vitro* expansion. However, continued culture of MSCs for tissue engineering applications requires proper stimulation to prevent premature cell aging, spreading and inactivity with increasing passage number [1-8]. To support and enhance the *in vitro* growth and activity of MSCs, the cell culture medium may be supplemented with various proteins and factors to mimic the physiologic environment in which cells optimally proliferate and differentiate [9-13].

The metabolism of cells in an organized environment is closely related to the intercellular metabolic interaction between different kinds of cells. However, when cells are isolated from their original tissues and cultured in a culture dish, their nutritional requirements should, as a matter of course, change and vary among each individual cell [14-17]. In fact, the stimulatory effect of various nutrients, especially amino acids, upon the growth rate of cells *in vitro* has been extensively investigated. Tyihak and Szende found that D-lysine exerted a tumor-promoting effect on cells, while D-aspartic acid, L-glutamic acid, D-arginine and L-lysine inhibited tumor growth [18,19]. Eagle observed the requirement of glycine for the growth of monkey kidney cells in the primary culture [20]. McCoy reported that the addition of serine and especially glycine promoted cell growth. However, cysteine, glycine, and serine did not influence the growth rate of human tumor cells at any of the concentrations tested [21].

In the case of mesenteric ischemia, the addition of glycine, a nonessential amino acid, induced the downregulation of pro-apoptotic bax and caspase-3, whereas anti-apoptotic bcl-2 was upregulated in the glycine-treated animals [22]. Tanaka et al., found that L-serine promoted neuronal survival and that L-serine and glycine upregulated the expression of the anti-apoptotic gene product Bcl-w, while reducing apoptosis [23]. It was reported elsewhere that various concentration of vitamin supplementations of hybridoma cells culture medium induced the down-regulation of bal-2 expression and reduced the rates of apoptosis [24]. Extensive studies on hamsters have also demonstrated that the inclusion of certain amino acids (asparagine, aspartate, glycine, histidine, serine, and taurine) in the culture medium stimulates the development of 1-cell embryos into the morula/blastocyst *in vitro*. In contrast, other amino acids (cysteine, isoleucine, leucine, phenylalanine, threonine, and valine) are known to strongly inhibit development [25,26].

Recently, amino acids exogenously supplemented were shown to affect mammalian embryonic development, and their beneficial effects have been examined in embryos from mice, hamsters, and cattle [27-29].

However, most studies on MSCs have been restricted to growth factors, cytokines, and hormones. Based on previous reports dem-

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onstrating the important roles of amino acids, we hypothesized that essential amino acids (EAs)/nonessential amino acids (NEAs) have some effects on the proliferation and differentiation of MSCs.

In this study, MSCs were cultured in six EA/NEA compositions based on two basal media--DMEM (basically containing EAs) and IMDM (basically containing EAs and NEAs)--in order to examine the effect of EAs/NEAs on the *in vitro* behavior of MSCs including proliferation, cytotoxicity, extracellular matrix (ECM) production, and differentiation.

MATERIALS AND METHODS

1. Primary Culture of Mesenchymal Stem Cells (MSC) from Bone Marrow

Mesenchymal stem cells (MSCs) from the bone marrow were isolated from human donors. Bone marrow aspirates were obtained from the iliac crest of healthy donors with the approval of the patients themselves and the Institutional Review Board of St. Mary's Hospital, Catholic University. Bone marrow aspirates were collected in a syringe containing 10,000 IU heparin in order to prevent coagulation. The mononuclear cell fraction was isolated by Ficoll (0.77 g/ml) density gradient centrifugation.

Mononuclear cells were plated into tissue culture flasks in expansion medium at a density of 10^5 cells/cm^2. The expansion medium consisted of DMEM (low glucose; Invitrogen Co.) and 10% fetal bovine serum (FBS; Cambrex Co.).

Upon reaching 80% confluency, the cells were trypsinized with 0.25% trypsin/1 mM EDTA (Sigma) and replated at a density of about 9,000 cells/cm^2. The cells were expanded for 2 to 6 passages. The MSCs (at the 5^{th} passage) were seeded at a density of 1×10^4 cells/well and divided into six groups: (A) DMEM, (B) DMEM+EAs, (C) IMDM, (D) IMDM+NEAs, (E) IMDM+EAs, (F) IMDM+EAs+NEAs. All media were supplemented with 10% FBS. The MSCs were then cultured for seven days. Essentially, DMEM contains EAs, and IMDM (Welgene Inc.) contains both EAs (equal molar concentration in DMEM) and NEAs as described in the manufacturer's data sheet. Each component of additional MEM EAs and NEAs (Sigma Co.) is summarized in Table 1.

Table 1. Each component of the additional MEM EAs and NEAs

	Essential amino acid (mM)	Nonessential amino acid (mM)
L-Alanine	-	0.1
L-Arginine	0.6	-
L-Asparagine·H_2O	-	0.1
L-Aspartic acid	-	0.1
L-Cystine	0.1	-
L-Glutamic acid	-	0.1
Glycine	-	0.1
L-Histidine	0.2	-
L-Isoleucine	0.4	-
L-Leucine	0.4	-
L-Lysine·HCl	0.4	-
L-Methionine	0.1	-
L-Phenylalanine	0.2	-
L-Proline	-	0.1
L-Serine	-	0.1
L-Threonine	0.4	-
L-Tryptophan	0.049	-
L-Tyrosine	0.2	-
L-Valine	0.4	-

2. Cell Proliferation Assay

In order to count the cells, single cell suspensions were obtained by incubating the cultures for 10 min at 37 °C with a 0.05% trypsin solution. Aliquots of the samples were mixed with trypan blue and the viable cells were counted by using a hemocytometer.

Population doubling level (PDL) was calculated by using the following equation,

$$PDL=\log(X_1/X_0)/\log 2$$

where X_0 is the initial cell number and X_1 is the final cell number.

3. Lactate Dehydrogenase (LDH) Assay

LDH activity was measured with an LDH-LQ kit (Asan Pharmaceutical Inc.). Briefly, after seven days of culture, aliquots of medium and working solution were mixed and incubated in darkness at room temperature for 30 min. The reaction was terminated by adding stop solution (1 N HCl), and the absorbance was measured at 570 nm.

4. Intracellular Collagen and Sulfated Glycosaminoglycans (GAG) Analysis

Total intracellular soluble collagen was measured by using a Sircol™ Collagen Assay Kit (Bioassay Inc.). Briefly, collagen samples were prepared from MSC cultures in various amino acid compositions. Following sample preparation, they were mixed with Sircol Dye reagent. After centrifugation (10,000 g for 10 min), the mixtures were dissolved in alkali reagent and the absorbance was measured at 540 nm.

The total intracellular sulfated GAG content was measured with a Blyscan™ Sulfated Glycosaminoglycans Assay Kit (Bioassay Inc.). GAG samples were prepared from MSC cultures in various amino acid compositions. The samples were mixed with Blyscan Dye Reagent and incubated for 30 min. After centrifugation (10,000 g for 10 min), visual inspection should reveal a dark purple residue within the test sample tubes. The samples were dissolved in dissociation reagent and the absorbance was measured at 656 nm.

5. Cell Surface Antigens Analysis by Fluorescence-activated Cell Sorter (FACS)

Antibodies against human antigens CD73 and CD90 were purchased from BD Sciences (San Jose, CA, USA). An antibody against CD105 was purchased from Ancell (Bayport, MN, USA). A total of 5×10^5 cells were resuspended in 200 μl PBS and incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies for 20 min at room temperature (or for 45 min at 4 °C). The fluorescence intensity of the cells was evaluated by a flow cytometer (FACScan; BD Sciences Inc.) and the data were analyzed using the CELLQUEST software (BD Sciences).

6. *In Vitro* Osteogenic Differentiation of MSCs

After reaching confluence, the media were changed to osteogenic medium and the MSCs were maintained for two weeks.

The osteogenic medium consisted of DMEM containing 10% FBS, 10 mM β-glycerophosphate (Sigma Co.), 50 μM L-ascorbate 2-phosphate (Sigma Co.) and 10^{-7} M dexamethasone (Sigma Co.)

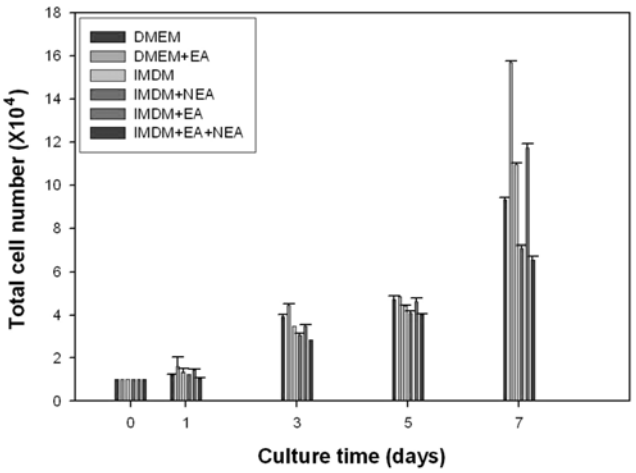

Fig. 1. Effect of essential and nonessential amino acid composition on the growth of MSCs according to culture time (days).

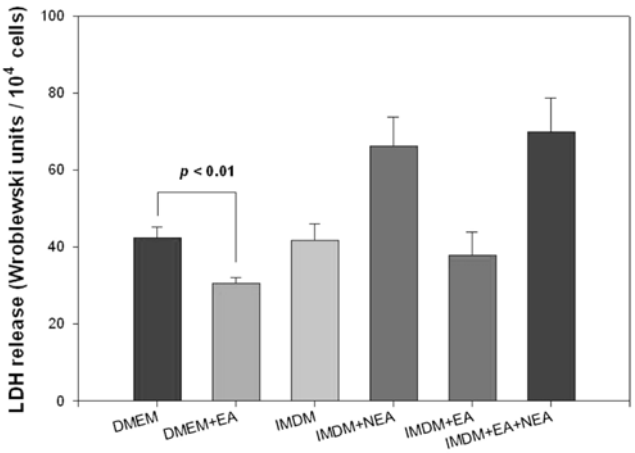

Fig. 2. Effect of essential and nonessential amino acid composition on the lactate dehydrogenase (LDH) release rate of MSCs (P5).

and was exchanged every three to four days. After 14 days, von Kossa staining was used to analyze the MSCs.

7. Histochemical Analysis

The degree of osteogenic differentiation was evaluated by von Kossa staining to detect any deposition of minerals, including calcium. For von Kossa staining, the cells were fixed with 10% formalin for 30 min and washed three times with 10 mM Tris·HCl, pH 7.2. The fixed cells were incubated with 5% silver nitrate for 5 min in daylight, washed twice with H_2O_2, and then treated with 5% sodium thiosulfate.

RESULTS AND DISCUSSION

1. Effect of EA/NEA Compositions on MSC Proliferation

To examine the effects of the essential/nonessential amino acid compositions on the proliferation of MSCs, the number of cells in each culture was counted on the 1st, 3rd, and 7th day after seeding. The initial seeding cell number was the same (1×10^4 cells) in each group. After 7 days of culture, the cell number was determined to be 9.32×10^4 cells in the A group, 15.72×10^4 cells in the B group, 10.95×10^4 cells in the C group, 7.09×10^4 cells in the D group, 11.72×10^4 cells in the E group, and 6.55×10^4 cells in the F group. In this case, the PDL values were 6.54 (A), 7.30 (B), 6.77 (C), 6.15 (D), 6.87 (E), and 6.03 (F), respectively. This result showed that the over-addition of EAs (B, E groups) and the appropriate level of NEAs (C group) enhanced the proliferation of MSCs, while the over-addition of NEAs significantly reduced the proliferation of MSCs. Although the over-addition of EAs could improve cell proliferation, it could not recover the reduction that resulted from the over-addition of NEAs (F group). The B group, in particular, showed significant improvement ($p<0.01$) in the proliferation of the MSCs in comparison to the A group, which was a conventional MSC culture condition.

2. Effect of EA/NEA Compositions on LDH Release from MSC Culture

LDH is a cytoplasmic catalytic enzyme related to the reversible conversion between pyruvic acid and latic acid. LDH is released through the cell membrane when a cell is damaged. Therefore, less LDH release means less cellular damage. The media were collected and analyzed after seven days in order to examine cellular damage according to EA/NEA compositions.

As shown in Fig. 2, the B group showed the lowest levels of LDH. The ascending order of LDH units was the same as the descending order of proliferation. Thus, it is thought that the increased proliferation resulted from less cellular damage. This result showed that the appropriate level of NEAs (D, E groups) had no effect on proliferation, that the over-addition of EAs (B group) reduced proliferation, and that the over-addition of NEAs significantly increased the LDH release. Although the over-addition of EAs could reduce LDH release, it could not recover the damage caused by the over-addition of NEAs (F group), which was consistent with the observed effects on cell proliferation. The B group showed significantly ($p<0.01$) reduced levels of LDH release in comparison to the A group, which is a conventional MSC culture condition.

3. Effect of EA/NEA Compositions on Collagen and GAG Production

Collagen and GAG are the main components of the extracellular matrix (ECM) involved in both cell proliferation and differentiation. The intracellular collagen and GAG content was analyzed after seven days of culture (Fig. 3). The collagen content of the groups, in ascending order, was B, E, C, A, D, F and the GAG content of the groups was B, E, D, C, A, F. The lack of significant difference among the A, C, and D groups indicates that these two patterns were similar.

These results showed that the over-addition of EAs significantly reduced collagen and GAG production, but that the NEAs did not affect that production. The production of collagen and GAG in the B group was significantly reduced ($p<0.01$) in comparison to the A group, which is the conventional MSC culture condition.

4. Effect of EA/NEA Compositions on MSC Surface Antigen Expression

To determine whether or not EA/NEA compositions alter MSC surface antigen expression, FACS analysis was performed for CD73, CD90, and CD105, which are some of the MSC markers. The MSCs in the A group were cultured by using a conventional culture condition and they served as the control group. There was no difference

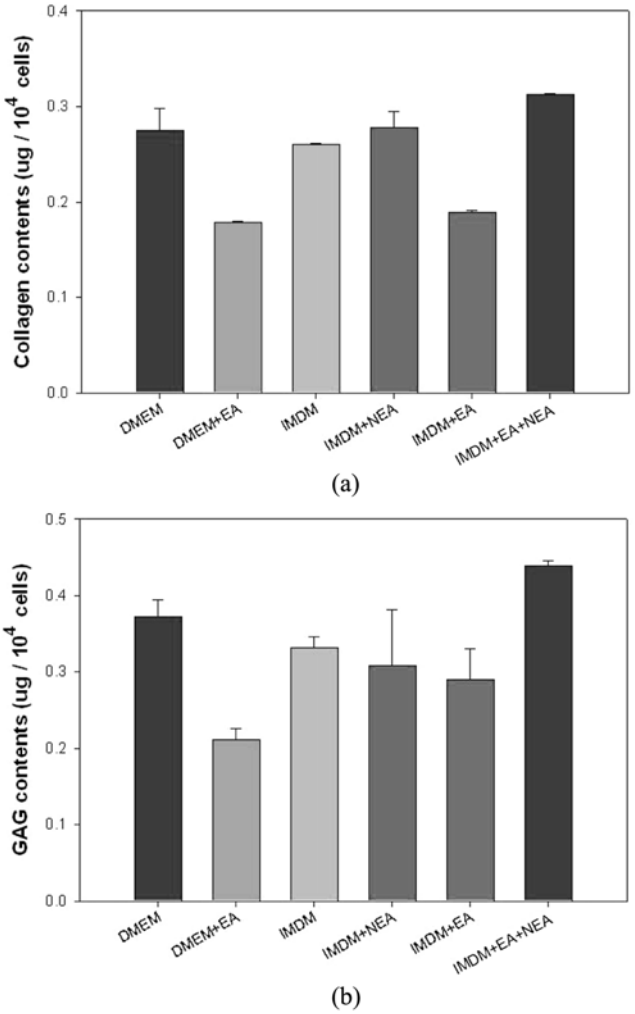

Fig. 3. Intracellular collagen and GAG analysis of MSCs (P5) cultured on various amino acid compositions for seven days; (a) Total collagen contents, (b) Total GAG contents.

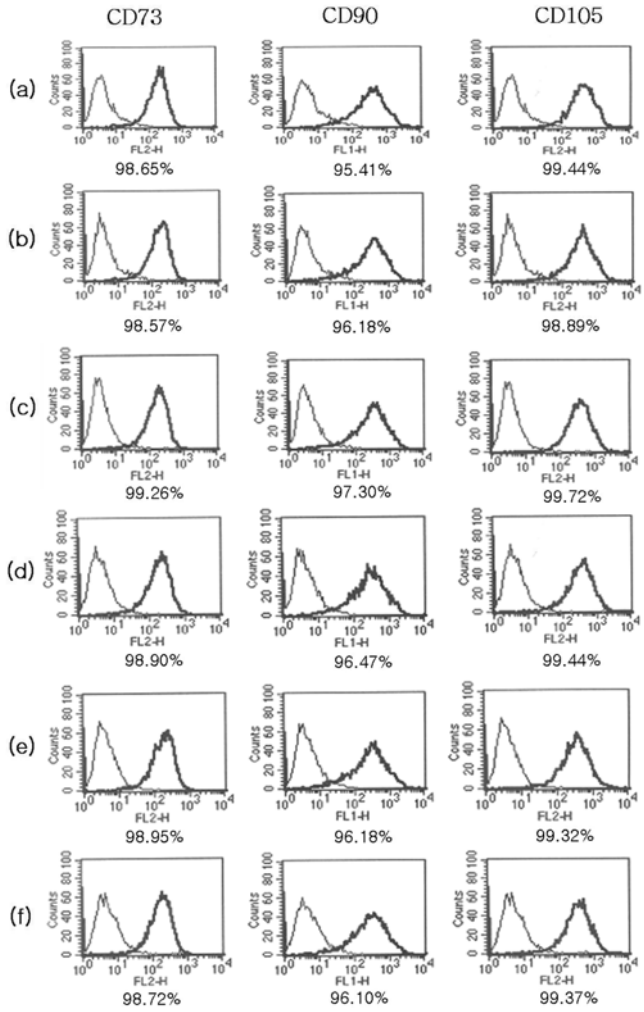

Fig. 4. FACS analysis of the surface marker after being cultured on various amino acid compositions. MSCs (P5) were labeled with FITC- or PE-conjugated antibodies and then analyzed in a flow cytometer: (a) DMEM, (b) DMEM+EA, (c) IMDM (d) IMDM+NEA, (e) IMDM+EA, (f) IMDM+EA+NEA.

in MSC surface antigen expression between the groups (Fig. 4), suggesting that the EA/NEA compositions did not induce the alteration of MSC surface antigen expression. The data from Fig. 4 is summarized in Table 2.

5. Effect of EA/NEA Compositions during Culture Following the Osteogenic Differentiation of MSCs

To confirm whether or not the EA/NEA compositions during culture alter the capacity for MSC differentiation, osteogenic differentiation was performed after culture. In all groups, calcium deposition was detected by von Kossa staining after two weeks of differentiation (Fig. 5). It generally takes MSCs (cultured in the A group, as control) four weeks to fully differentiate into osteoblasts under osteogenic conditions. However, osteogenic differentiation was performed for two weeks when calcium deposition began to occur in order to clarify the osteogenic difference among the MSCs (cultured in different EA/NEA compositions) under osteogenic conditions. As shown in Fig. 5, the MSCs cultured in the A, B, and D groups showed weak staining and those cultured in the C and E groups showed strong

Table 1. Immunophenotype of MSCs cultured in various amino acid compositions. CD73, CD90 and CD105 were highly expressed on cell surface and remained unchanged after culture

Specific marker of MSC	Media					
	DMEM	DMEM+EA	IMDM	IMDM+NEA	IMDM+EA	IMDM+EA+NEA
CD73	98.65%	98.57%	99.26%	98.90%	98.95%	98.72%
CD90	95.41%	96.18%	97.30%	96.47%	96.18%	96.10%
CD105	99.44%	98.89%	99.72%	99.44%	99.32%	99.37%

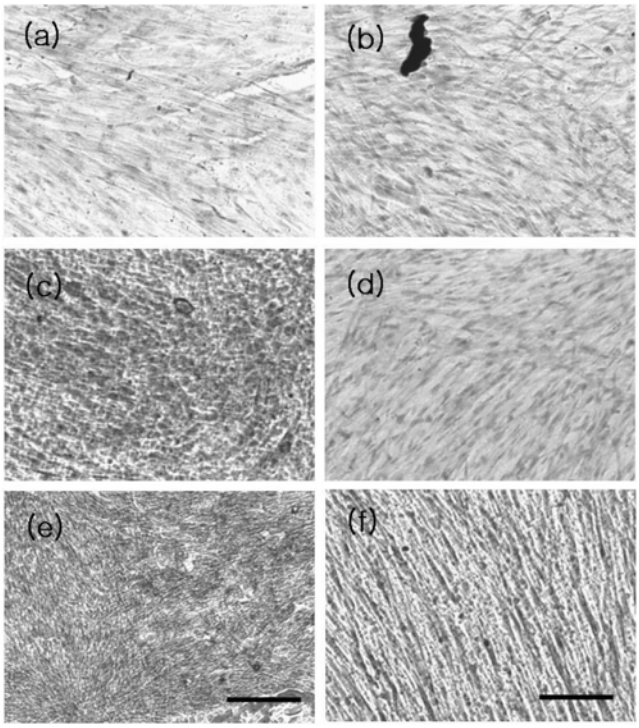

Fig. 5. Histological analysis of MSCs maintained in osteogenic medium for 14 days. MSCs were stained with von Kossa: (a) DMEM, (b) DMEM+EA, (c) IMDM, (d) IMDM+NEA, (e) IMDM+EA, (f) IMDM+EA+NEA media (bar 100 μm).

staining. The high calcium deposition shown in the MSCs in the F group resulted from necrotic disruption. The MSCs in the C group showed the highest calcium deposition. This result showed that the EA/NEA composition during culture affected the osteogenic capacity of the cells under the same differentiation condition.

6. Correlation among Cell Proliferation, LDH Release, ECM Production and Osteogenic Differentiation

The over-addition of EAs and the appropriate level of NEAs enhanced MSC proliferation, but the over-addition of NEAs significantly reduced MSC proliferation. This result is consistent with the finding that the release of LDH under the appropriate level of NEAs had no effect on the cells, that the over-addition of EAs induced, and that the over-addition of NEAs remarkably induced cellular damage.

The over-addition of EAs significantly reduced the production of ECM components; however, the NEAs had no effect on the production of collagen and GAG. Considering cell proliferation and LDH release, a slight range of ECM may be necessary for MSC proliferation. Therefore, we hypothesize that too much ECM probably induced differentiation/maturation through a high degree of cell-matrix interaction rather than via proliferation. Although some MSC surface antigens (CD73, CD90, CD105) showed similar expression levels regardless of the EA/NEA compositions, they are not exactly MSC-specific and do not represent enough MSC stemness or multipotency. Under the same osteogenic conditions, the degree of calcium deposition was different among the MSCs after they were cultured under different EA/NEA compositions. It is thought that the NEAs induced spontaneous osteogenic differentiation during culture. In addition to osteogenic capacity, other capacities, such as chondrogenesis and adipogenesis, remain unexplored. Therefore, further investigation is necessary to confirm the results of this study.

Strikingly, the B group (over-addition of EAs; DMEM+EAs) showed significant improvement in MSC proliferation and significant reduction in cellular damage without the reduction of osteogenic capacity in comparison to the A group (control).

CONCLUSIONS

This study reveals that the EA/NEA compositions during culture can modulate MSC proliferation and differentiation and, especially, that EAs serve as a potent positive modulator in the proliferation of MSCs without causing a loss in the differentiation capacity of the cells. However, it is not clear which molecular mechanisms are related to the response and which EA(s) induce the selective response. Thus, further investigation is necessary to obtain a more complete understanding of the molecular biological mechanism.

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