Osteoblast-like cell responses to ion products released from magnesium- and silicate-containing calcium carbonates

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Abstract

BACKGROUND: Inorganic ions released from bioceramics and bioactive glasses have been reported to influence osteogenic cell functions. Cell responses depend on types of the ions provided, for example, silicate ion has been found to up-regulate their proliferation, differentiation and mineralization.

OBJECTIVE: Mouse osteoblast-like cells (MC3T3-E1) were cultured in media containing silicate and calcium ions with/without magnesium ion to evaluate their combined effects on the cell's functions.

METHODS: The cells were cultured in the media containing the extract of silicate-containing vaterite (SiV) and magnesium- and siloxane-containing one (MgSiV) and normal medium and then their adhesion, proliferation, differentiation and mineralization were evaluated.

RESULTS: The adhesion of the cells was enhanced when they were cultured in the medium containing MgSiV-extract. Their proliferation and differentiation were up-regulated in both media containing MgSiV-extract and SiV-extract. In particular, the MgSiV-extract significantly enhanced their differentiation than the SiV-extract. This was supported by the mineralization test's results, which showed a large amount of mineral deposit was observed in the cells cultured in the MgSiV-extract medium.

CONCLUSIONS: Providing the three kinds of ions was effective for up-regulating the cell's

mineralization compared to providing silicate and calcium ions without magnesium ion.

1. Introduction

Ions released from bioactive glasses and ceramics have been reported to influence functions of cells, such as osteoblasts and mesenchymal stem cells, and regarded to play one of the important role in bioactivities of such materials *in vitro* and *in vivo* [1]. Up-regulation of cell activities by the ions have been explained in resent reports. In particular, the ionic dissolution products released from bioactive glasses and glass-ceramics have enjoyed an increasing interest in bioceramics research field [2-6]. A trace amount of silicate ions released from bioactive glasses have been found to enhance proliferation, differentiation and mineralization of osteogenic cells. Calcium ions (Ca^{2+}) are one of the component materials of bone and teeth and also enhance such functions of osteoblasts [7]. In our previous research, siloxane-containing vaterite (SiV) particles were developed as materials providing silicate and calcium ions [8-11]. Although SiV releases silicate ions with $T^0 \sim T^3$ species which are different ionic state to those of bioactive glasses, mouse osteoblast-like cells (MC3T3-E1 cells) cultured on the surfaces of a SiV-containing biodegradable polymer composite film exhibited enhanced proliferation and differentiation in comparison with those on vaterite and polymer composites, which release only calcium ion [8, 9].

Cell responses to the ions depend on ion types. For example, copper was reported to advance synergetic stimulating effects on angiogenesis and zinc was found to possess anti-inflammatory effect and stimulate bone formation [1]. Magnesium enhances cell adhesion onto materials and angiogenic

function of endothelial cells [12, 13]. Zreiqat *et al*. reported that human bone-derived cells possessed the up-regulated expression of integrin family, which is adhesion proteins, when they were cultured on magnesium-modified alumina compared with unmodified alumina (Mg-free) [12]. Cell adhesion to biomaterial surfaces is one of the important processes for achievement of satisfactory metabolisms of cells on the surfaces, especially for adherent cells like osteoblasts. To promote cell adhesion would be effective for achieving enhanced proliferation and following biological reactions of such cells. We, therefore, hypothesized that bioceramics having the ability of providing silicate, calcium and magnesium could be good candidate for materials used in bone regeneration. In our previous work, magnesium and siloxane-containing vaterite (MgSiV) was developed [14]. MgSiV particles were developed as a new type of vaterite-based inorganic particle with the ability of enhancing osteoblast's activities and bone formation. MgSiV is synthesized by a conventional carbonation process in the same way for SiV. The results of the immersion tests for MgSiV and SiV using Tris-HCl buffer solution (pH 7.4) described that MgSiV released Mg^{2+} ion and almost the same amounts of silicate and Ca^{2+} ions as those of SiV for more than 7 days [14]. The silicate ions released from MgSiV are believed to stimulate the functions of osteoblast-like cells like SiV, since they are supposed to have a similar ionic state to that released from SiV.

The aim of the present work is to estimate the responses of MC3T3-E1 cells to the ionic products released from MgSiV and SiV and examine the combined effects of several kinds of ions on the

functions of the cells. Although individual effects of each ion on the cell functions have been reported by many researchers, there were few reports on the combined effects of silicate, Ca^{2+} and Mg^{2+} ions on the functions. As described above, MgSiV and SiV release three and two kinds of ion in combination through their degradation, respectively. We focused on the combined effects of Mg^{2+} ion with Ca^{2+} and silicate ions on the adhesion, proliferation, differentiation and mineralization of MC3T3-E1 cells.

2. Materials and methods

2.1. Preparation of MgSiV particles

SiV particles were prepared by a carbonation process in methanol solvent, following the published report[9]. MgSiV particles were prepared by the same method apart from adding Mg^{2+} ion source [14]. As Mg^{2+} ion source, 0.2 mol of magnesium hydroxide (Wako Pure Chemicals Inc., Osaka, Japan) was added to a solvent consisting of 2.0 L methanol (Wako Pure Chemicals Inc.) and 200 mL distilled water while stirring at 21 °C. Carbon dioxide (CO_2) gas was bubbled to the slurry at a feed rate of 2.0 L/min for 20 min. Subsequently, 1.8 mol calcium hydroxide (Kishida Chemical Co., Ltd., Japan) and 60 mL 3-aminopropyltriethoxysilane (APTES, Dow Corning Co., USA) were mixed into the slurry under CO_2 gas flow. The gas was added over 40 min, resulting in the formation of a precursor gel. The resulting gel was aged at room temperature for 12 h and then dried at 110 °C for 24 h to

remove the residual solvent. The obtained agglomerated powder was ground to form MgSiV particles.

SiV particles containing no magnesium were also prepared as a control in the same manner. SEM

images of MgSiV and SiV particles obtained were shown in Fig. 1.

2.2. Preparation of ion extracts

The SiV or MgSiV particles were sterilized with ethylene oxide gas at 44 °C for 20 h. The ion

extracts were prepared by immersing 300 mg of the particles in 30 mL of α-modified minimum

essential medium (α-MEM; MEMα with l-glutamine and phenol red, Wako, Japan) supplemented with

10% fetal bovine serum (FBS) and incubated in 5% (v/v) CO_2 at 37 °C for 24 hours. An α-MEM which

contains no particles was also incubated under the same conditions. The ion extract derived from SiV

or MgSiV is denoted by "SiV-extract" or "MgSiV-extract", respectively. The concentrations of Ca, Si,

Mg and P elements in α-MEM and the ion extracts were measured by inductively coupled plasma

atomic emission spectroscopy (ICP-AES; ICPS-500, Shimadzu, Kyoto, Japan). This measurement was

repeated for a total of three repetitions.

2.3. Cell culture test

The effects of the ion extracts on the initial adhesion ability of osteoblasts were determined with

adhered cellular numbers on a culture plate and their morphology. Murine osteoblast-like cells

(MC3T3-E1 cells) suspended in α-MEM or each ion extract at a density of 1.0×10^5 cells/mL were seeded in 24-well culture plates (1 mL of the cell suspension for each well). The culture plates were placed in a CO_2 incubator for 3 hours. The adhered cellular numbers in the culture well were estimated using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan), following its instructions. The numbers of live cells on the samples were colorimetrically estimated by measuring the absorbance of the resulting media at 450 nm with a microplate scanning spectrophotometer (Sunrise Remote; Tecan, Kanagawa, Japan). The cells were fixed with a 2.5% (v/v) glutaraldehyde solution for 10 min, and then stained with Giemsa's solution. The stained cells were observed with an optical microscope (Biorevo BZ-9000; Keyence, Osaka, Japan). Three parallel samples of each medium were cultured.

For evaluation of proliferation ability, MC3T3-E1 cells suspension was prepared using α-MEM at a density of 3.0×10^4 cells/mL, and seeded in the 24-well culture plates. After the incubation for 24 hours, the culture media were replaced with α-MEM or the ion extracts. Subsequently, the media were replaced with fresh ones once in two days up to 7 days. The proliferation ability was evaluated after treatment using a Cell Counting Kit-8, following its instructions. The numbers of live cells on the samples were colorimetrically estimated by measuring the absorbance of the resulting media at 450 nm with a microplate scanning spectrophotometer.

To evaluate the differentiation ability, the cells were pre-cultured in the same condition with the evaluation of proliferation. After 7 days, the culture media in the wells were replaced with a

differentiation medium, which is α-MEM supplemented with 0.05 mM L-ascorbic acid-2-phosphate, 100 nM dexamethasone and 10 mM β-glycerophosphate as differentiation agents and 1 % penicillin/streptomycin, or the medium containing the ion extracts. Subsequently, the media were replaced with fresh ones once in two days up to 28 days. Alkaline phosphatase (ALP) activity of the cells was analyzed using a *p*-nitrophenyl phosphate (pNPP) tablet sets (Lab Assay ALP Kit; Wako, Japan) 7, 14, 21 and 28 days after the replacing with the differentiation medium. ALP activity was evaluated after measuring the absorbance of *p*-nitrophenol product formed at 405 nm with the spectrophotometer. The value of ALP activity was expressed as μmol of pNPP/min/cells.

The mineralization of MC3T3-E1 cells were evaluated by an Alizarin red (Alizarin Red S; Wako, Japan) staining method. The Alizarin red forms insoluble orange-red lakes, identifying points of insoluble cations that are incorporated within mineralized tissues. The cell culture was conducted in the same condition with ALP assay for 28 and 35 days. After the above time points, the cultured samples were washed with phosphate buffer solution (PBS) and subsequently fixed with 70 % ethanol for 60 min at 4 °C. The samples were stained with the Alizarin red solution dissolved in 50 mM acetic acid for 10 min and washed 5 times with deionized water. The resulting samples were observed with an optical microscope.

2.4. Statistical Analysis

The data were presented as the mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using Student's *t*-test and single-factor ANOVA (SPSS 21 software; IBM, USA) followed by Tukey's multiple comparison test. Values of $p < 0.05$ were considered to be significant.

3. Results and discussion

3.1. Ion concentrations in the extracts

After soaking for 24 h, Ca, Si, Mg and P concentrations in MgSiV-extract were originally 146, 65, 76 and 35 ppm, respectively. In our previous report, most of Mg and Si in MgSiV and SiV were released in 1 day after soaking in Tris buffer solution (pH=7.40). The resulting samples after soaking in the solution for 7 days consisted predominantly of alagonite, which is one of the polymorphs of calcium carbonate [14]. Thus, MgSiV releases Mg and Si immediately and most of Ca in MgSiV remains as calcium carbonate in aqueous solutions including culture media. In the present work, Ca concentration of MgSiV and SiV extracts was adjusted to 140 ppm by diluting with α-MEM. The resulting concentrations of Ca, Si, Mg and P in α-MEM and the ion extracts used for the cell culture test were shown in Table 1. There was a significant difference only in Mg concentration between MgSiV-extract and SiV-one.

3.2. The initial cellular adhesion

Fig. 2(a) shows the cellular numbers adhered on culture-wells in 3 hours. The adhered cellular number cultured in SiV-extract was approximately 3.0×10^4 cells/well that was almost the same with that in α-MEM. In contrast, significantly larger number of the cells adhered on the well in MgSiV-extract. The adhered cellular number cultured in MgSiV-extract was approximately 4.1×10^4 cells/well.

The morphology of the cells adhered in α-MEM and the ion extracts were indicated in Fig. 2(b-d). The cells cultured in α-MEM and SiV-extract exhibited less-spread and circular shape (Fig. 2(b) and (c)), while those cultured in MgSiV-extract exhibited spindle-like elongated shape (Fig. 2(c)). The cells cultured in MgSiV-extract might have good extensibility of their pseudopodium. These results indicate that Mg^{2+} ions released from MgSiV promoted the initial cellular adhesion.

3.3. Proliferation ability

Figure 3(a) shows the results of the proliferation assay for MC3T3-E1 cells cultured for 7 days. The cellular numbers increased with a culture time for all samples. The numbers of live cells cultured in SiV- or MgSiV-extract were larger than that in α-MEM at every time point during 7 days. At the initial stage of proliferation (from 1 day to 3 days), the cells in MgSiV-extract exhibited the highest proliferation ability, although there were no significant differences in the values between MgSiV and SiV-extracts. Mg^{2+} ions released from MgSiV influenced the cellular proliferation, especially at initial stage. The cellular numbers cultured in SiV-extract for 7 days were almost the same as those in MgSiV-

extract. Appropriate extracellular Mg^{2+} ion levels, such as 19 ppm (the physiological concentration of the ions is 19 to 24 ppm), were essential to the stimulations of osteoblast proliferation by platelet-derived growth factor (PDGF), whereas Ca^{2+} ion levels do not influence the stimulation [15]. PDGF has been found to promote proliferation and migration in a variety of cell types including osteoblastic cells. Although it is still unclear how the stimulation of osteoblast proliferation by PDGF changes when larger amounts of Mg^{2+} ions are provided, the up-regulated proliferation in the early stage in the present experiment might be relate to the PDGF stimulations. Further investigation is needed to clarify more detail of the proliferation behaviors influenced by the ratio of Ca^{2+} and Mg^{2+} ion concentrations in the extracts.

3.4. Alkaline phosphatase (ALP) activity

The ALP activities in MC3T3-E1 cells cultured in α-MEM and the ion extracts for 28 days were indicated in Fig. 3(b). ALP activity is one of the established markers for the osteoblast phenotype. It is generally accepted that increase in the ALP activity reflects a shift to a more differentiated bone cells and the activity reaches its peak directly before start of mineralization of the cells [16]. The cells cultured in SiV- and MgSiV-extract exhibited significantly higher ALP activity than that in α-MEM at every time point during 28 days. It is considered that the differentiation of MC3T3-E1 cells was strongly enhanced by the extracellular silicate ions. Moreover, the activity level of MgSiV-extract

reached its peak at 14 days of culture, while that of SiV-extract reached at 21 days. This suggests that the differentiation of MC3T3-E1 cells was substantially promoted by the additionally released Mg^{2+} ions in addition to silicate ions from MgSiV.

3.5. Mineralization

Figure 4 shows the results of Alizarin red staining of mineralized nodules that were formed by MC3T3-E1 cells after the cultivation for 28 and 35 days. The degree of redness in the images represents the quantity of Ca^{2+} ion incorporated into the cells. After the cultivation for 28 days, all the samples possessed almost no mineralized tissues (Fig. 4(a), (c) and (e)) On the other hand, the cells cultured for 35 days in MgSiV-extract showed well-mineralized nodules (Fig. 4(f)), while those in α-MEM and SiV-extracts indicated no red-stained tissues (Fig. 4(b) and (d)). An enhancement of mineralized nodule formation in the cells was observed only when they were cultured in MgSiV-extract.

Magnesium has been regarded to control bone calcification and crystallization processes [17, 18]. Wuthier et al. reported that the highest contents of magnesium were contained in calcified cartilage, trabecular, and cortical bone. This must indicate that magnesium plays an active role in calcification and mineralization processes. Leidi et al., however, revealed that the culture medium containing higher amount of magnesium ions (120 ppm) compared to a conventional one (24 ppm)

inhibited the differentiation and mineralization of osteoblast-like cells (SaOS-2) [19]. Serre *et al.*

reported that magnesium-substituted hydroxyapatite and collagen composites leaded a decrease of

proliferation and calcification activities of osteoblast-like cells. In particular, the composites

containing higher amount of magnesium ions suppressed a toxic effect on the cells [20]. These reports

seem to show completely opposite results to those obtained in the present work. This might be because

there are differences in the cell type used and the amount of magnesium ion in culture media among

these reports. For example, Yoshizawa *et al.* reported that the mineralization of human bone marrow

stromal cells was enhanced by providing ~ 240 ppm of magnesium ions, whereas 2,400 ppm of the

ions did not up-regulate them [21].

Another possible reason why there are such differences in the cell responses is a combination

of ions provided to the cells. Silicate, Ca^{2+} and Mg^{2+} ions were provided to the cells at the same time

in the present work, the other reports did only Mg^{2+} ion. Conventional culture media contain Ca^{2+} and

Mg^{2+} ions. The present work aimed to clarify what would happen to the cells when additional amounts

of these ions were provided to them with silicate ion, since some bioactive glasses and bioceramics,

such as SiV and MgSiV, release not only single ion but also several ones at the same time through

their dissolution or degradation *in vitro* and *in vivo*. The differentiation and mineralization of the cells

were up-regulated by providing both Mg^{2+} and silicate ions, as shown in Figs. 3 and 4. This must

indicate combined effects of the two ions on the cell functions. We will examine the combined effects

of several kinds of ions on cell functions with systematic procedure in future work.

The differences in the initial cellular morphology between MgSiV- and SiV-extracts were shown in Fig. 2(b-d). Cellular morphologies are known to be related to gene expression [22]. Several factors, such as roughness, wettability and functional groups on substrate surfaces on which cells are cultured, have been reported to influence cellular morphologies. These factors, however, would be the same for all conditions in the present work, because we used the same well-plates. The cells cultured in MgSiV-extract which showed well-elongated shapes resulted in good mineralization ability. This means the additional amount of Mg^{2+} ions promoted the elongation of the cells followed by the up-regulated mineralization. Thus, to enhance cell elongation might be effective for up-regulating mineralization. We, however, also expect that combined effects of the ions would be involved in the up-regulation of mineralization observed in the present work, since the proliferation and the ALP level were enhanced for not only the MgSiV-extract but also the SiV-one, as shown in Fig. 3.

4. Conclusion

Cytocompatibility of magnesium- and siloxane-containing vaterite (MgSiV) was examined by culture tests using MC3T3-E1 cells for its ion extract. At the initial stage of cell culture test, the cells in MgSiV-extract showed better elongation of their pseudopodium and had excellent adhesion ability compared with that in conventional α-MEM and siloxane-containing vaterite (SiV)-extract. The

proliferation ability of the cells cultured in MgSiV-extract was higher than that in SiV-extract after 3 days of culture, while the ability in the two extracts resulted in the same level after 7 days. The level of ALP activity of the cells cultured in MgSiV-extract increased at the earlier time point of the culture than those in α-MEM and SiV-extract. This result implies that the differentiation of the cells was promoted in MgSiV-extract. Moreover, the cells cultured in MgSiV-extract exhibited well-mineralized nodules by Alizarin red staining after culture for 35 days. The combination of Si, Ca and Mg provided was effective for up-regulating the cell's mineralization.

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Table

Table 1. Concentrations (ppm) of Ca, Si, Mg and P elements in α–MEM and the ion extracts used for the cell culture analysis.

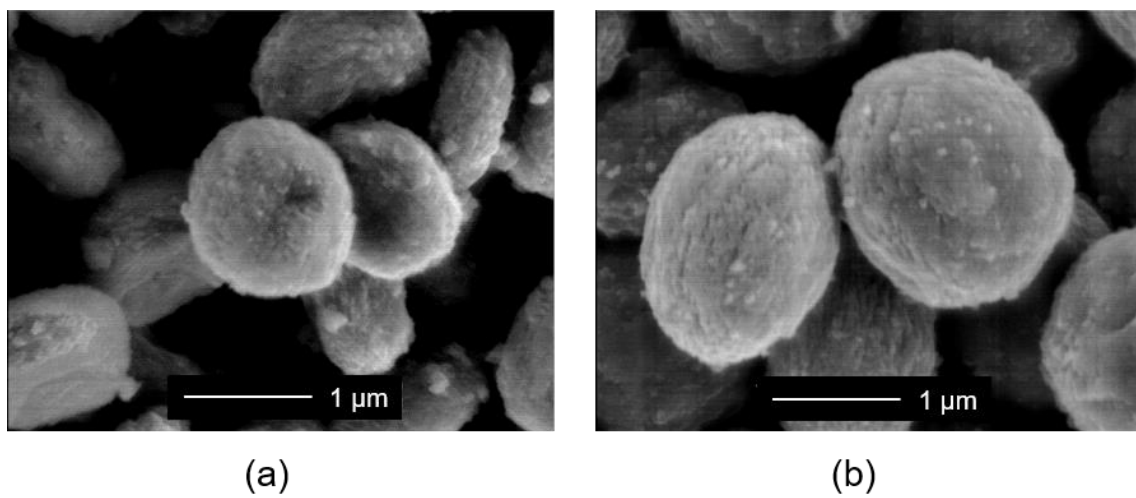|	Ca	Si	Mg	P
α-MEM	78	0	28	40
SiV-extract	140	57	25	38
MgSiV-extract	140	58	71	36

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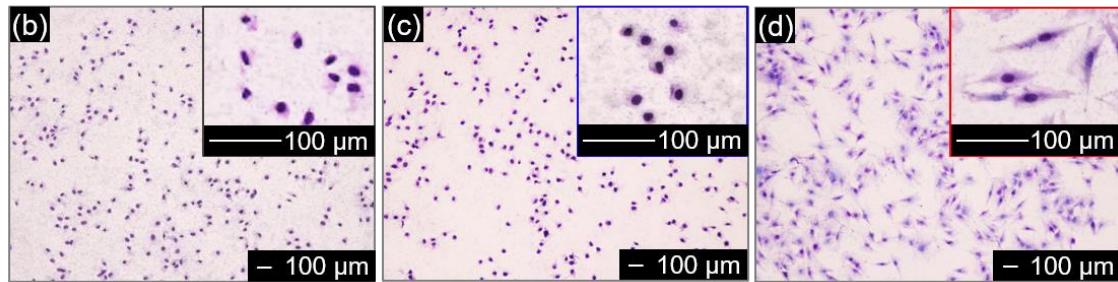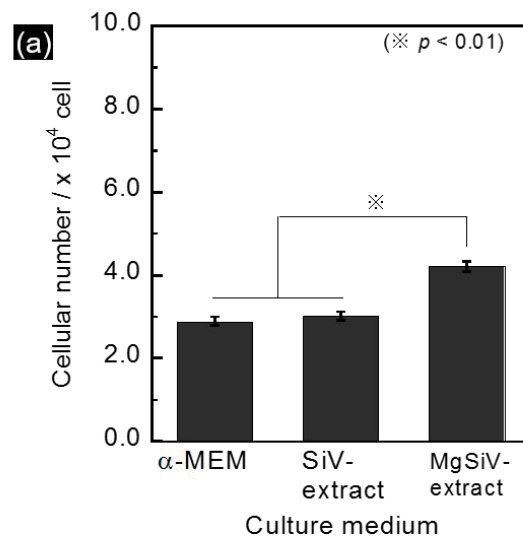
Fig. 1. SEM images of (a) MgSiV and (b) SiV particles.

Fig. 2. (a) Numbers of the cells adhered on culture-wells in 3 hours. (b-d) Morphology of the cells adhered on culture-wells in (b) α-MEM, (c)SiV-extract and (d)MgSiV-extract in 3 hours.

Fig. 3. (a) Numbers of the cells cultured in α-MEM and the extracts for 1–7 days. (b) ALP activities of the cells cultured in α-MEM and the extracts for 7–28 days.

Fig. 4. Optical micrographs of Alizarin red stained mineralized nodules formed by MC3T3-E1 cells after the cultivation for 28 and 35 days. (a) cultured in α-MEM for 28 days, (b) cultured in α-MEM for 35 days, (c) cultured in SiV-extract for 28 days, (d) cultured in SiV-extract for 35 days, (e) cultured in MgSiV-extract for 28 days and (f) cultured in MgSiV-extract for 35 days.

Figures

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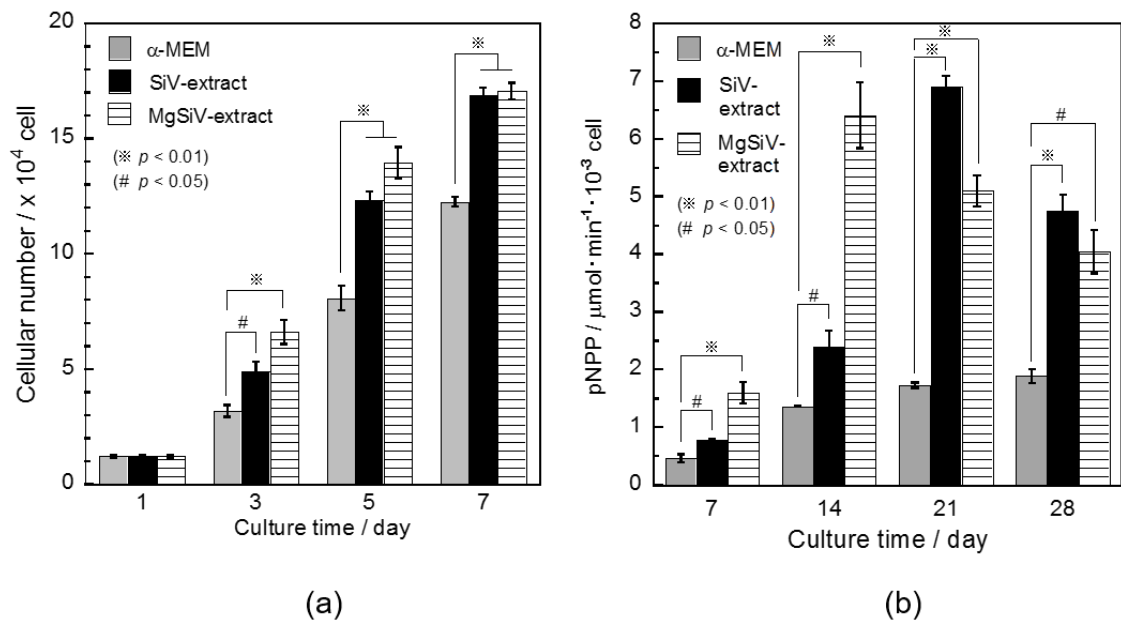
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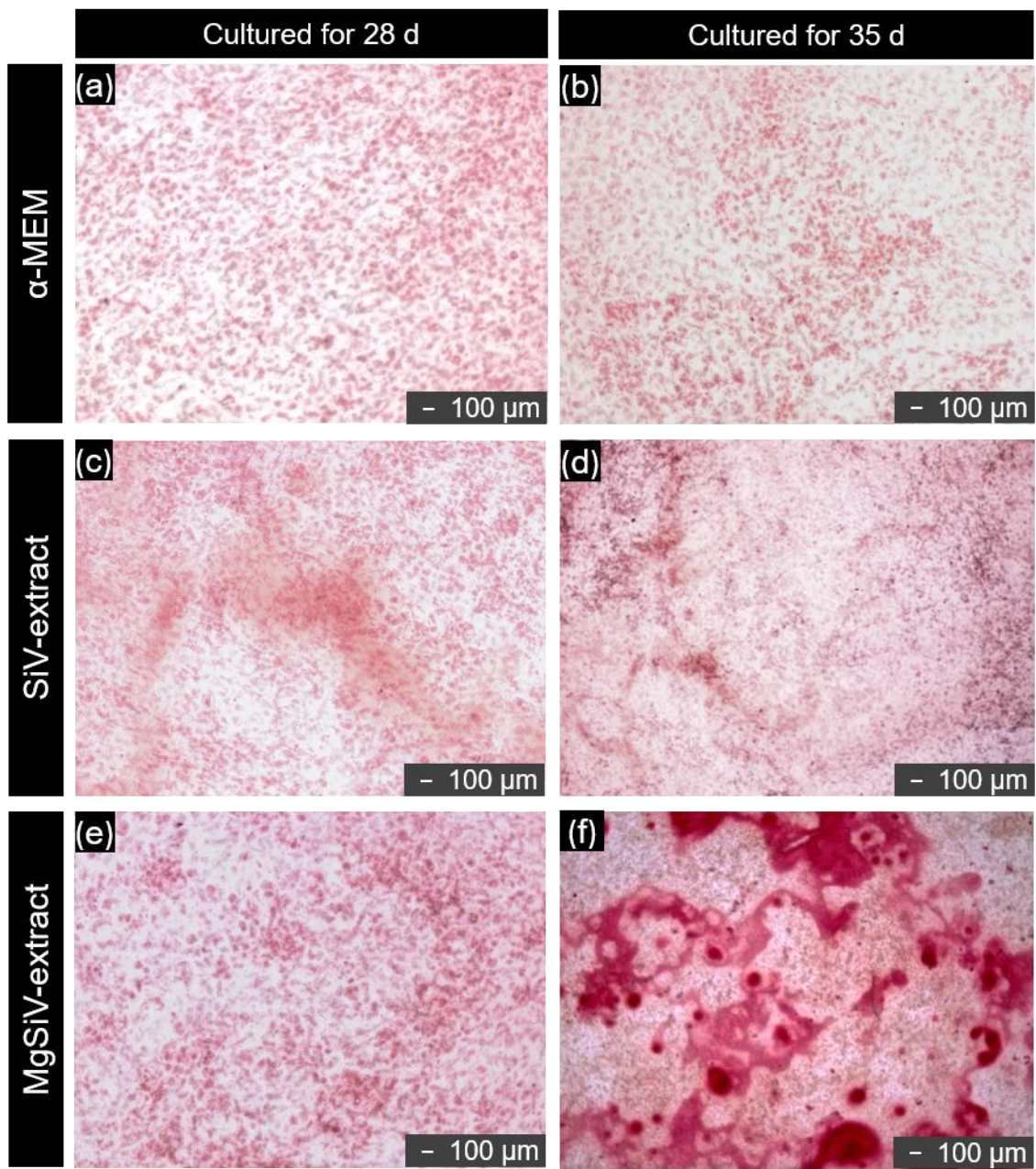
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