

# Zinc Restored the Decreased Vascular Smooth Muscle Cell Viability under Atherosclerotic Calcification Conditions

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**ABSTRACT:** Zinc is considered to be involved in maintaining healthy vascular condition. Atherosclerotic calcification of vascular smooth muscle cells (VSMCs) occurs via the mechanism of cell death; therefore, cell viability is a critical factor for preventing VSMC calcification. In this study, we tested whether zinc affected VSMC viability under both normal physiological non-calcifying (0 mM P) and atherosclerotic calcifying conditions (3 and 5 mM P), since VSMC physiological characters change during the VSMC calcification process. The study results showed that an optimal zinc level (15  $\mu$ M) restored the decreased VSMC viability which was induced under low zinc levels (0 and 1  $\mu$ M) and calcifying conditions (3 and 5 mM P) at 9 and 15 days culture. This zinc-protecting effect for VSMC viability is more prominent under atherosclerotic calcifying condition (3 and 5 mM P) than normal condition (0 mM P). Also, the increased VSMC viability was consistent with the decreased Ca and P accumulation in VSMC cell layers. The results suggested that zinc could be an effective biomineral for preventing VSMC calcification under atherosclerotic calcifying conditions.

**Keywords:** zinc, atherosclerosis, vascular smooth muscle cells (VSMCs), cell viability, VSMC calcification

## INTRODUCTION

Vascular calcification is observed in atherosclerotic plaque and it is a critical sign to transit to the severe stage of atherosclerosis. The blood vessel wall is composed of mainly three layers: the intimal endothelial cell layers, the medial vascular smooth muscle cell (VSMC) layers which give the flexibility to the blood vessel for proper blood flow, and finally adventitia fibroblast layers (mostly collagenous fibers). Vascular calcification mainly occurs in VSMC layers and eventually causes the blood vessel wall to lose flexibility and becomes stiff (1).

One of the primary mechanisms for VSMC calcification is VSMC apoptosis (the programmed cell death) (2-4). Therefore, the impaired cell viability in VSMCs is considered favorable condition for stimulating vascular calcification. The cellular apoptotic bodies, which were derived from the cultured human VSMCs, contain concentrated and crystallized Ca and this creates favorable conditions for producing a nucleating deposition for vascular calcification (5). Inorganic phosphate ( $P_i$ ) is also the nucleator for VSMC calcification by combining with Ca ions (6).

It is well documented that cellular zinc depletion causes cell apoptosis in various cell types (7). The main

mechanism that cellular zinc depletion can cause cell death is via the activation of pro-apoptotic enzyme caspase-3, and zinc deficiency has been regarded as contributing factor for the incidence of atherosclerosis (8). Recently we reported that a zinc-deficient diet induced VSMC apoptosis in rat aorta origin tissue samples and this VSMC apoptosis was induced via dephosphorylation (activation for apoptosis) of pro-apoptotic protein Bcl-2-associated death promoter protein (BAD) (9).

In this study, we tested whether zinc compensated the lowered VSMC viability under atherosclerotic calcifying condition, which was induced by the addition of calcifying agent,  $P_i$ .

## MATERIALS AND METHODS

### Chelexing zinc in FBS

To deplete zinc in media (mainly from fetal bovine serum, FBS), zinc in FBS was chelexed using chelex-100 resin (Bio-Rad, Berkeley, CA, USA), following the manufacturer's instruction. Briefly 5 g chelex-100/100 mL of FBS was mixed in a roller shaker at 4°C for 12 h. Then, the mixture was decanted. After 30 min, the supernatants were sterile filtered using a 0.2  $\mu$ m filter, while

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the zinc-chelexed portion was precipitated. After chelexing zinc in FBS, the supernatant (zinc-depleted FBS) was used for cell culture with the addition of the designated zinc level as  $ZnCl_2$ .

#### A7r5 cell culture and zinc treatment

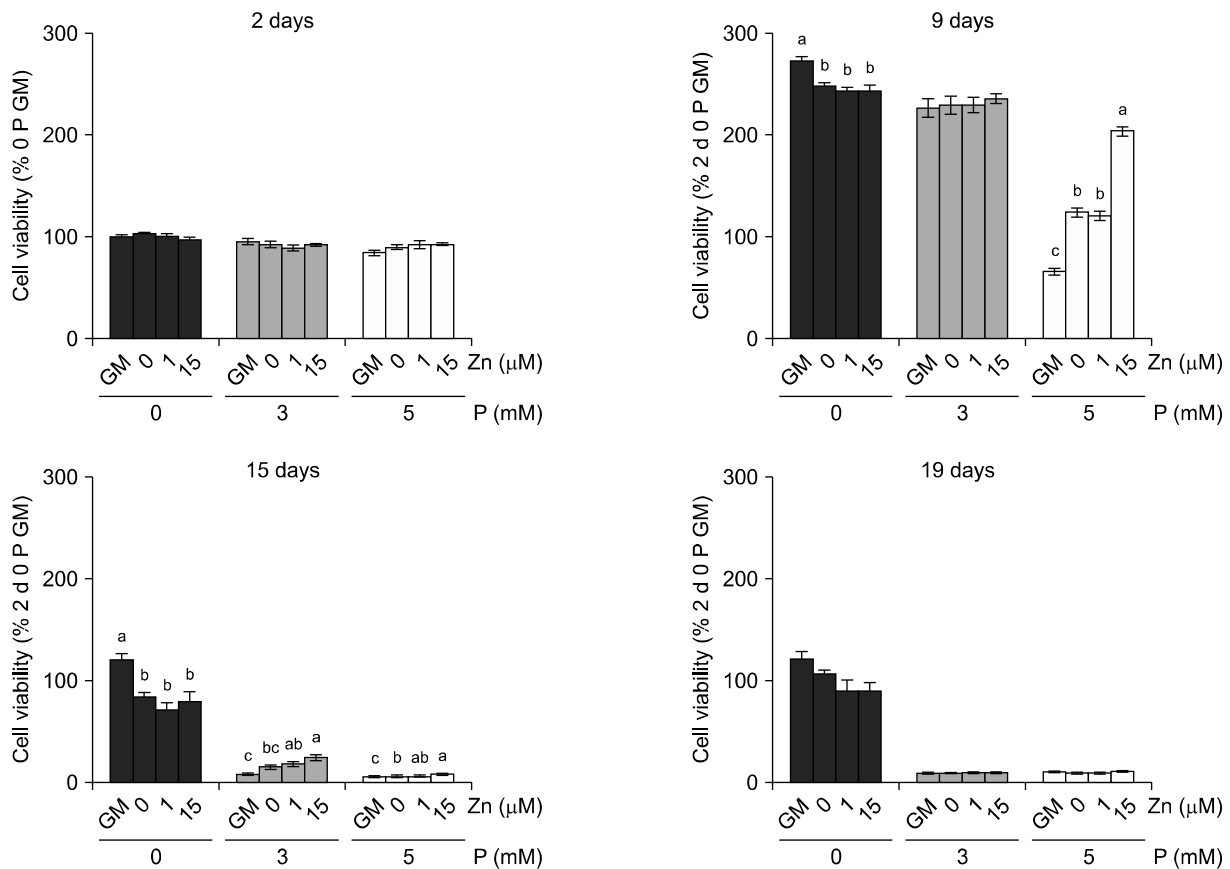
A7r5 cells were commercially obtained as rat aorta VSMC line model (American Type Culture Collection, Manassas, VA, USA). A7r5 cells were cultured in growth media (GM) [Dulbecco's Modified Eagle's Medium (DMEM)+10% FBS+100 units/mL penicillin+100  $\mu$ g/mL streptomycin] at 37°C with 5%  $CO_2$  until 90% cells were confluent. Then, the cells were cultured with the zinc-chelexed FBS plus various zinc levels (0, 1, and 15  $\mu$ M Zn) under normal non-calcifying physiological condition (0 mM P as monosodium phosphate,  $NaH_2PO_4$ ) or calcifying atherosclerotic conditions (3 or 5 mM) up to 22 days. GM was used as normal control within each of the 0, 3, and 5 mM P treatments. Cell culture reagents were purchased from Gibco (Grand Island, NY, USA) and Sigma (St. Louis, MO, USA).

#### Cell viability measurement

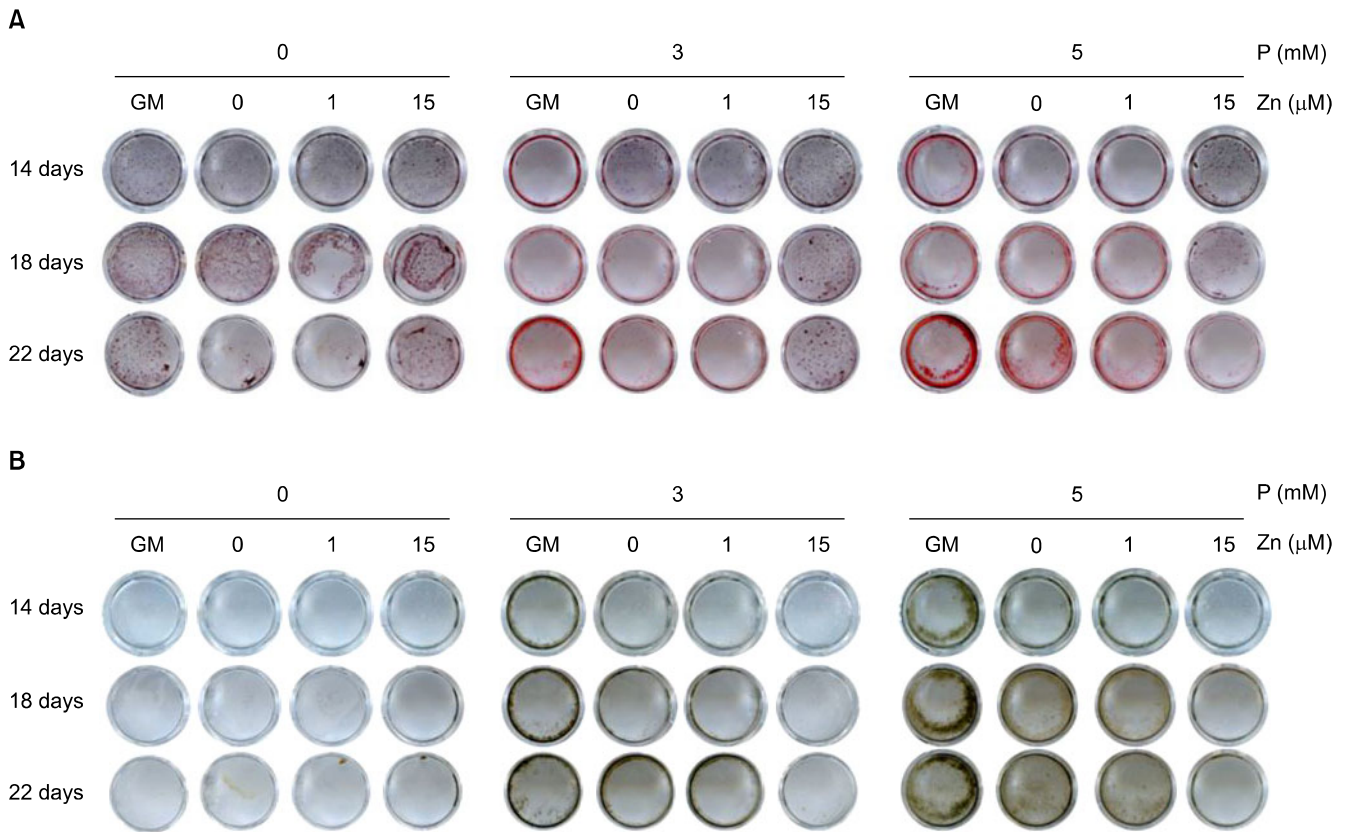
Cell viability was determined by the MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] (Sigma) assay. This assay measures the formazan, which is the product of dehydrogenase as the reduced form of tetrazolium MTT by metabolically active cells. The assay was conducted using the protocol instructions, and the formazan products were measured at 570 nm using an absorbance reader (Sunrise™ Absorbance Reader, Tecan, Port Melbourne, VIC, Australia).

#### Alizarin red S staining for Ca deposition

Ca deposition on extracellular matrix was measured by Alizarin red S (Sigma) staining where Alizarin red S dye binds with Ca ions in cell layers. The cells were washed with phosphate buffered saline (PBS) and fixed with 2% formaldehyde (Sigma) for 15 min at 4°C. Then, the cells were stained with 1% Alizarin red S (pH 4.2) for 30 min at room temperature and then the cells were washed with deionized water. Ca deposits were colored red.



**Fig. 1.** Zinc restored vascular smooth muscle cell viability is more prominently under atherosclerotic calcifying conditions (3 and 5 mM P) than under physiological non-calcifying conditions (0 mM P). Zn compensated A7r5 cell viability under atherosclerotic calcifying condition (5 mM P) than normal physiological non-calcifying condition (0 mM P) at days 9 and 15. This restoring pattern was shown as zinc concentration-dependent manners. Growth media (GM) was used as normal control culture. Cell viability measured by the MTT assay and presented as percentage of cell viability at 0 mM P GM at 2 days. Data was analyzed by one-way ANOVA at  $P < 0.05$ , followed by Tukey's *post hoc* test. Different letters (a-c) indicate significant differences among zinc groups within P treatment. Mean  $\pm$  SEM (n=8).



**Fig. 2.** A7r5 cell Ca (A) and P (B) accumulation also decreased as zinc compensated cell viability. Optimal zinc level (15  $\mu\text{M}$ ) in A7r5 cells decreased Ca and P accumulation and it is more prominent under atherosclerotic conditions (3 and 5 mM P). A7r5 cells were cultured under the same conditions in Fig. 1 and Ca and P depositions were measured using Alizarin Red S and von Kossa staining, respectively. Representative image of  $n=3$ .

#### von Kossa staining for P deposition

von Kossa staining was used to assess P accumulation as  $\text{P}_i$ . Phosphate ions co-precipitates Ca ions to make hydroxyapatite for calcification such as in bone and atherosclerotic plaques. After washing with PBS and fixing with 2% formaldehyde, cells were stained with 3% silver nitrate (Sigma) and exposed to light for 30 min. Phosphate deposits were colored dark brown.

#### Statistical analysis

Values are presented as mean  $\pm$  SEM. Statistical significance was analyzed by one-way ANOVA, followed by Tukey's test as *post hoc* statistical power test, using the Statistical Package for Social Sciences (SPSS version 21, IBM Corporation, New York, NY, USA).  $P < 0.05$  was considered for the mean comparison to be significant.

## RESULTS

**Zinc compensated the decreased vascular smooth muscle cell viability and it is more prominent under atherosclerotic calcifying condition than under normal physiological non-calcifying condition**

Low zinc levels (0 and 1  $\mu\text{M}$ ) for VSMC culture induced the decreased cell viability and the addition of phosphate also induced severely impaired cell viability. Optimal zinc addition (15  $\mu\text{M}$ ) to A7r5 cells restored the decreased cell viability, compared to the addition of low zinc levels (0 and 1  $\mu\text{M}$ ), especially under atherosclerotic calcifying conditions (3 and 5 mM P), during culture period of 9 and 15 days (Fig. 1). This zinc-recovering effect for the decreased VSMC viability was more prominent under atherosclerotic calcifying condition (3 and 5 mM P) than under normal non-calcifying condition (0 mM P).

#### Ca and P accumulation in VSMCs was also decreased as zinc compensated cell viability

Ca and P deposition, a critical sign for VSMC calcification, was also decreased as the addition of zinc increased. It was more prominent under atherosclerotic conditions (3 and 5 mM P) (Fig. 2). This decreased Ca and P accumulation was consistent with the pattern of zinc-compensated VSMC viability under atherosclerotic conditions. Especially, the pattern of the decreased Ca and P accumulation by the addition of optimal zinc level (15  $\mu\text{M}$ ) was consistent with zinc compensated VSMC via-

bility in Fig. 1.

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## DISCUSSION

The decreased cell viability in VSMC culture is closely related to VSMC calcification, since cell death provides the favorable conditions for nucleation, promoting calcification by accumulating the apoptotic bodies and the disrupted cell membrane debris (10). Biomineral zinc is considered to be involved in healthy vascular conformation (8). Zinc protects cell death, because it has anti-apoptotic functions via various cellular pathways (8,9). In our previous studies, we reported that zinc protects VSMC from apoptosis (9) and also compensated VSMC dysfunction via producing a dual-acting humoral factor (11). In this study, we questioned whether zinc modulated VSMC viability and it was different under non-calcifying (normal physiological condition, 0 mM P) and calcifying (atherosclerotic condition, 3 and 5 mM P) conditions, since during VSMC calcification the cells have changed physiological characters.

The study findings showed that the optimal zinc level (15  $\mu$ M) compensated VSMC viability which was impaired under low zinc level and by the addition of phosphate. This zinc-restoring effect for the impaired cell viability is more prominent under atherosclerotic calcifying conditions (3 and 5 mM P), compared to the normal non-calcifying condition (0 mM P). This finding implies that zinc can be more effective in protecting the damaged cell viability in atherosclerotic conditions, where blood vessel calcification is already occurring.

This zinc protective effect for the impaired VSMC viability is confirmed in Ca and P accumulation, where Ca and P deposition is decreased more in optimal zinc status (at 15  $\mu$ M Zn) than in low zinc status (0 and 1  $\mu$ M Zn), during the VSMC culture period of up to 22 days.

The findings from this study suggest that zinc can be more effective in preventing cell death under atherosclerotic stages where VSMC calcification is already progressed. This finding would be the first report in our knowledge. The further questions for the mechanism of how zinc can prevent VSMC calcification need to be clarified.

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## AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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