

# Stimulating Effect of a Novel Synthesized Sulfonamido-Based Gallate ZXHA-TC on Primary Osteoblasts

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interest.

**Purpose:** This study is intended to investigate the effects of plants or plant-derived antioxidants on prevention of osteoporosis through the maintenance of reactive oxygen species (ROS) at a favorable level. **Materials and Methods:** In this study, a novel antioxidant, namely 3,4,5-Trihydroxy-N-[4-(5-hydroxy-6-methoxy-pyrimidin-4-ylsulfamoyl)-phenyl]-benzamide (ZXHA-TC) was synthesized from gallic acid and sulfadimoxine. Its effect on osteoblast metabolism was investigated via the detection of cell proliferation, cell viability, production of ROS, and expression of osteogenic-specific genes including runt-related transcription factor 2 (*RUNX2*), bone sialoprotein (*BSP*), osteocalcin (*OCN*), alpha-1 type I collagen (*COL1A1*), and osteogenic-related proteins after treatment for 2, 4, and 6 days respectively. **Results:** The results showed that ZXHA-TC has a stimulating effect on the proliferation and osteogenic differentiation of primary osteoblasts by promoting cell proliferation, cell viability, and the expression of genes *BSP* and *OCN*. Productions of bone matrix and mineralization were also increased by ZXHA-TC treatment as a result of up-regulation of *COL1A1* and alkaline phosphatase (*ALP*) at the early stage and down-regulation of both genes subsequently. A range of  $6.25 \times 10^{-3}$   $\mu\text{g/mL}$  to  $6.25 \times 10^{-1}$   $\mu\text{g/mL}$  is the recommended dose for ZXHA-TC, within which  $6.25 \times 10^{-2}$   $\mu\text{g/mL}$  showed the best performance. **Conclusion:** This study may hold promise for the development of a novel agent for the treatment of osteoporosis.

**Key Words:** Gallic acid, sulfonamido, osteoporosis, osteogenesis, osteoblast

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## INTRODUCTION

Osteoporosis, a public health concern, is a systemic skeletal disease characterized by micro-architectural deterioration of bone with resultant low bone mass, bone fragility, and increased fracture risk.<sup>1-4</sup> As reported by the National Osteoporosis

Foundation, osteoporosis affects approximately 10 million individuals<sup>5</sup> and over 2 million fractures per year<sup>6</sup> in the USA. It is estimated that up to 50% of women and 25% of men over the age of 50 years will experience an osteoporotic fracture in their remaining lifetime.<sup>5</sup> Osteoporotic fractures can cause considerable pain, disability, loss of independence, depression, deterioration in quality of life, and even death.<sup>4,7,8</sup> These negative effects not only impose a great economic burden on family and country but also cause great anxiety for the patients.

There is an increasing awareness that plants and plant-derived compounds may play an important role in the prevention of osteoporosis.<sup>9</sup> Many bioactive compounds from plants, such as daidzein and its derivative,<sup>10-14</sup> (-)-Epigallocatechin-3-gallate (EGCG),<sup>15</sup> were found to act as estrogen receptor agonists with beneficial outcomes in osteoporosis. Their prevention of osteoporosis may be attributed partly to their antioxidant properties. In the process of osteoporosis, excessive reactive oxygen species (ROS) and inflammatory responses stimulate differentiation and function of osteoclasts and simultaneously suppress osteoblastic proliferation and differentiation.<sup>16</sup> Thus, antioxidants that can maintain ROS at a favorable level by reducing excessive ROS may hold promise in the prevention of osteoporosis.

Gallic acid (GA) and its derivatives comprise a group of polyphenol compounds that have been known to affect several pharmacological and biochemical pathways and have strong anti-oxidative<sup>17</sup> and anti-inflammatory<sup>18</sup> effects. Ad-

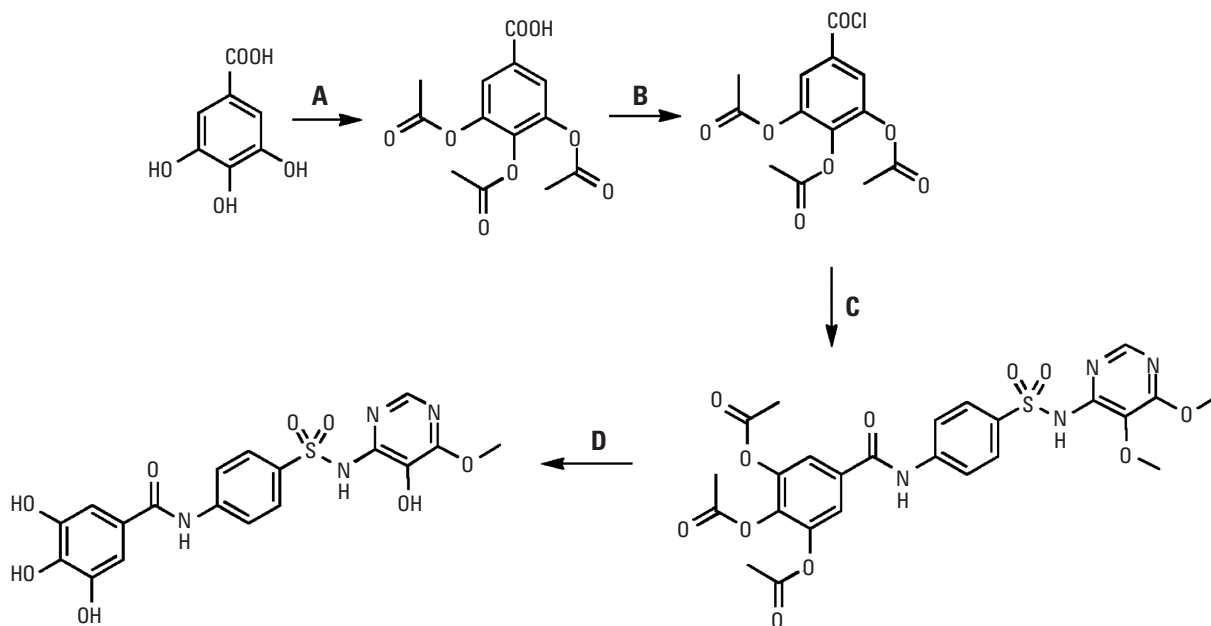
ditionally, GA has been reported to have an anti-osteoporosis effect.<sup>19</sup> However, GA was reported to suppress cell proliferation<sup>20-22</sup> and to be more hydrophilic than its esters,<sup>17</sup> which may influence its anti-osteoporosis effect. Therefore, the introduction of certain lipophilic compounds may improve the bioactivity of GA and broaden its application. As recommended by Nuti, et al.,<sup>23</sup> modification by sulfonamide groups may enhance the hydrophobicity and bioactivity of GA and therefore support the growth of cells. It is the character of sulfonamide to modify antibiotic ability and hydrophobicity by replacing hydrogen atoms on the para-position of the amino with a different heterocyclic structure.

In this study, we synthesized sulfonamido-based gallate ZXHA-TC and investigated its effect on the osteoblasts isolated from rat calvaria by detecting cell proliferation, the expression of osteogenic specific genes and proteins.

## MATERIALS AND METHODS

### Synthesis of ZXHA-TC

ZXHA-TC was prepared from GA and sulfadimoxine. The synthetic route is presented in Fig. 1 in detail. After the reactions, an appropriate amount of distilled water was added to the mixture, and the precipitated raw product was separated by vacuum filtration. The raw product was recrystallized in a tetrahydrofuran-methanol solvent system.



**Fig. 1.** The synthetic route of ZXHA-TC was presented. Reagents and conditions. (A) Acetyl oxide, oil bath, 120°C. (B) SOCl<sub>2</sub>, oil bath, 80°C. (C) Sulfadimoxine, THF, pyridine, ice bath. (D) HCl, THF, 60°C.

### Extraction and culture of primary osteoblasts

Primary osteoblasts were harvested from the bilateral parietal bone of 3 to 7 days' newborn Sprague Dawley rats by enzymatic digestion. After rats were put to death by cervical dislocation, the bilateral parietal bones were stripped clearly with sterile gauze in a sterile environment. After the connective tissue around the parietal bone was removed by 0.25% trypsin-ethylene diamine tetraacetic acid (EDTA) (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China), the bone was cut into pieces about 1×1 mm in a sterile vial and then digested with 1 mg/mL collagenase type I (Gibco-BRL, Carlsbad, CA, USA) in serum-free  $\alpha$ -modified Eagle's medium ( $\alpha$ -MEM, Gibco-BRL) for 3 h. After centrifugation at 1000 rpm for 5 min, isolated osteoblasts were suspended in  $\alpha$ -MEM containing 10% (v/v) fetal bovine serum (FBS, Gibco-BRL) and 1% (v/v) antibiotics (penicillin 100 U/mL, streptomycin 100 U/mL). Cultures were maintained in a 5%-CO<sub>2</sub> incubator (Thermo Scientific™ Forma Series II Water-Jacketed, Santa Ana, CA, USA) at 37°C with the culture medium changed every other day. At 80–90% confluence after about 7 days of culture, primary cells were used for subsequent experiments.

### Cell treatment

ZXHA-TC was dissolved in sodium hydroxide solution (NaOH, Sigma, St. Louis, MO, USA), its PH value was adjusted to 7.0 as stock solution, and it was then stored at 4°C. The stock solution of ZXHA-TC was then added to the cell cultures in various concentrations. Culture medium containing these various concentrations of ZXHA-TC was replaced every other day.

### Concentration screening and cytotoxicity assay

To detect the effect of ZXHA-TC on primary osteoblasts, concentration screening was assessed by the 3-(4,5)-dimethylthiazoliazoyl-3,5-di-phenyltetrazolium-romide (MTT; Sigma) method. Briefly, cells were detached by 0.25% trypsin-EDTA, suspended in  $\alpha$ -MEM containing 10% (v/v) FBS and 1% (v/v) antibiotics (penicillin 100 U/mL, streptomycin 100 U/mL) and seeded into a 96-well plate at a density of 1×10<sup>3</sup> cells/well. Additionally, the outside circle wells were sealed with phosphate buffered saline (PBS). After 24 hours of culture, the medium was replaced with 200  $\mu$ L culture medium containing ZXHA-TC ranging from 0  $\mu$ g/mL to 2.56×10<sup>5</sup>  $\mu$ g/mL. The solution of MTT in PBS was added to each well with a final concentration of 5 mg/mL after 3 days of interference. After 4 hours of incubation, the supernatant

was discarded, and 200  $\mu$ L Dimethyl sulfoxide (DMSO, Sigma) was added for formazan-crystal solubilization. After continuous gentle shaking for 10 minutes in a dark environment to dissolve formazan-crystal thoroughly and evenly, the absorbance value was measured at 570 nm with a microplate reader (Thermo Scientific Multiskan GO Microplate Spectrophotometer, Helsinki, Finland). All samples were done in triplicate, and results were shown as absorbance values. The detection of cytotoxicity was started by seeding 5×10<sup>3</sup> cells/well in 24-well plates and then treating with the concentrations of ZXHA-TC that had been selected by the previous concentration screening assay. After 2, 4, and 6 days, a solution of MTT in PBS was added to each well, with a final concentration of 5 mg/mL. After 4 hours of incubation, the supernatant was discarded and 1 mL DMSO was added for formazan-crystal solubilization. After continuous gentle shaking for 10 minutes in the dark to dissolve the DMSO thoroughly and evenly, samples at 200  $\mu$ L were randomly extracted from each of three parallel wells at the same concentration three times and transferred to 96-well plates. The absorbance value was measured at 570 nm with the microplate reader, and the results were shown as optical-density absorbance values.

### Cell viability assay

Cell viability was determined by fluorescein diacetate [FDA; Life Technologies (AB & Invitrogen), Carlsbad, CA, USA]-propidium iodide [PI; Life Technologies (AB & Invitrogen)] staining at 2, 4, and 6 days. Briefly, FDA and PI stock solutions were added to the cells at final concentrations of 5  $\mu$ g/mL and 20  $\mu$ g/mL, respectively, and incubated in a dark environment for 5 min at 37°C. Images were captured and statistically analyzed via laser scanning confocal microscope (Nikon A1, Tokyo, Japan).

### Cell proliferation assay

Cell proliferation was detected with a 5-bromo-2-deoxyuridine (BrdU) cell proliferation detection kit (Sigma), following the manufacturer's instructions. Briefly, primary osteoblasts were seeded in a 10-cm diameter culture dish. After 24 hours, cells were treated with ZXHA-TC at different concentrations for 4 days. Afterwards, cells were detached, suspended at a concentration of 1×10<sup>6</sup> cells in 500  $\mu$ L per tube, fixed, rinsed, denatured, labeled with BrdU, and detected by flow cytometry (BD Biosciences, Newark, NJ, USA), with excitation and emission settings at 488 nm and 520 nm, respectively.

### Cell actin cytoskeleton detection

After being cultured for 2, 4, and 6 days, cells were washed with PBS two times and fixed with 4% paraformaldehyde (Cytoskeleton, Denver, CO, USA) for 10 minutes. Subsequently, the cells were treated with 0.5% Triton-X (Gibco-BRL) for 5 min and then incubated in a dark environment with rhodamine phalloidin (Sigma) for 30 min. Eventually, after rinsing with PBS, the cells were incubated away from light with Hoechst 33258 (Sigma) for 5 min. Images were captured and statistically analyzed by a laser scanning confocal microscope.

### Real-time polymerase chain reaction (RT-PCR) assay

A real-time polymerase chain reaction (RT-PCR) assay was performed to detect the expression of runt-related transcription factor 2 (*RUNX2*), bone sialoprotein (*BSP*), osteocalcin (*OCN*) and alpha-1 type I collagen (*COL1A1*). At 2, 4, and 6 days, total RNA was extracted with an RNA extraction kit (Beijing ComWin Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. An equal amount of RNA (300 ng) was used as a template and was reverse transcribed into cDNA using a reverse transcription kit (Fermentas company, Pittsburgh, PA, USA), which was then amplified by using an SYBR-Green mix kit (Roche company, Berlin, Germany) on a real-time fluorescence quantitative instrument (realplex 4, Eppendorf Corporation, Hamburg, Germany). The primers used for PCR were designed as shown in Table 1. The dissociation curve of each primer pair was analyzed to confirm the primer specificity. Marker gene expressions were analyzed by the  $2^{-\Delta\Delta CT}$  method using  $\beta$ -actin. Each sample was repeated three times for each gene.

### ALP activity assay and ALP staining

Alkaline phosphatase (ALP) activity assay and ALP staining were carried out using ALP detection reagent kit and ALP staining kit (both purchased from Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) respectively following the manufacturer's instructions. After 2, 4,

and 6 days of culturing primary osteoblasts, culture medium was collected and slices were fixed for staining. After being centrifuged at 2500 rpm for 10 min, the supernatant of the medium was harvested for subsequent assay. Briefly, after adding buffer solution, matrix solution, water bath, and coloring, the absorbance value was measured at 520 nm with the microplate reader. Subsequently, the activity value was calculated with a computational formula. All samples were done in triplicate. Slices were washed with PBS, and staining was processed following the manufacturer's instructions. The staining and subsequent related staining were all observed and photographed by an inverted phase contrast microscope (Olympus, Tokyo, Japan) and its related image acquisition system (Nikon, Tokyo, Japan).

### Intracellular reactive oxygen species (ROS) assay

Production of intracellular ROS was assayed with a ROS detection reagent kit (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) according to the manufacturer's instructions. Briefly, primary osteoblasts were seeded in concentrations of  $2 \times 10^4$  cells/well in 6-well plates and cultured with different concentrations of ZXHA-TC after 24 hours. All samples were done in triplicate. After 2, 4, and 6 days of treatment, culture medium was replaced with  $10 \mu\text{M}$  2,7-dichlorofluorescein diacetate and incubated for 30 min at  $37^\circ\text{C}$  in the dark environment. After being washed with PBS three times, cells were detached using 0.25% trypsin-EDTA and suspended in a culture medium at a density of  $1 \times 10^5$  cells/mL. Samples at  $100 \mu\text{L}$  were randomly extracted from each of three parallel wells at the same drug concentration three times and transferred to 96-well plates. Oxidative burst was detected with a microplate fluorescence reader (FLx800, Biotek, Montpelier, VT, USA) with excitation and emission setting at 485 nm and 525 nm respectively.

### Statistical analysis

Data were presented as mean  $\pm$  standard deviation. All data were evaluated by a one-way analysis of variance. A least

**Table 1.** Primers for Real-Time Polymerase Chain Reaction

Gene name	Forward primer	Reverse Primer
$\beta$ -actin	5'-CCCATCTATGAGGGTTACGC-3'	5'-TTTAATGTACGCACGATTTC-3'
RUNX2	5'-CCAAGTGCCAGGTTCAACG-3'	5'-GGGATGAGGAATGCGCCCTA-3'
BSP	5'-CCGGGAGAACAATCCGTGCC-3'	5'-AAAGCACTCGCCATCCCCAA-3'
OCN	5'-CAGGTGCAAAGCCCAGCGAC-3'	5'-TGGGGCTCCAAGTCCATTGTT-3'
COL1A1	5'-CATGAGCCGAAGCTAACCC-3'	5'-CTCCTATGACTTCTGCGTCTGG-3'

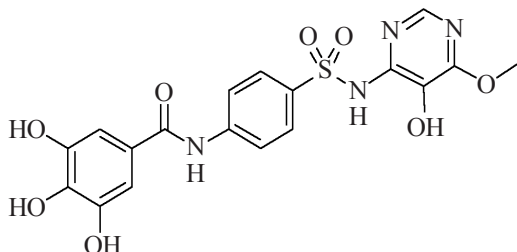
$\beta$ -actin, beta-actin; RUNX2, runt-related transcription factor 2; BSP, bone sialoprotein; OCN, osteocalcin; COL1A1, alpha-1 type I collagen.

significance difference test for multiple comparisons was performed for further evaluation of the data.  $p < 0.05$  was considered statistically significant.

## RESULTS

### Preparation of ZXHA-TC

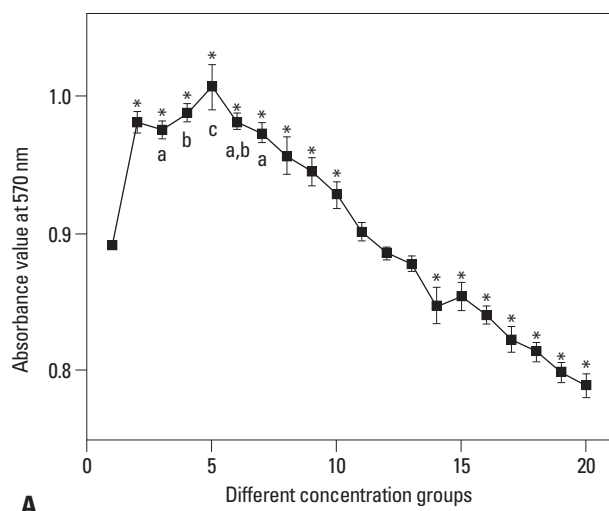
3,4,5-Trihydroxy-N-[4-(5-hydroxy-6-methoxy-pyrimidin-4-ylsulfamoyl)-phenyl]-benzamide,



white crystals, mp:  $>270^{\circ}\text{C}$ , ESI-MS: 447.1[M-H]<sup>-</sup>; <sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>)  $\delta$  10.58 (s, 1H, -SO<sub>2</sub>-NH), 10.25 (s, 1H, -CO-NH), 7.93–7.86 (dd, J=9.0 Hz, 4H, 4×Ar-H), 7.78 (s, 1H, Py-H), 6.94 (s, 2H, 2×Ar-H), 3.67 (s, 3H, -OCH<sub>3</sub>); <sup>13</sup>C-NMR (125 MHz, DMSO)  $\delta$  166.05, 157.98, 145.58, 144.00, 143.54, 137.31, 134.92, 129.85, 128.28, 124.40, 119.34, 107.44, 59.02.

### Concentration screening and cytotoxicity assay

As shown in Fig. 2A, ZXHA-TC indicated low cytotoxicity at the concentration ranging from  $6.25 \times 10^{-5}$  to  $2.5 \times 10^2$   $\mu\text{g}/\text{mL}$

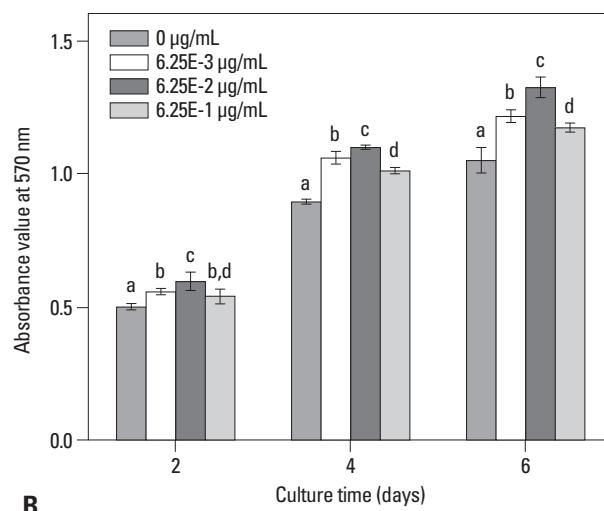


mL and presented no significant differences with the control group from  $5 \times 10^2$  to  $2 \times 10^3$   $\mu\text{g}/\text{mL}$ . Within this range, ZXHA-TC ranged from  $6.25 \times 10^{-3}$  to  $6.25 \times 10^{-1}$   $\mu\text{g}/\text{mL}$  significantly promoted cell growth ( $p < 0.05$ ). In contrast, ZXHA-TC ranged from  $4 \times 10^3$  to  $2.56 \times 10^5$   $\mu\text{g}/\text{mL}$  displayed an inhibitive effect on primary osteoblasts. Therefore, ZXHA-TC concentrations of  $6.25 \times 10^{-3}$   $\mu\text{g}/\text{mL}$ ,  $6.25 \times 10^{-2}$   $\mu\text{g}/\text{mL}$ , and  $6.25 \times 10^{-1}$   $\mu\text{g}/\text{mL}$  were chosen for further investigation.

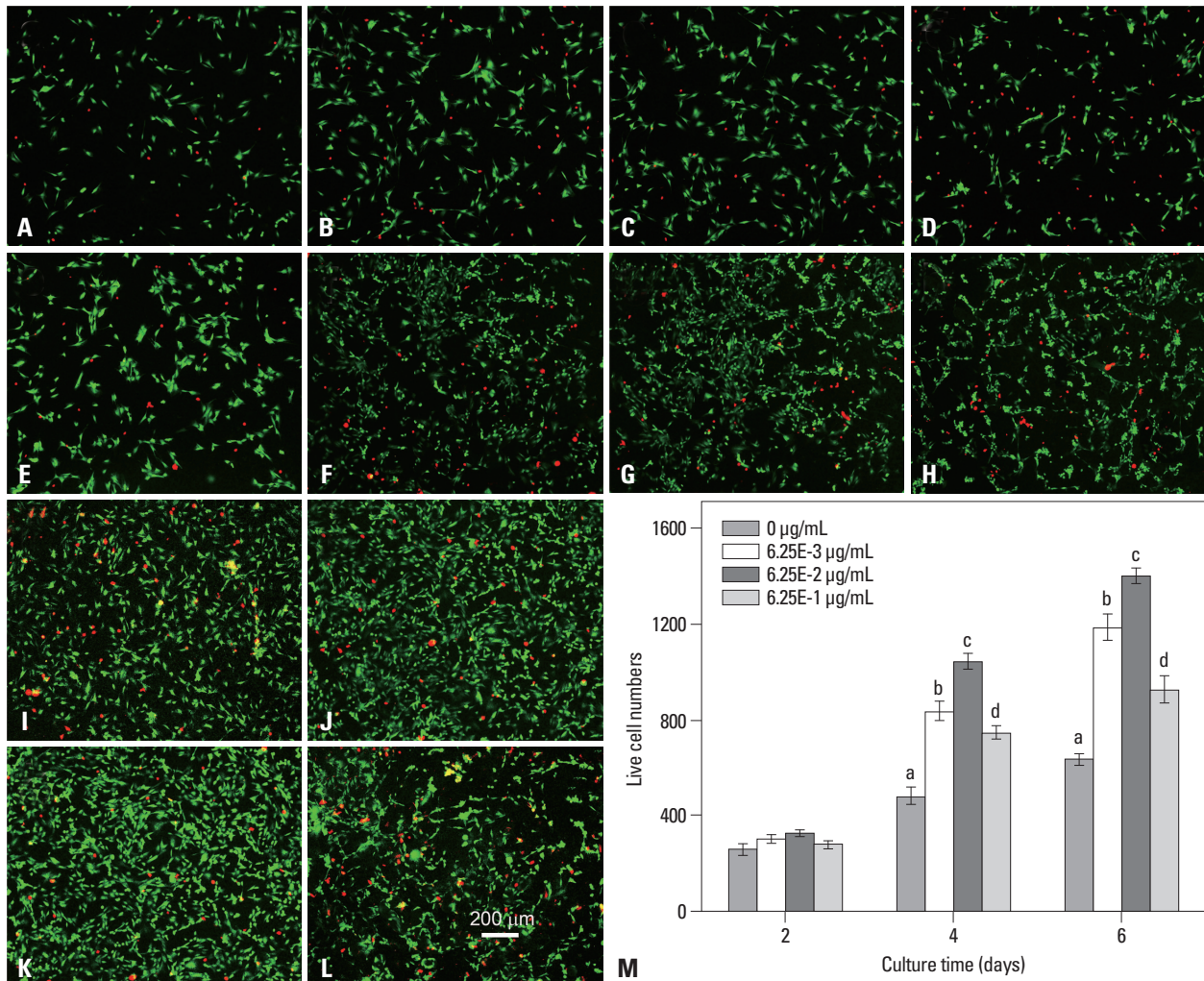
As shown in Fig. 2B, cytotoxicity was treated with ZXHA-TC at different concentrations (0  $\mu\text{g}/\text{mL}$ ,  $6.25 \times 10^{-3}$   $\mu\text{g}/\text{mL}$ ,  $6.25 \times 10^{-2}$   $\mu\text{g}/\text{mL}$ , and  $6.25 \times 10^{-1}$   $\mu\text{g}/\text{mL}$ ), and primary osteoblasts treated with ZXHA-TC grew significantly faster than that of the control in the same culture period. Among the three concentrations,  $6.25 \times 10^{-2}$   $\mu\text{g}/\text{mL}$  exhibited the strongest promoting effect on cell growth.

### Cell viability assay

Cell viability was determined by FDA-PI staining (Fig. 3), in which viable cells were stained green and dead cells were stained red. The results demonstrated that ZXHA-TC exerted a potent effect on primary osteoblast survival. The FDA-PI staining images indicated that there were more live cells in ZXHA-TC groups than in the control, which was consistent with the results of cell proliferation. The results implied that ZXHA-TC had a positive effect on cell growth. Among the experimental groups, a concentration of  $6.25 \times 10^{-2}$   $\mu\text{g}/\text{mL}$  was superior to others.



**Fig. 2.** MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was used to analyze the cytotoxicity of primary osteoblasts treated with ZXHA-TC at different concentrations. (A) A range of  $0$ – $2.56 \times 10^5$   $\mu\text{g}/\text{mL}$  of ZXHA-TC was chosen. The result shows that ZXHA-TC at the concentrations ranging from  $6.25 \times 10^{-5}$  to  $2.5 \times 10^2$   $\mu\text{g}/\text{mL}$  showed low cytotoxicity, among which  $6.25 \times 10^{-3}$   $\mu\text{g}/\text{mL}$  to  $6.25 \times 10^{-1}$   $\mu\text{g}/\text{mL}$  have a significantly positive effect on cell viability ( $p < 0.05$ ). (B) The effect of ZXHA-TC at the concentrations of  $6.25 \times 10^{-3}$   $\mu\text{g}/\text{mL}$ ,  $6.25 \times 10^{-2}$   $\mu\text{g}/\text{mL}$ , and  $6.25 \times 10^{-1}$   $\mu\text{g}/\text{mL}$  on primary osteoblasts was detected ( $n=9$ ). Different letters indicate that the two groups are significantly different from each other ( $p < 0.05$ ), and similar letters indicate no significant difference. Treatment of ZXHA-TC promoted cell viability in a dose-dependent manner; particularly at the concentration of  $6.25 \times 10^{-2}$   $\mu\text{g}/\text{mL}$ , ZXHA-TC enhanced cell viability the most. \*Significant difference ( $p < 0.05$ ,  $n=3$ ).



**Fig. 3.** Cell viability was determined by fluorescein diacetate-propidium iodide staining, in which viable cells were stained green and dead cells were stained red. (A-D) Staining of primary osteoblasts treated with ZXHA-TC at concentrations of 0 µg/mL,  $6.25 \times 10^{-3}$  µg/mL,  $6.25 \times 10^{-2}$  µg/mL, and  $6.25 \times 10^{-1}$  µg/mL at 2 days. (E-H) Staining of primary osteoblasts treated with ZXHA-TC at concentrations of 0 µg/mL,  $6.25 \times 10^{-3}$  µg/mL,  $6.25 \times 10^{-2}$  µg/mL, and  $6.25 \times 10^{-1}$  µg/mL at 4 days. (I-L) Staining of primary osteoblasts treated with ZXHA-TC at concentrations of 0 µg/mL,  $6.25 \times 10^{-3}$  µg/mL,  $6.25 \times 10^{-2}$  µg/mL, and  $6.25 \times 10^{-1}$  µg/mL at 6 days. (M) Statistical analysis of the data from the staining pictures from A-L (n=3). As time elapsed, more and more dead cells were found in each group. Comparatively, more viable cells were found in the ZXHA-TC-treated groups, which indicated the positive effect on the primary osteoblasts. Scale bar=200 µm.

### Cell proliferation assay

To measure cell proliferation, cells were stained with anti-BrdU FITC and analyzed using flow cytometry. As shown in Fig. 4, compared with the control, ZXHA-TC has a positive effect on cell proliferation, especially at the concentration of  $6.25 \times 10^{-2}$  µg/mL.

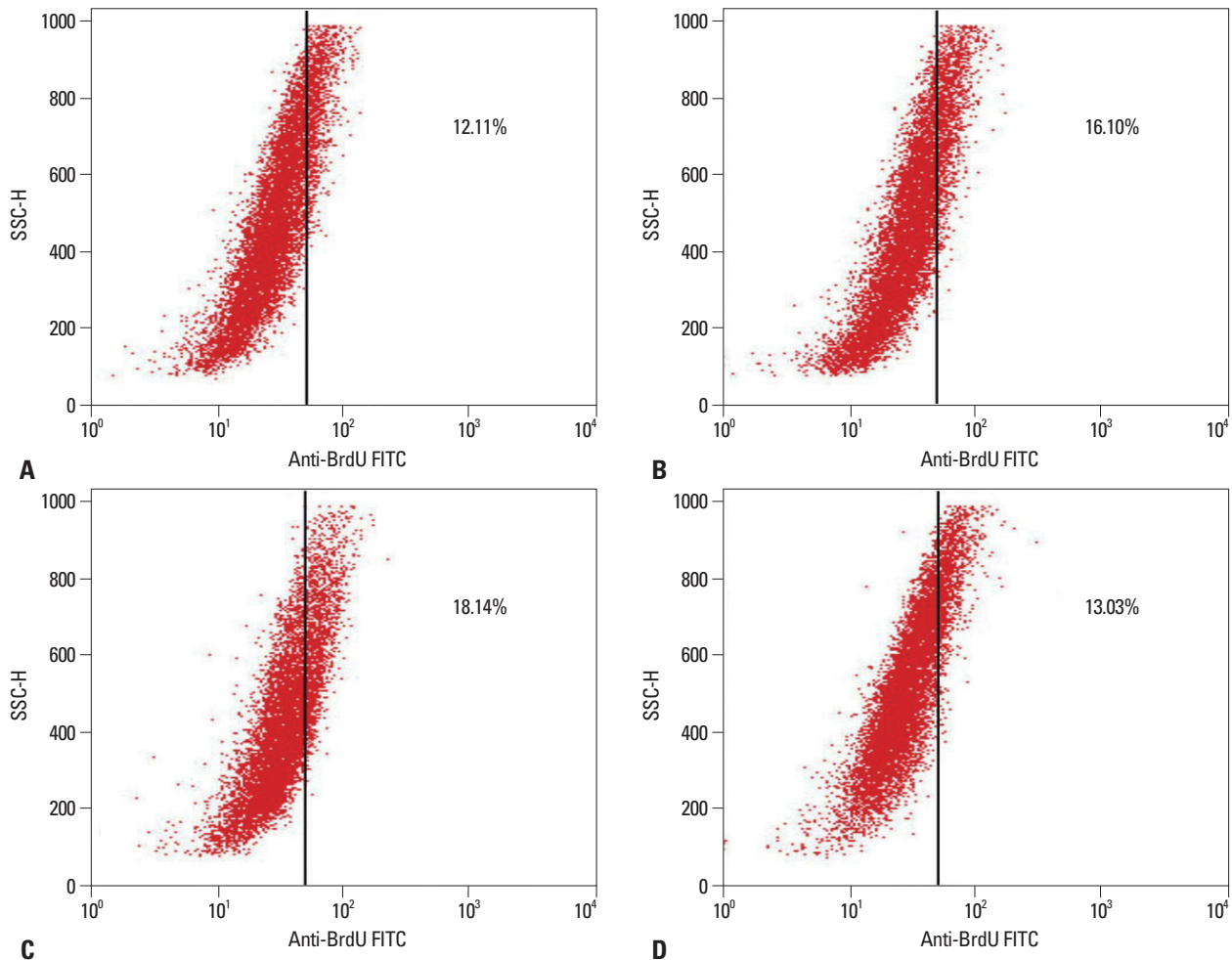
### Distribution of actin cytoskeleton

The attachment and spreading of osteoblasts was assessed by detecting the actin filaments with rhodamine phalloidin and Hoechst 33258 staining. As shown in Fig. 5, the cells in ZXHA-TC-treated groups grew in clumps with densely-distributed extracellular matrix, which indicates that cells in these groups communicated more closely than those in the control. Particularly, a ZXHA-TC concentration of  $6.25 \times$

$10^{-2}$  µg/mL exhibited more distributed actin filaments than others.

### Gene expression

The effect of ZXHA-TC on osteoblasts was further investigated through gene expression of *RUNX2*, *BSP*, *OCN*, and *COL1A1* after culturing cells for 2, 4, and 6 days. As shown in Fig. 6, *BSP* and *OCN* were all notably promoted by ZXHA-TC at the three observation points. The results indicated that ZXHA-TC could up-regulate the expressions of genes *BSP* and *OCN* continuously. However, the expressions of genes *RUNX2* and *COL1A1* were up-regulated remarkably from 2 to 4 days and then down-regulated gradually thereafter. Among all groups, the concentration of  $6.25 \times 10^{-2}$  µg/mL performed better than others, as demonstrated by the high-



**Fig. 4.** Cell proliferation was detected with a BrdU cell proliferation detection kit by flow cytometry. (A-D) Represent the results of primary osteoblasts treated with ZXHA-TC at concentrations of 0  $\mu\text{g/mL}$ ,  $6.25 \times 10^{-3}$   $\mu\text{g/mL}$ ,  $6.25 \times 10^{-2}$   $\mu\text{g/mL}$ , and  $6.25 \times 10^{-1}$   $\mu\text{g/mL}$ , respectively, at 4 days. The results showed that ZXHA-TC promoted cell growth markedly, especially at the concentration of  $6.25 \times 10^{-2}$   $\mu\text{g/mL}$ .

est expressions of *BSP* and *OCN*, both of which are osteogenic-specific marker genes.

#### ALP activity assay and ALP staining

As shown in Fig. 7A, despite no significant differences found between the ZXHA-TC-treated groups and the control at 2 days, primary osteoblasts treated with three concentrations all exhibited higher ALP activity than those in the control at 4 and 6 days. Overall, ALP activity tended to increase from 2 to 4 days and decrease slightly thereafter. This trend was further confirmed by ALP staining, shown in Fig. 7B-M. The quantitative and qualitative assays both demonstrated that ZXHA-TC was beneficial to osteogenic differentiation, and those at the concentration of  $6.25 \times 10^{-2}$   $\mu\text{g/mL}$  performed prominently.

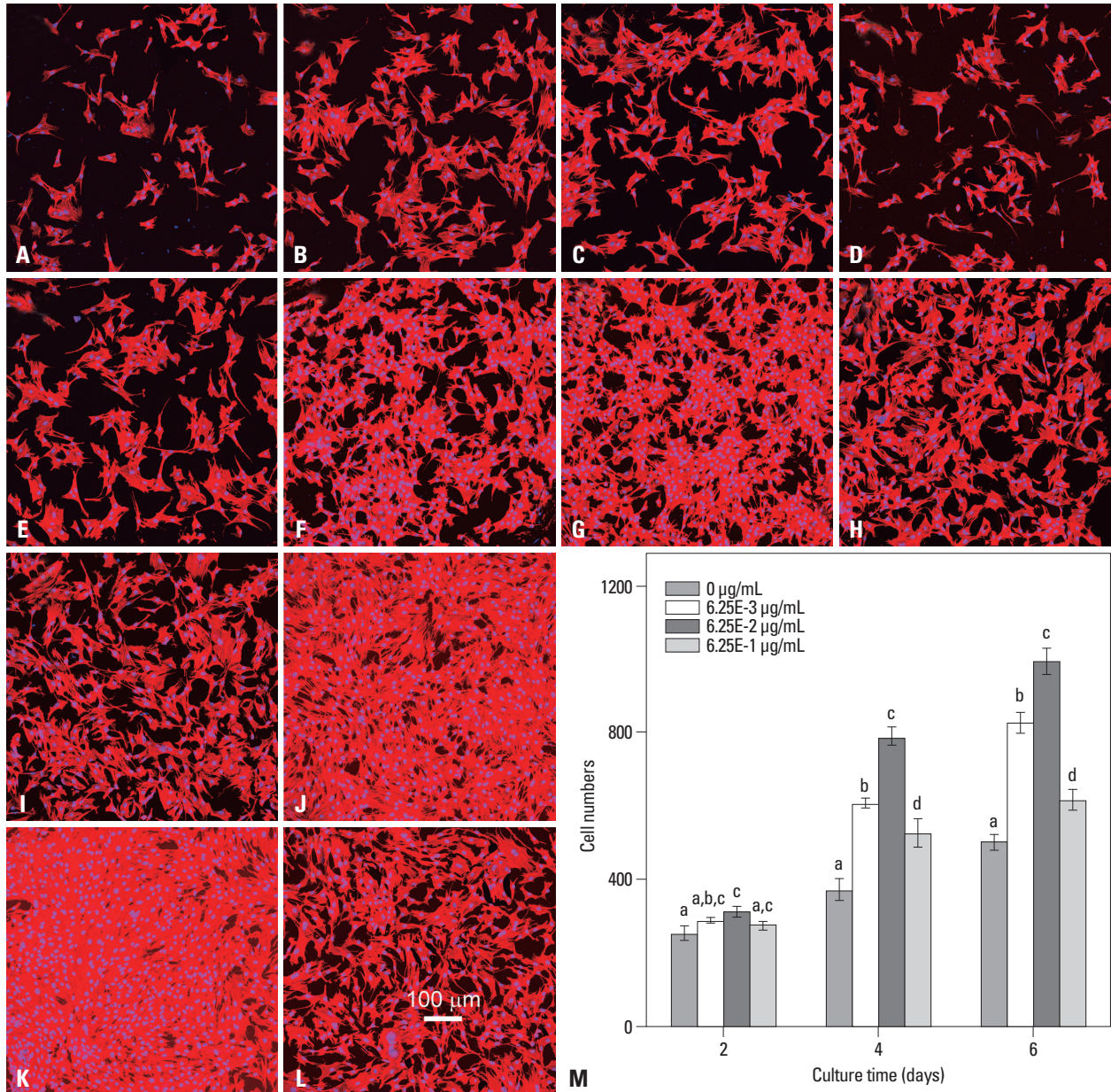
#### Intracellular ROS assay

To detect the level of intracellular ROS, the microplate fluo-

rescence reader was adopted to assess the intensity of fluorescence indirectly. As shown in Fig. 8, although no significant differences were found between the ZXHA-TC-treated groups and the control at 2 days, the level of intracellular ROS was significantly reduced compared with the control, and the concentration of  $6.25 \times 10^{-2}$   $\mu\text{g/mL}$  performed outstandingly.

## DISCUSSION

GA has been reported to have a strong antioxidant effect and is known to impact several pharmacological and biochemical pathways.<sup>17</sup> However, its inferior bioactivity has limited its clinical usage.<sup>17,20</sup> Moreover, the hydrophilicity of GA has hindered its application as a potent antioxidant, as appropriate hydrophobicity may be essential for promoting antioxidant abilities.<sup>17</sup> GA derivatives were report-



**Fig. 5.** Actin filament was detected with rhodamine phalloidin-Hoechst 33258 staining, in which the actin filament and nuclei were stained red and blue respectively. (A-D) Staining of primary osteoblasts treated with ZXHA-TC at concentrations of 0 µg/mL,  $6.25 \times 10^{-3}$  µg/mL,  $6.25 \times 10^{-2}$  µg/mL, and  $6.25 \times 10^{-1}$  µg/mL at 2 days. (E-H) Staining of primary osteoblasts treated with ZXHA-TC at concentrations of 0 µg/mL,  $6.25 \times 10^{-3}$  µg/mL,  $6.25 \times 10^{-2}$  µg/mL, and  $6.25 \times 10^{-1}$  µg/mL at 4 days. (I-L) Staining of primary osteoblasts treated with ZXHA-TC at concentrations of 0 µg/mL,  $6.25 \times 10^{-3}$  µg/mL,  $6.25 \times 10^{-2}$  µg/mL, and  $6.25 \times 10^{-1}$  µg/mL at 6 days. (M) Statistical analysis of the data from the staining pictures from A-L (n=3). Cells in ZXHA-TC-treated groups grew in clumps, with the density of the groups at the concentration of  $6.25 \times 10^{-2}$  µg/mL being most salient, which indicates that cells in these groups communicate more closely than those in the control. Scale bar=100 µm.

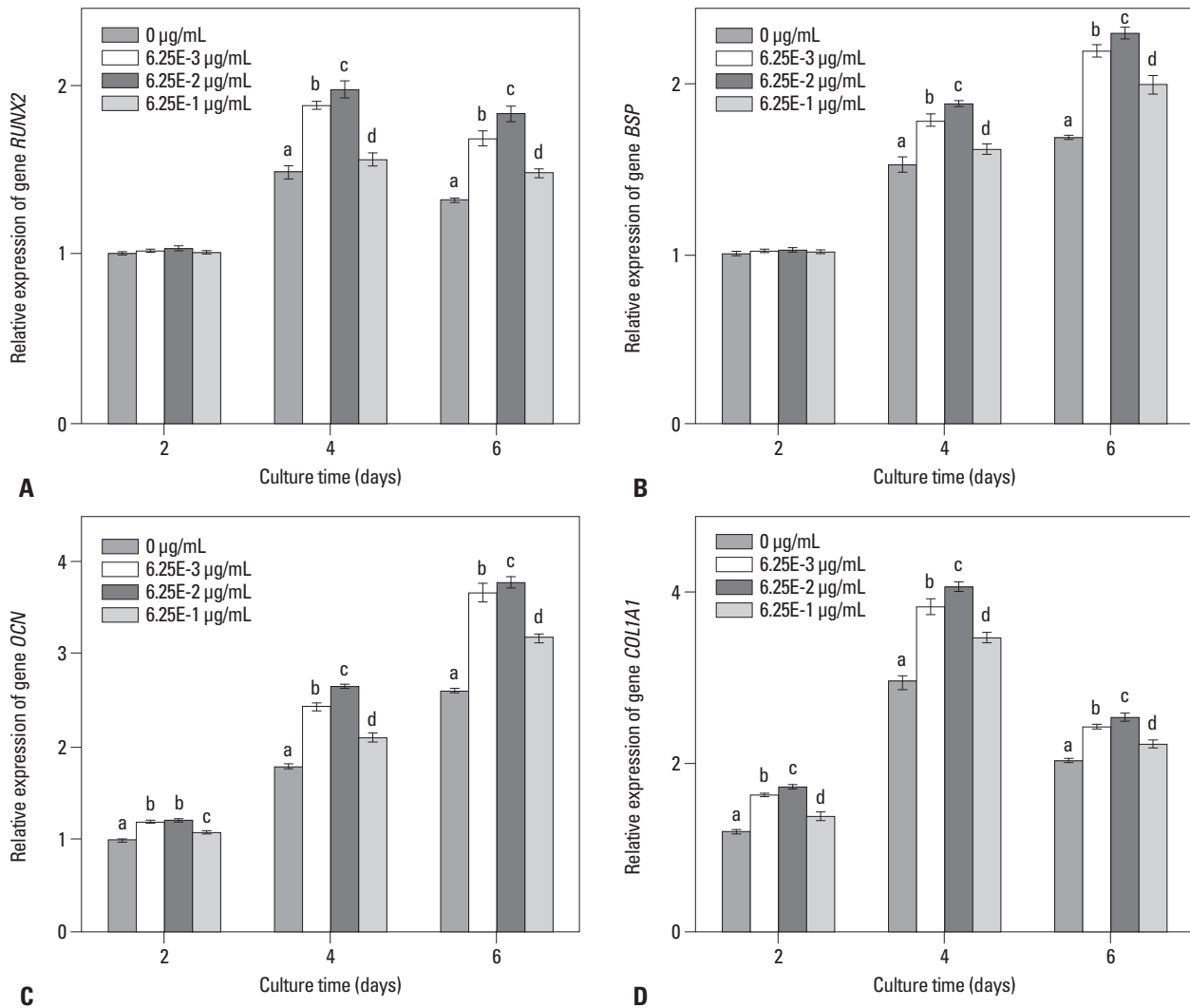
ed to have no toxicity to human erythrocytes even at higher concentrations.<sup>24</sup> To improve the pharmacological effect and biological properties of GA,<sup>25-28</sup> modification is of significance. In this study, ZXHA-TC was synthesized by coupling sulfonamide groups with GA, and the effects of ZXHA-TC on osteoblast growth and metabolism were investigated.

As evidenced by concentration screening and cytotoxicity assay, ZXHA-TC concentrations ranging from  $6.25 \times 10^{-5}$

to  $2.5 \times 10^2$  µg/mL were found to show no cytotoxicity, with a promoting effect at the range of  $6.25 \times 10^{-3}$  to  $6.25 \times 10^{-1}$  µg/mL (Fig. 2). Further exploration of cell viability, cell proliferation, and PCR analysis revealed that a ZXHA-TC concentration of  $6.25 \times 10^{-2}$  facilitated cell growth the most in all groups (Figs. 3-6).

Osteogenic specific genes *RUNX2*, *BSP*, *OCN*, and *COL1A1* were evaluated to determine the effect of the compound on osteoblasts. Expressions of *RUNX2* and *CO-*



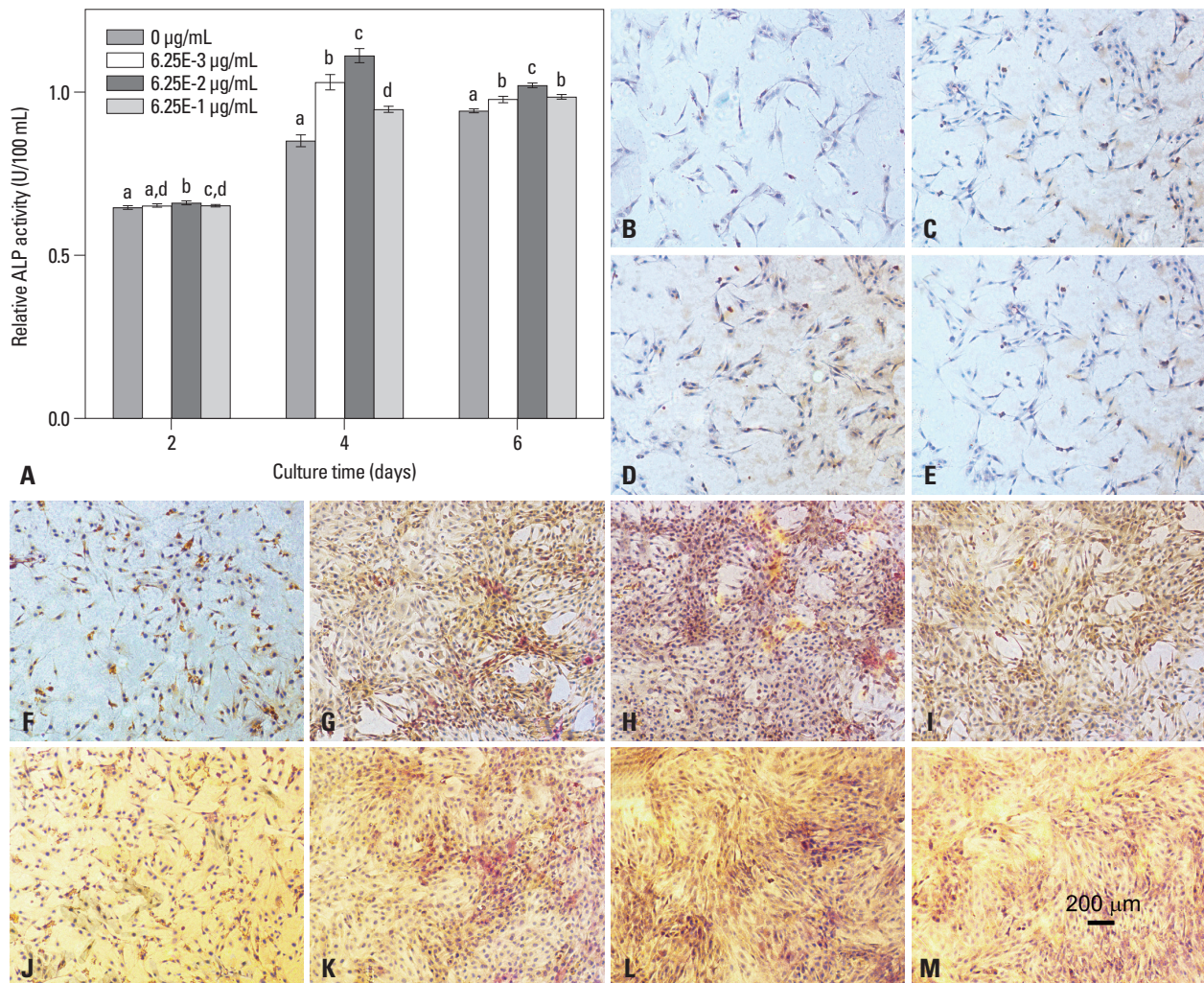


**Fig. 6.** A quantitative real-time polymerase chain reaction was used to analyze the progression of the expression of osteogenic genes *RUNX2* (A), *BSP* (B), *OCN* (C), and *COL1A1* (D) in primary newborn osteoblasts cultured in different groups for 2, 4, and 6 days. Compared with the control, expressions of the four genes were all up-regulated. However, the expressions of genes *BSP* and *OCN* were both up-regulated over time, and those of genes *RUNX2* and *COL1A1* were up-regulated from 2 to 4 days and down-regulated from 4 to 6 days. The minimum value was set to 1, and values are expressed as mean $\pm$ 2 SD. The bars with different letters at the same time are significantly different from each other ( $p < 0.05$ ;  $n = 3$ ), and those with similar letters show no significant difference. *RUNX2*, runt-related transcription factor 2; *BSP*, bone sialoprotein; *OCN*, osteocalcin; *COL1A1*, alpha-1 type I collagen.

*L1A1* were up-regulated remarkably from 2 to 4 days and down-regulated gradually from 4 to 6 days (Fig. 6). As the master regulator essential for osteoblast development and maturation, *RUNX2* plays a crucial role in the early stage of bone calcification, favoring bone formation and calcification.<sup>29</sup> However, over-expression of *RUNX2* in the late stage inhibits osteoblast maturation.<sup>30,31</sup> Thus, ZXHA-TC-oriented early up-regulation and subsequent down-regulation of *RUNX2* may be beneficial to the overall process of osteogenic differentiation. *COL1A1*, known as a major specific marker of bone matrix, is highly expressed during bone formation<sup>32</sup> though down-regulated in the process of osteoblast differentiation.<sup>33</sup> The expression of *COL1A1* increased in the early period (Fig. 6D) after treatment with

ZXHA-TC, indicating the positive role of ZXHA-TC on the formation of bone matrix. As a result, *BSP* and *OCN*, which are specific markers in mineralized tissues,<sup>34-37</sup> were up-regulated by ZXHA-TC continuously during the culture period (Fig. 6B and C), indicating that ZXHA-TC is beneficial to osteogenic differentiation and mineralization.

ALP produced by osteoblasts is found to be involved in the degradation of inorganic pyrophosphate, which provides sufficient local phosphate or inorganic pyrophosphate for mineralization to occur.<sup>32</sup> Commonly used as a marker of osteogenesis, ALP activity increases during the differentiation stage and declines when mineralization occurs.<sup>38,39</sup> In this study, ALP activity in the ZXHA-TC-treated groups increased early yet decreased later (Fig. 7A), contrary to the



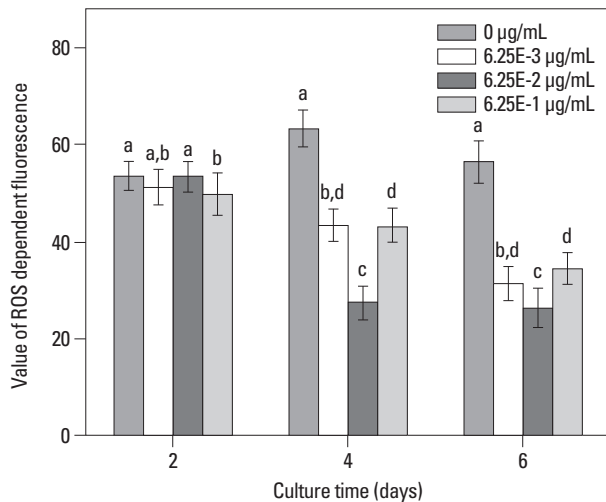
**Fig. 7.** Time-course of ALP activity and ALP staining of primary osteoblasts at different concentrations ( $0 \mu\text{g/mL}$ ,  $6.25 \times 10^{-3} \mu\text{g/mL}$ ,  $6.25 \times 10^{-2} \mu\text{g/mL}$ , and  $6.25 \times 10^{-1} \mu\text{g/mL}$ ) of ZXHA-TC are exhibited. (A) Relative ALP activity (units/100 mL) was expressed as mean $\pm$ 2 SD, and the activity in  $6.25 \times 10^{-2} \mu\text{g/mL}$  was significantly higher than other groups. ALP activity in the ZXHA-TC-treated groups increased from 2 to 4 days and decreased slightly from 4 to 6 days. However, the activity in the control increased over time. The bars with different letters at the same time are significantly different from each other ( $p < 0.05$ ;  $n = 3$ ), and those with similar letters indicate no significant difference. (B-E) Staining of primary osteoblasts treated with ZXHA-TC at concentrations of  $0 \mu\text{g/mL}$ ,  $6.25 \times 10^{-3} \mu\text{g/mL}$ ,  $6.25 \times 10^{-2} \mu\text{g/mL}$ , and  $6.25 \times 10^{-1} \mu\text{g/mL}$  at 2 days. (F-I) Staining of primary osteoblasts treated with ZXHA-TC at concentrations of  $0 \mu\text{g/mL}$ ,  $6.25 \times 10^{-3} \mu\text{g/mL}$ ,  $6.25 \times 10^{-2} \mu\text{g/mL}$ , and  $6.25 \times 10^{-1} \mu\text{g/mL}$  at 4 days. (J-M) Staining of primary osteoblasts treated with ZXHA-TC at concentrations of  $0 \mu\text{g/mL}$ ,  $6.25 \times 10^{-3} \mu\text{g/mL}$ ,  $6.25 \times 10^{-2} \mu\text{g/mL}$ , and  $6.25 \times 10^{-1} \mu\text{g/mL}$  at 6 days. ALP staining in the ZXHA-TC-treated groups was strengthened from 2 to 4 days and was weakened slightly from 4 to 6 days, and the concentration of  $6.25 \times 10^{-2} \mu\text{g/mL}$  performed best, which was in accordance the ALP activity results. Scale bar=200  $\mu\text{m}$ . ALP, alkaline phosphatase.

control, in which a continuous increase was observed during the whole period. This was further verified by ALP staining (Fig. 7B-M).

ROS have been appreciated as regulators of many cellular processes. However, over-expression of intracellular ROS may not only induce chromosomal damage<sup>40</sup> but also suppress osteoblastic proliferation and differentiation.<sup>16</sup> Thus, it is necessary to maintain ROS at a moderate level in order to benefit cellular processes. The maintenance of the ROS level may be essential in the prevention of osteoporosis. ZXHA-TC was found to have a prominent effect on reducing the level of intracellular ROS, especially at the con-

centration of  $6.25 \times 10^{-2} \mu\text{g/mL}$  (Fig. 8). All these results demonstrate that ZXHA-TC is beneficial to the overall process of osteogenic differentiation.

In summary, the effect of ZXHA-TC on primary osteoblasts was well-documented as verified by analysis of cell proliferation, cell viability, and RT-PCR results. The recommended dose of ZXHA-TC ranges from  $6.25 \times 10^{-3}$  to  $6.25 \times 10^{-1} \mu\text{g/mL}$ , particularly  $6.25 \times 10^{-2} \mu\text{g/mL}$ . The evidences found in this study indicate that ZXHA-TC may have a positive effect on osteogenic differentiation *in vitro*, implying it as a potential agent for the treatment of osteoporosis in a clinical application.



**Fig. 8.** Production of intracellular ROS was detected through the intensity of fluorescence indirectly with a microplate fluorescence reader. After being treated with different concentrations of ZXHA-TC (0 µg/mL,  $6.25 \times 10^{-3}$  µg/mL,  $6.25 \times 10^{-2}$  µg/mL, and  $6.25 \times 10^{-1}$  µg/mL) for 2, 4, and 6 days, primary osteoblasts were labeled with new synthesized DNA and assayed. The level of intracellular ROS was markedly reduced by ZXHA-TC, especially at the concentration of  $6.25 \times 10^{-2}$  µg/mL. ROS, reactive oxygen species.

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