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Effects of Different Concentrations of Collagenous Peptide from Fish Scales on Osteoblast Proliferation and Osteoclast Resorption

Chung-Hsuan Hu[1, #], Chun-Hsu Yao[2, 3, 4, 5, #], Tzu-Min Chan[6], Teng-Le Huang[6], Yang Sen[1], Chih-Yang Huang[2, 7, 8], and Chien-Yi Ho[2, 9]

[1]*Institute of Dermatology and Department of Dermatology, No.1 Hospital, Anhui Medical University, Hefei 230032, Anhui, People's Republic of China*
[2]*Biomaterials Translational Research Center, China Medical University Hospital, Taichung 40202, Taiwan, R.O.C.*
[3]*Department of Biomedical Imaging and Radiological Science, China Medical University, Taichung 40202, Taiwan, R.O.C.*
[4]*School of Chinese Medicine, China Medical University, Taichung 40402, Taiwan, R.O.C.*
[5]*Department of Biomedical Informatics, Asia University, Taichung 41354, Taiwan, R.O.C.*
[6]*Department of Medical Education and Research, China Medical University Beigan Hospital, Yunlin 65152, Taiwan, R.O.C.*
[7]*Department of Biotechnology, Asia University, Taichung 41354, Taiwan, R.O.C.*
[8]*Graduate Institute of Basic Medical Science, China Medical University, Taichung 40202, Taiwan, R.O.C.*
and
[9]*Department of Family medicine, China Medical University hospital, Taichung 40202, Taiwan, Republic of China*

Abstract

The incidence of osteoporosis has increased among the elderly population. Establishing a model of bone remodeling for screening new drugs is critical to identify safe and effective treatments for osteoporosis. In this study, we established a platform to investigate the therapeutic effects of collagenous peptides extracted from scales of two kinds of fish, namely, sparidae and chanos. These peptides were prepared using seven concentrations of collagenous peptide: 100, 80, 60, 40, 20, 10 and 1 mg/ml. Experimental results indicated that collagenous peptides promoted the proliferation of osteoblasts and inhibited the proliferation of mature osteoclasts; the effective concentration of collagenous peptide-sparidae was 10 mg/ml and that of collagenous peptide-chanos was 40 mg/ml. These findings demonstrate that, to a certain extent, collagenous peptides extracted from fish scales can be used to prevent osteoporosis to assist bone remodeling.

Key Words: collagenous peptides, osteoblasts, osteoclasts, osteoporosis, proliferation

Introduction

Bone is a unique connective tissue composed of calcified colloid mesh compounds, and it comprises a mineral component and an organic component with a mineral-to-organic ratio of 3:1 (1, 21). The three types

Corresponding authors: [1] Dr. Yang Sen, Institute of Dermatology and Department of Dermatology, No.1 Hospital, Anhui Medical University, Hefei 230032, Anhui, People's Republic of China. and [2] Dr. Chien-Yi Ho, Biomaterials Translational Research Center, China Medical University Hospital, No. 91 Hsueh-Shih Road, Taichung 40402, Taiwan, R.O.C. and [3] Dr. Chih-Yang Huang, Department of Biotechnology, Asia University, Taichung 41354, Taiwan, R.O.C. Tel: +886-4-22053366#3325, E-mail: samsam172@yahoo.com.tw
[#]Contributed equally to this work.
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of bone cells are osteoblasts, which are involved in bone formation; osteoclasts, which are responsible for bone resorption; and osteocytes. Osteoporosis reflects the balance result between bone modeling and remodeling. Bone modeling indicates the acquisition of the appropriate bone skeletal fragment with bone proliferation and resorption at the end of bone surfaces. Bone remodeling indicates the turnover of mature skeletal bone with the mechanism of coupled and balanced bone resorption and formation along the bone surface (13, 19, 20, 22, 26), and is defined as continuous renewal of bone tissue throughout an individual's life (7, 15). Effective bone remodeling requires coordination between osteoclasts and osteoblasts; and any imbalance can reduce or increase the rate of bone formation (5, 6, 13, 20, 22, 28). In clinical therapy, conventional hormone replacement therapy (HRT) can be utilized to treat or relieve disease symptoms caused by estrogen deficiency (30). However, HRT increases the risk of breast cancer, particularly in patients who have previously undergone HRT for more than a decade. Long-term HRT patients also exhibit significantly higher incidence rates of vein thrombosis, and the incidence of breast cancer in patients has even led the National Institute of Health to terminate a clinical HRT study prematurely. Therefore, establishing a platform to evaluate the factors that influence osteoblast proliferation and osteoclast resorption for treating or preventing osteoporosis is important. Type I collagen is the main structural component of bone formation; collagen and hydroxyapatite coating on the bone surface produce better bone remodeling than hydroxyapatite coating (18). In a study by Lee (14), the implant surface was coated with hydroxyapatite, collagenous peptides and bone morphogenetic protein-2. Results showed that only the collagenous peptides coating group promoted significantly greater peri-implant bone formation (14, 18). Although collagenous peptides play an important role in bone formation, sufficient evidence based on a study of different concentrations of collagenous peptides on bone remodeling is lacking. Collagen from cattle may be uncertain in safety because of possibility of bovine spongiform encephalopathy. Approximately half of the scales of fish (sparidae) are full of collagenous peptide. Chanos in particular is abundant in freshwater aquaculture, can be obtained easily, and is less expensive. This study presents the effect of different concentrations of collagenous peptide extracted from fish scales on osteoblast and osteoclast imbalance during bone remodeling.

Materials and Methods

Collagenous Peptide Extractions from Fish Scales

Collagenous peptides were obtained from Taiwan Fertilizer Co., Ltd. They are considered a food grade product and are ISO22000-certified. Collagenous peptide-sparidae and collagenous peptide-chanos were first dissolved in sterile water, and formulae were prepared using seven concentrations of each substance (collagenous peptide: 1,000, 800, 600, 400, 200, 100 and 10 mg/ml). The formulae were co-cultured with osteoblasts or osteoclasts by using a drug-to-medium ratio of 1:9 prior to relevant staining procedures and biochemical analyses. The effects of the tested substances on viability, differentiation capacity, nodule mineralization, proliferation and activity of osteoblasts are considered in that order.

Osteoblast Culture and Cell Viability Test with MTT

Human MG-63 osteosarcoma cell line was obtained from the Bioresource Collection and Research Center (number 60279) of the Food Industry Research and the Development Institute (FIRDI, Hsinchu, Taiwan, R.O.C). Cells were cultured in Modified Eagle's Medium (MEM) (Gibco, Life Technologies Corporation, Grand Island, NY, USA), 10% fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit Haemek, Kibbutz, Israel). Cultured osteoblasts were seeded in 24-well plates at a density of 1×10^4 cells/well. After a day, the culture medium was replaced with various concentrations of collagenous peptide, n = 6. After two days of incubation, the supernatant was removed. A solution of 5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT Assay) (USB Corporation, Cleveland, OH, USA) was added in each well. The culture plates were incubated at 37°C for 4 h to form insoluble formazan crystals. The solution was removed, and 500 μl/well dimethyl sulfoxide was added. Approximately 200 μl of the dissolved solutions was pipetted into 96-well plates, and the optical density values were measured using an ELISA reader (SpectraMax 340pc, Molecular Devices, Sunnyvale, CA, USA) at wavelengths of 570 and 650 nm.

Alkaline Phosphatase Assay (ALP) and Staining for Osteoblast Differentiation

After culturing for two days, the culture plates were washed three times with PBS. Approximately 200 μl/well of the ALP assay kit (procedure number N2770, Sigma, St. Louis, MO, USA) was then added, and the plates were incubated at 37°C for 30 min. Approximately 200 μl of the yellow p-nitrophenol product from each well was pipetted into 96-well plates, and the optical density value was measured using an ELISA reader at a wavelength of 405 nm. For staining, after culturing for two days, the culture plates were washed three times with PBS. Approximately 200 μl/

Table 1. Osteoblast proliferation test with different concentrations of collagenous peptide by MTT assay

(mg/ml)	Control	0.1	1	10	20	40	60	80	100
O.D. value Sparidae	0.96 ± 0.04	0.97 ± 0.02	0.92 ± 0.03	1.56 ± 0.04**	1.54 ± 0.03**	1.34 ± 0.04**	1.27 ± 0.15**	1.25 ± 0.30**	1.37 ± 0.09**
O.D. value Chanos	0.93 ± 0.03	0.97 ± 0.03	0.98 ± 0.05	1.00 ± 0.05*	1.07 ± 0.06**	1.21 ± 0.05**	1.04 ± 0.06**	0.62 ± 0.03**	0.56 ± 0.12**

Results are expressed as O.D. values of the control (*$P < 0.05$; **$P < 0.01$).

Table 2. Osteoblast differentiation test with different concentrations of collagenous peptide by ALP assay

(mg/ml)	Control	1	10	20	40	60	80	100
O.D. value Sparidae	0.164 ± 0.003	0.171 ± 0.005	0.168 ± 0.007	0.172 ± 0.005	0.186 ± 0.004**	0.203 ± 0.002**	0.200 ± 0.013**	0.175 ± 0.002**
O.D. value Canos	0.194 ± 0.019	0.189 ± 0.008	0.224 ± 0.010**	0.226 ± 0.019**	0.264 ± 0.021**	0.245 ± 0.010**	0.231 ± 0.002**	0.216 ± 0.010**

Results are expressed as O.D. values of the control (**$P < 0.01$).

well of the stationary liquid (acetone-citrate-formaldehyde) was then added for 30 seconds. Then, 400 μl of the ALP staining kit (procedure number 86R, Sigma) was added for 15 min in the dark at room temperature. Finally, 200 μl hematoxylin solution was added as a counterstain.

Assessment of Mineralized Nodules with Von Kossa Stain

The culture medium was added with 50 μg/ml L-ascorbic acid (A8960, Sigma), 10 mM β-glycerol phosphate (Calbiochem), and 10 nM dexamethasone-water soluble (D2915, Sigma). The culture medium was changed every three days. After culturing for 21 days, the cultures were fixed with 2% glutaraldehyde for 20 min. Then, 500 μl of 5% silver nitrate (Showa Chemical Co. LTD., Tokyo, Japan) was added. The culture plates were placed under UV light for one hour u ntil a brown silver phosphate sediment appeared, and then washed with 5% sodium thiosulfate (Sigma-Aldrich Laborchemikalien GmbH) for 2-3 min. Mineralized nodules were observed by using an inverted optical microscope.

Osteoclast Culture and Cell Viability Test with MTT Assay

Murine macrophage RAW 264.7 cell line was obtained from the Bioresource Collection and Research Center (number 60001) of FIRDI. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Life Technologies Corporation, Grand Island, NY, USA), 10% FBS. Cultured RAW 264.7 cells were seeded in 48-well plates at a density of 2×10^3 cells/well. After a day, the culture medium was replaced with 50 ng/ml RANKL (Alexis Biochemicals, Lausen, Switzerland) in α-minimal essential medium (α-MEM) (Gibco) with 5% FBS for six days. Various concentrations of collagenous peptide were also added to the cells on days 1 to 6 or days 6 to 8. Osteoclast proliferation and differentiation were examined by MTT assay.

Evaluation of Tartrate-Resistant Acid Phosphatase (TRAP) Activities for Osteoclast Differentiation

After 6 or 8 days of culture, osteoclasts were observed by using TRAP stain (procedure number 387A, Sigma). Briefly, 200 μl/well of the stationary liquid (acetone-citrate-formaldehyde) was added for 30 sec. Then, 300 μl of TRAP stain was added, and the plate was incubated at 37°C for one hour. Finally, 200 μl hematoxylin solution was added as a counterstain.

Statistical Analysis

Statistical comparisons among the groups were performed by using one-way analysis of variance (SPSS 16.0). Tukey's test was then used as *post hoc* test.

Results

Effects of Osteoblast Proliferation

The effect of collagenous peptide-sparidae on osteoblast proliferation strengthened with increasing concentration, with the most pronounced effect being achieved at a concentration of 10 mg/ml. Moreover, osteoblast viability strengthened as the concentration of collagenous peptide-chanos increased from 10 mg/ml to 60 mg/ml, but worsened as the concentration increased to 80 mg/ml. The concentration of collagenous peptide-chanos that maximized osteoblast proliferation was 40 mg/ml (Table 1).

Effects of Osteoblast Differentiation

The osteoblast activity tested by ALP assay indicated that collagenous peptide-sparidae concentrations between 40 and 100 mg/ml promoted ALP activity; the concentration of 60 mg/ml had the strongest ef-

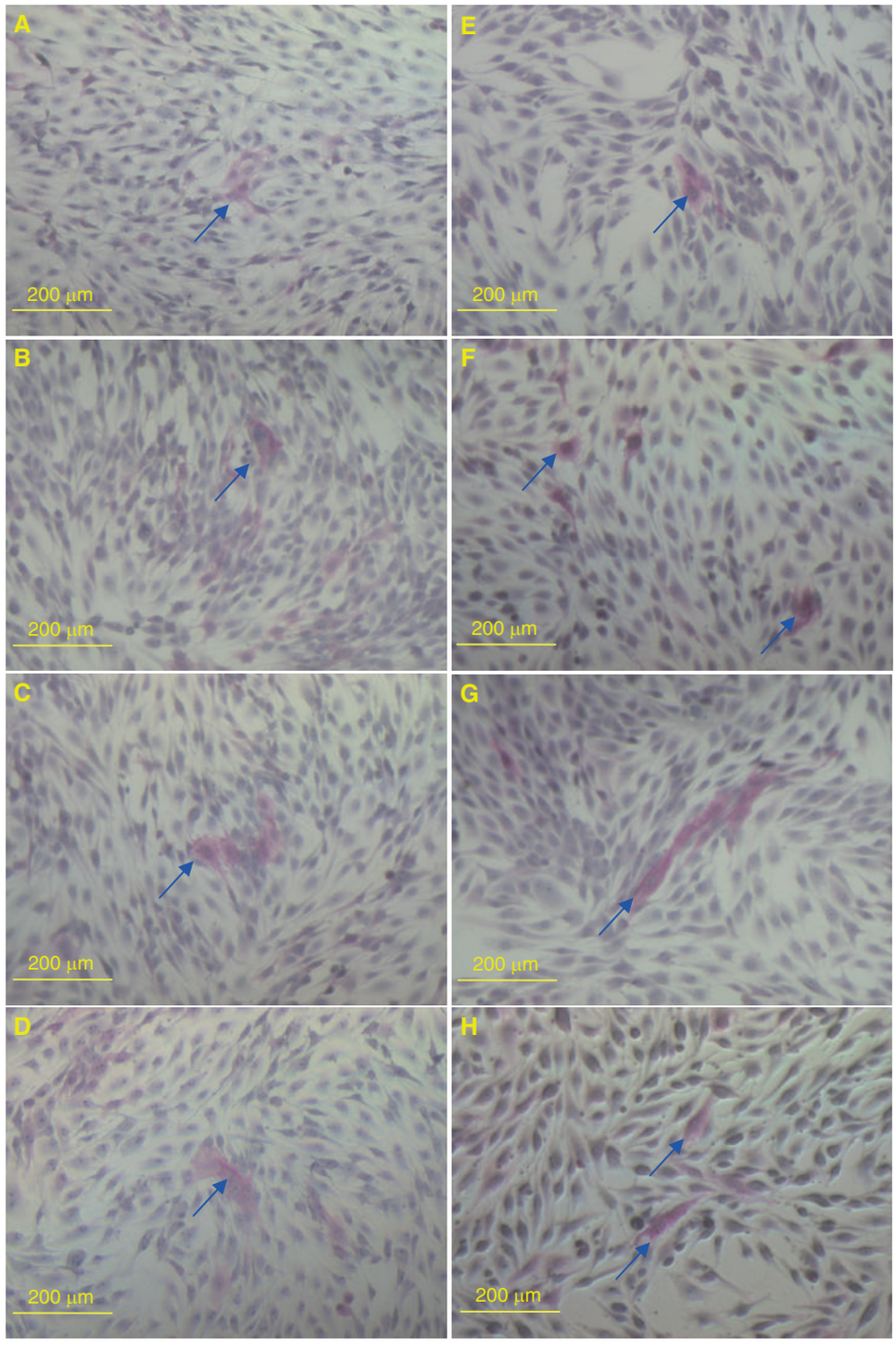

Fig.1. ALP staining of osteoblasts treated with different concentrations of collagenous peptide-sparidae in (A) the control group and at (B) 1 mg/ml, (C) 10 mg/ml, (D) 20 mg/ml, (E) 40 mg/ml, (F) 60 mg/ml, (G) 80 mg/ml and (H) 100 mg/ml concentrations. Arrows indicate accumulation of ALP.

fect. Collagenous peptide-chanos concentrations between 10 and 100 mg/ml promoted ALP activity; 40 mg/ml had the strongest effect (Table 2). The osteoblast differentiation that was tested using ALP stain revealed that collagenous peptide-sparidae concentrations between 40 and 100 mg/ml promoted osteoblast differentiation better than the control group. Furthermore, collagenous peptide-chanos concentrations between 10 and 100 mg/ml resulted in better osteoblast differentiation. Among the two fish col-

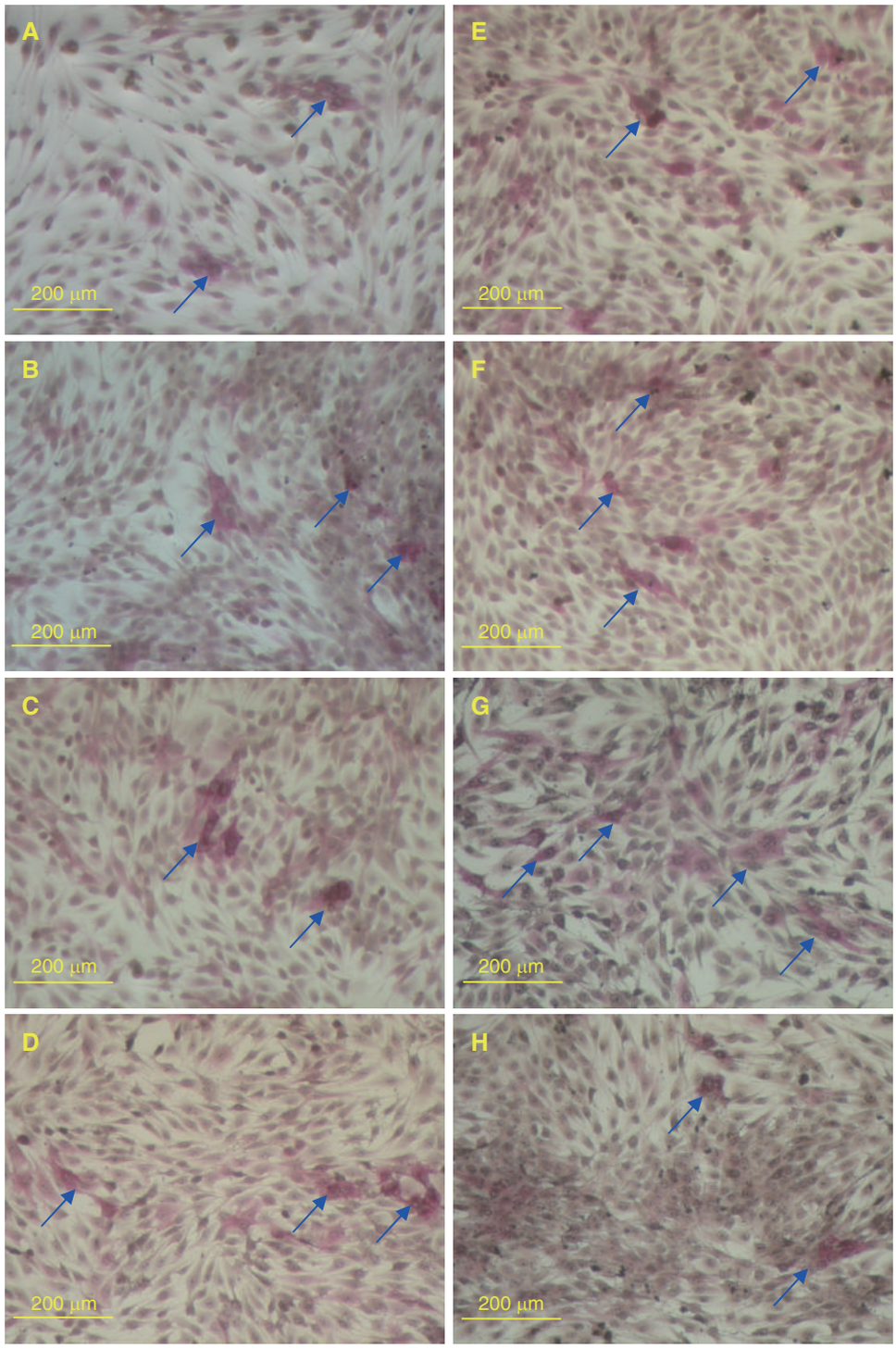

Fig. 2. ALP staining of osteoblasts treated with different concentrations of collagenous peptide-chanos in (A) the control group and at (B) 1 mg/ml, (C) 10 mg/ml, (D) 20 mg/ml, (E) 40 mg/ml, (F) 60 mg/ml, (G) 80 mg/ml and (H) 100 mg/ml. Arrows indicate accumulation of ALP.

lagenous peptides, collagenous peptide-chanos exhibited higher osteoblast activity (Figs 1 and 2).

Effects of Osteoblast Nodule Mineralization with Von Kossa Stain

Osteoblast nodule mineralization was the best long-term bone formation index. Observations under an optical microscope revealed mineral nodules in specimens with collagenous peptide concentrations between 1 and 100 mg/ml. The maximum quantity of

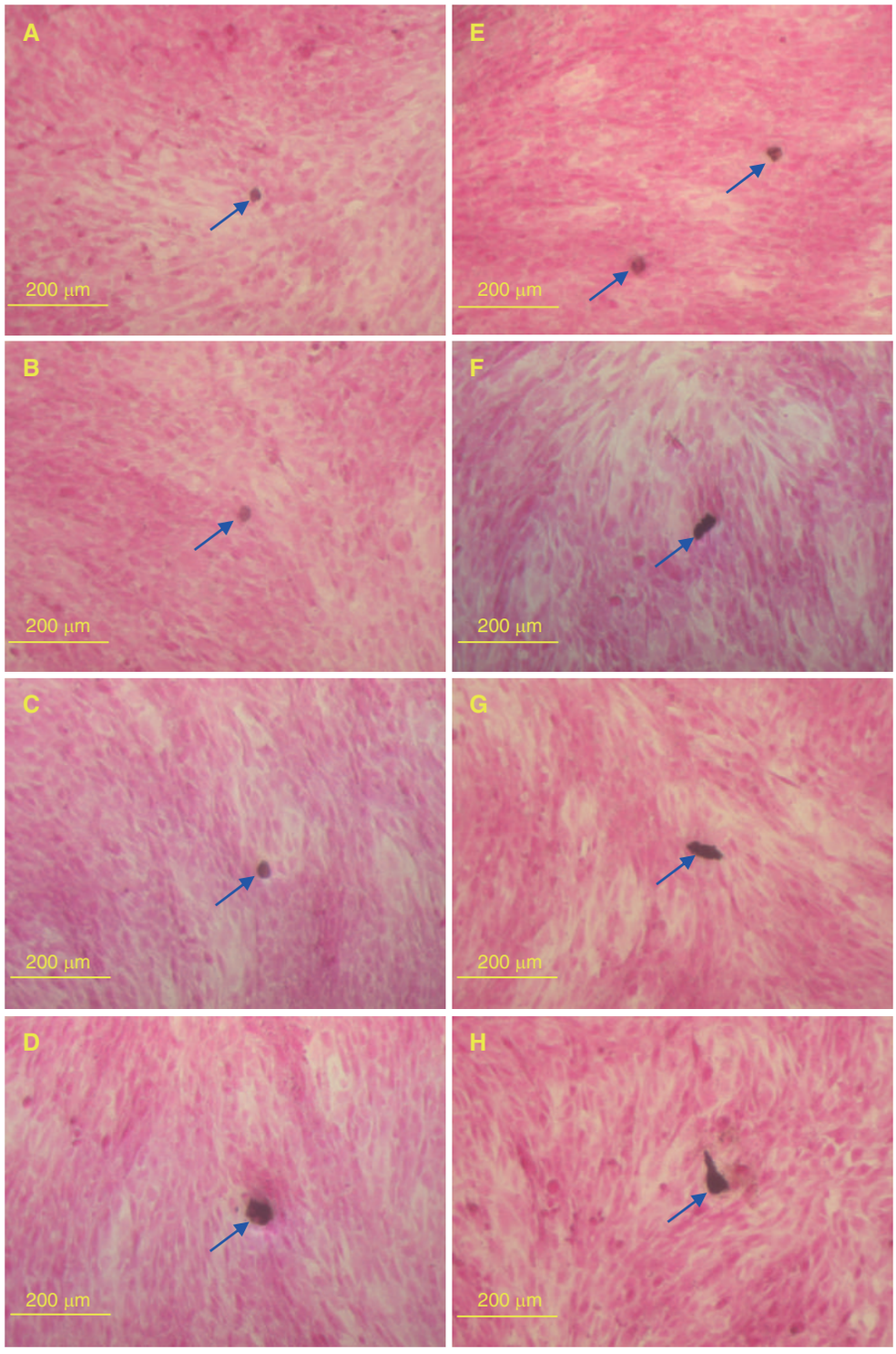

Fig. 3. Von Kossa's staining of osteoblasts treated with different concentrations of collagenous peptide-sparidae in (A) the control group and at (B) 1 mg/ml, (C) 10 mg/ml, (D) 20 mg/ml, (E) 40 mg/ml, (F) 60 mg/ml, (G) 80 mg/ml and (H) 100 mg/ml. Arrows indicate deposition of mineralized matrix.

mineral nodules was achieved with the collagenous peptide-sparidae concentration of 20 mg/ml and the collagenous peptide-chanos concentration of 60 mg/ml (Figs. 3 and 4).

Effects of Osteoclast Proliferation

This section focused on osteoclasts that were co-cultured with various concentrations of collagenous

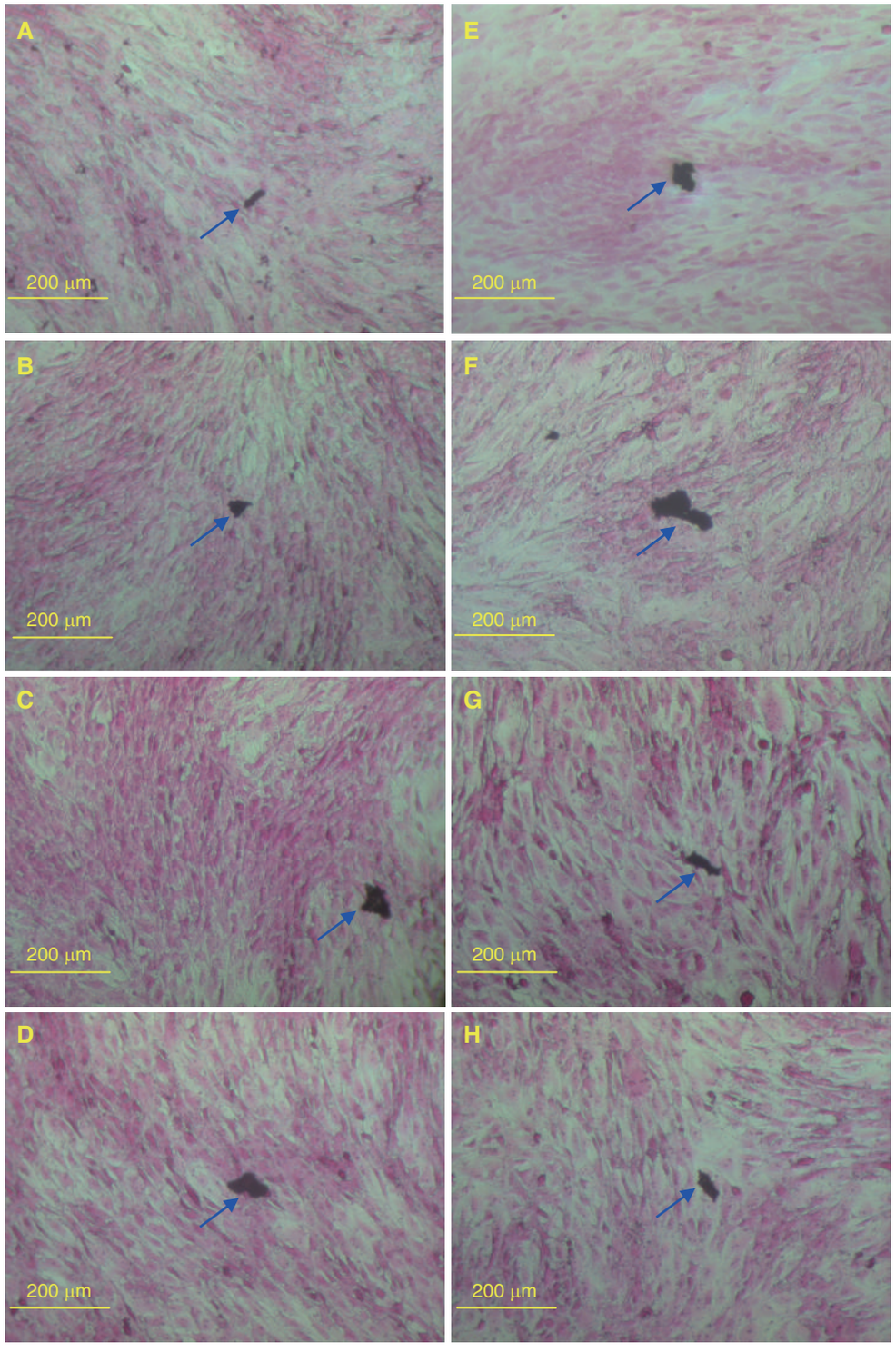

Fig. 4. Von Kossa's stain of osteoblasts treated with different concentrations of collagenous peptide-*c*hanos in (A) the control group and at (B) 1 mg/ml, (C) 10 mg/ml, (D) 20 mg/ml, (E) 40 mg/ml, (F) 60 mg/ml, (G) 80 mg/ml and (H) 100 mg/ml. Arrows indicate deposition of mineralized matrix.

peptide-sparidae and collagenous peptide-chanos that were added on days 1 to 6 and on days 6 to 8, respectively. On days 1 to 6, collagenous peptide-sparidae concentrations between 1 and 60 mg/ml promoted osteoclast proliferation significantly, whereas concentrations between 60 and 100 mg/ml suppressed osteoclast proliferation. On days 6 to 8, collagenous peptide-sparidae concentrations between 60 and 100

Table 3. Osteoclast proliferation test with different concentrations of collagenous peptide administered on days 6 to 8 by MTT assay

(mg/ml)	Control	1	10	20	40	60	80	100
O.D. value Sparidae	3.167 ± 0.132	3.256 ± 0.069	3.288 ± 0.067	3.269 ± 0.039	2.982 ± 0.118	2.571 ± 0.213**	2.394 ± 0.143**	2.363 ± 0.154**
O.D. value Canos	3.282 ± 0.090	0.444 ± 0.044**	0.545 ± 0.085**	0.302 ± 0.056**	0.198 ± 0.030**	0.112 ± 0.028**	0.118 ± 0.061**	0.066 ± 0.019**

Results are expressed as O.D. values of the control (**$P < 0.01$).

mg/ml inhibited osteoclast proliferation significantly. On days 1 to 6, collagenous peptide-chanos concentrations between 1 and 10 mg/ml promoted osteoclast proliferation significantly. By contrast, concentrations between 20 and 100 mg/ml suppressed osteoclast proliferation. On days 6 to 8, concentrations between 1 and 100 mg/ml inhibited osteoclast proliferation significantly.

The results of the MTT assays revealed that during the proliferation of osteoclast precursor cells between days 1 and 6, particular concentrations of collagenous peptide-sparidae (1 mg/ml to 60 mg/ml) and collagenous peptide-chanos (1 mg/ml to 10 mg/ml) promoted osteoclast proliferation. However, in mature osteoclasts that were added collagenous peptide between days 6 and 8, both collagenous peptide-sparidae and collagenous peptide-chanos inhibited osteoclast proliferation (Table 3).

Effects of Osteoclast Differentiation with TRAP Staining

This section focused on osteoclasts that were co-cultured with various concentrations of collagenous peptide-sparidae and collagenous peptide-chanos, added on days 1 to 6 and on days 6 to 8, with TRAP staining. Results of TRAP staining demonstrated that during the differentiation of osteoclast precursor cells with the substance added on days 1 to 6, collagenous peptide-sparidae did not promote differentiation. In mature osteoclasts with the substance added between days 6 and 8, collagenous peptide-sparidae also did not significantly affect osteoclast activity. Comparisons with the control group revealed that 10 mg/ml collagenous peptide-chanos promoted osteoclast differentiation. By contrast, concentrations between 20 mg/ml and 100 mg/ml inhibited differentiation on days 1 to 6. Comparisons with the control group showed that collagenous peptide-chanos between 1 and 100 mg/ml inhibited osteoclast differentiation on days 6 to 8 (Figs. 5 and 6).

Discussion

Adequate concentrations of collagenous peptide are assumed to improve osteoblast proliferation and osteoclast inhibition. However, few studies discuss the effects of different concentrations of collagenous peptide on bone remodeling. Yamada indicates that fish collagen peptide has a positive effect on osteoblastic cells and suggests potential benefits of collagenous peptide fish scale in bone tissue engineering (8, 25). In our study, collagenous peptide-sparidae at 10 mg/ml and collagenous peptide-chanos at 40 mg/ml achieved the best effect on osteoblast proliferation.

Collagenous peptide also enhances the differentiation capacity of osteoblasts (2-4, 9-11). Collagenous peptide-sparidae at 40 to 100 mg/ml and collagenous peptide-chanos at 1 to 100 mg/ml have identical pharmaceutical properties on osteoblast differentiation. Mizuno *et al*. showed that type I collagen could induce osteoblastic differentiation of bone marrow cells mediated by collagen-$\alpha 2\beta 1$ integrin interaction (17, 18).

High concentrations of collagenous peptide-sparidae (80 mg/ml to 100 mg/ml) and collagenous peptide-chanos (20 mg/ml to 100 mg/ml) inhibited the proliferation of osteoclast precursor cells significantly on days 1 to 6. On days 6 to 8, collagenous peptide-chanos had significantly better inhibition abilities of mature osteoclasts at higher concentrations (12, 16, 21, 24, 27, 29). Schultz *et al.* described collagen as osteoclast-associated receptor ligands, and collagenous peptides can induce the activity of NF-κB-dependent osteoclast genesis (25).

During the differentiation of osteoclast precursor cells on days 1 to 6, collagenous peptide-sparidae did not promote differentiation, and only a specific concentration of collagenous peptide-chanos (10 mg/ml) facilitated the differentiation. In mature osteoclasts on days 6 to 8, collagenous peptide-sparidae did not affect osteoclast activity significantly, and collagenous peptide-chanos suppressed osteoclast activity. The results appear inconsistent with previous findings that indicate that intrafibrillar mineralization of collagen releases osteopontin and influences the activity of osteoclasts (23).

In summary, osteoblasts are differentiated to improve bone proliferation, but the activity of osteoclasts is inhibited by collagenous peptide from fish scales. These results demonstrate that collagenous peptide from fish scales can have potential effects, to a certain extent, on osteoporosis, which balances osteoblast and osteoclast abilities. A limitation of this study is that this is an *in vitro* study. Further advanced study must be conducted *in vivo*.

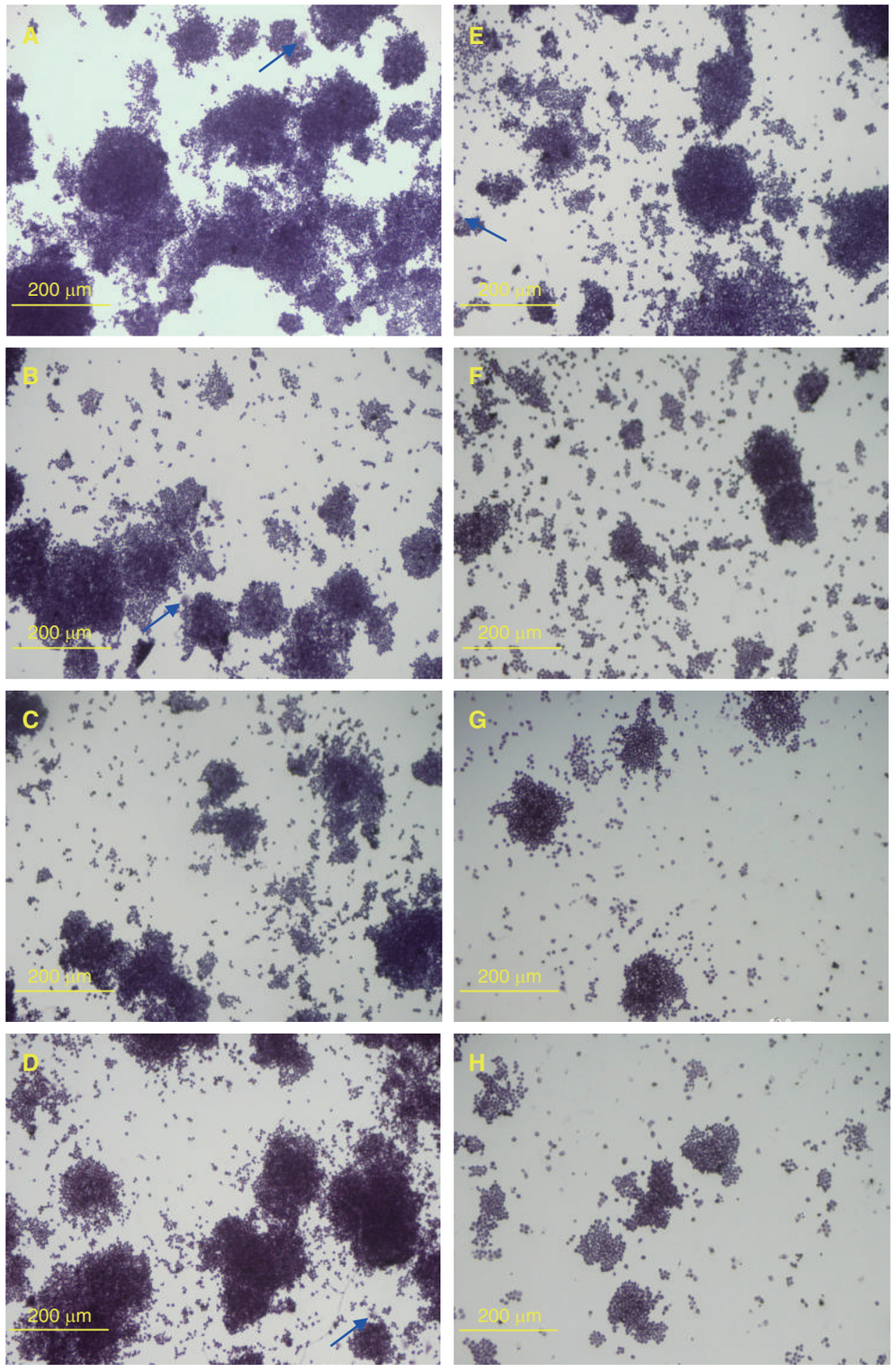

Fig. 5. TRAP staining of osteoblasts treated with different concentrations of collagenous peptide-sparidae at day 6 to 8 in (A) the control group and at (B) 1 mg/ml, (C) 10 mg/ml, (D) 20 mg/ml ,(E) 40 mg/ml, (F) 60 mg/ml, (G) 80 mg/ml and (H) 100 mg/ml. Arrows indicate osteoclasts.

Declaration of Conflicting Interest

The authors declare no conflicts of interest.

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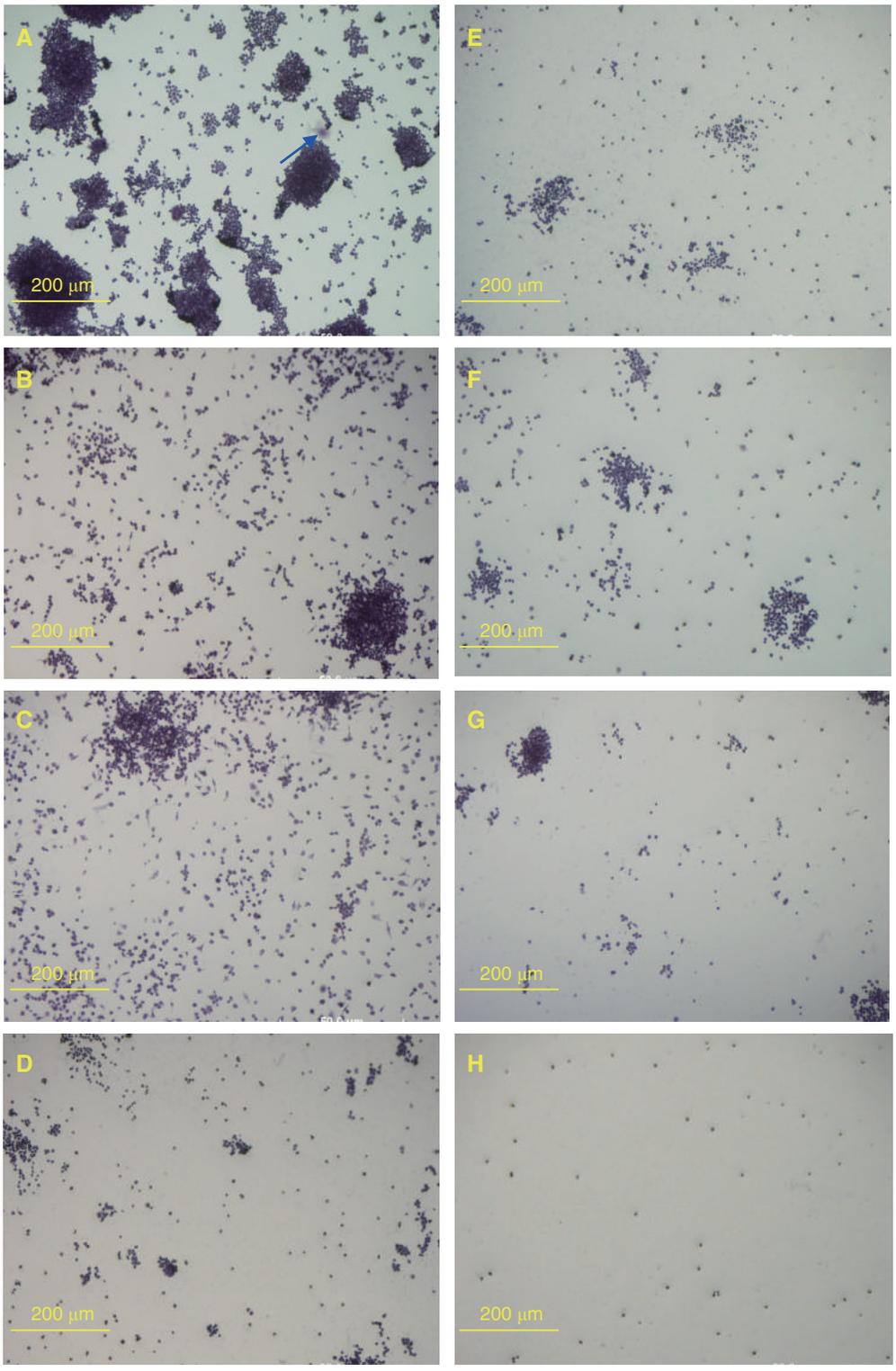

Fig. 6. TRAP staining of osteoblasts treated with different concentrations of collagenous peptide-chanos at day 6 to 8 in (A) the control group and at (B) 1 mg/ml, (C) 10 mg/ml, (D) 20 mg/ml, (E) 40 mg/ml, (F) 60 mg/ml, (G) 80 mg/ml and (H) 100 mg/ml. Arrows indicate osteoclasts.

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