We determined the effects of yolk water-soluble protein (YSP) on bone resorption. YSP potently suppressed osteoclastogenesis from bone marrow-derived precursor cells driven by tumor necrosis factor-α (TNF-α). YSP (200 μg/ml) abolished the formation of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts. Furthermore, TNF-α induced TRAP activity was greatly inhibited by YSP (100 μg/ml) treatment. Our results suggest that YSP has therapeutic potential for bone-erosive diseases.

Key words: yolk soluble protein; osteoclasts; differentiation; bone resorption; tartrate-resistant acid phosphatase

Osteoporosis is one of the most serious clinical disorders in elderly people, particularly for postmenopausal women. It results from an imbalance in the bone-remodeling process, with bone resorption exceeding bone formation.\(^1\) The activity of osteoclasts, which resorb bone, relative to bone formation, dictates the development of osteoporosis.\(^2\) Hence, inhibitors of osteoclast formation and of osteoclast activation are therapeutically promising in the treatment of osteoporosis.

We have reported that delipidated egg yolk soluble protein (YSP) promotes longitudinal bone growth in adolescent rats\(^3\) and stimulates bone formation in MC3T3-E1 pre-osteoblastic cells.\(^4\) YSP stimulated proliferation, differentiation, and mineralization of osteoblasts.\(^5\) In the present study, we extended this work to examine the effects of YSP on TNF-α induced osteoclastogenesis.

Egg yolk water-soluble protein (YSP) was prepared as described previously,\(^6\) and was provided by Pharmfoods International Co., (Kyoto, Japan). Bone marrow cells prepared from the femur of male ICR mice were cultured in α-modified minimal essential medium (α-MEM, Gibco, Rockville, MD) containing 10% heat-inactivated FBS. After 3 d of culture, floating cells were removed and attached cells were used as osteoclast precursors. Induction of differentiation of osteoclasts was achieved by culturing the cells with TNF-α (10 ng/ml). A TNF-α untreated control group was tested at the same time.

Cytotoxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. After treatment with YSP (0–5,000 μg/ml) for 2 d, an MTT solution (0.5 mg/ml) was added to each well, and the cells were further incubated for 4 h at 37°C. Absorbance at a test wavelength of 595 nm with a reference wavelength of 690 nm was measured using a microplate reader (Biotek, Winooski, VT). The optical density was calculated as the difference between the absorbance from reference wavelength and from test wavelength.

Cytochemical staining of TRAP-positive cells was performed. After culture for 7 d, cells adherent to the 24-well plates were stained for tartrate-resistant acid phosphatase (TRAP), a marker enzyme of osteoclasts.\(^6\) Briefly, cells were washed with Hank’s balanced salt solution and fixed with 10% neutralized formalin-salt as a stain for the reaction product in the presence of 10 mM sodium tartrate. Numbers of osteoclasts were determined by counting TRAP-positive cells.

TRAP activity was determined with cells treated with TNF-α (10 ng/ml) and various concentrations of YSP (25–200 μg/ml). After 7 d of culture, cells were lysed with 2% Triton X-100 for measurement of TRAP activity and protein concentration. TRAP activity in cell lysate was determined with 50 mM citrate buffer (pH 4.5) containing p-nitro phenyl phosphate (5.5 mM) and 10 mM sodium tartrate as a substrate. An aliquot of cell lysate was added to the substrate solution and incubated at 37°C for 30 min. The reaction was stopped by adding 50 μl of 1 N NaOH, and the optical density at 405 nm was measured using a microplate reader. Protein concentrations were determined using a BCA protein

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Abbreviations: YSP, yolk water-soluble protein; TRAP, tartrate-resistant acid phosphatase; TNF-α, tumor necrosis factor-α
assay kit (Bio-Rad, Hercules, CA).

Results were expressed as mean \pm SE. The data were analyzed by one-way ANOVA, followed by Dunnett’s post-hoc analysis using SPSS. Differences were considered significant at \( P < 0.05 \).

Osteoporosis is a disorder resulting from an imbalance between the rates of bone formation and bone resorption. Osteoclasts are the only cell type capable of resorbing mineralized bone. The intervention of osteoclast-mediated bone resorption is considered a therapeutic approach in the treatment of osteoporosis.

To determine the effects of YSP on osteoclast formation, \textit{in vitro} osteoclastogenesis was investigated by culturing bone marrow cells in the presence of the osteoclastogenic cytokine TNF-\( \alpha \). Before examining the effects of YSP on osteoclast differentiation, the potential cytotoxicity of YSP was tested, because cell toxicity would decrease cell numbers during culture and consequently reduce TRAP-positive cell formation irrespective of its effects on differentiation \textit{per se}. YSP did not show cytotoxicity at concentrations of up to 5,000\( \mu \)g/ml (data not shown). Therefore, YSP has a selective effect on osteoclastogenesis.

Many cytokines, including receptor activator of NF-\( \kappa \)B ligand (RANKL), lipopolysaccharide, IL-1\( \alpha \), and TNF-\( \alpha \), critically regulate both osteoclast differentiation and activation.\(^6,7\) TNF-\( \alpha \) has been found to play a pivotal role in the pathogenic mechanisms of osteoporosis and rheumatoid arthritis. Overexpression of TNF-\( \alpha \) in rheumatoid arthritis synovium and the results of TNF-\( \alpha \) blockade in animal models of arthritis argue for the importance of this cytokine.\(^8\)

The differentiation process of osteoclasts from progenitor cells of hematopoietic origin has not yet been precisely described, but it is generally accepted that the expression of several genes, including TRAP, is stimulated. TRAP is regarded as an important cytochemical marker of osteoclasts.\(^9\)

To assess the effect of YSP on osteoclastogenesis, bone marrow cells were induced to undergo osteoclastogenesis by culturing in the presence of TNF-\( \alpha \) (10ng/ml). The addition of TNF-\( \alpha \) induced TRAP-positive osteoclasts (Fig. 1A, normal, vs. B, control), and YSP (200\( \mu \)g/ml) significantly decreased the formation of TRAP-positive osteoclasts, to approximately 20\% of the level of TNF-\( \alpha \) treated control group (Fig. 1C, D). YSP (100\( \mu \)g/ml) failed to affect osteoclast formation (Fig. 1D).

Therapeutic inhibition of bone resorption can be achieved by reducing the differentiation rate of osteo-

![Fig. 1. Effects of YSP on Osteoclast Differentiation.](image)
clasts and thus decreasing the number of resorbing cells. It is also possible to inhibit their resorptive activity without affecting osteoclast formation. Hence, TRAP activity was further assessed. TNF-α significantly increased TRAP activity, to $100.0 \pm 7.1\%$ as compared with the blank group ($35.9 \pm 11.5\%$), and YSP treatment (100 and 200 μg/ml) significantly reduced TNF-α induced TRAP activity, to $18.7 \pm 10.1$ and $32.1 \pm 11.3\%$ respectively (Fig. 2). YSP (25 and 50 μg/ml) failed to reduce TRAP activity induced by TNF-α ($92.1 \pm 4.8$ and $108.4 \pm 5.9\%$, Fig. 2).

In this study, we found that egg yolk water-soluble protein (YSP) inhibited bone resorption. YSP showed an inhibitory effect on TNF-α induced osteoclastogenesis and TRAP activity. These results, along with our previous study showing the stimulating nature of YSP toward the function of osteoblastic cells, suggest that egg yolk water-soluble protein might be a therapeutic candidate for osteoporosis and other bone resorption/formation imbalance-associated pathological conditions.

**Acknowledgment**

This study was supported by Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea (204017-02-2-HD110).

**References**


