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Ameliorative effects of phosphorylated peptide from Antarctic krill (*Euphausia superba*) against H_2O_2-induced damage in MC3T3-E1 cells

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Abstract

Phosphorylated peptide from Antarctic krill (P-AKP) was prepared by the dry-heating method with sodium pyrophosphate in order to improve its antioxidant activity and osteogenic activity. P-AKP exhibited more competitive DPPH• and OH• scavenging activities compared to the native Antarctic krill peptide (AKP). In hydrogen peroxide (H_2O_2)-induced oxidative damage of MC3T3-E1 cells, both AKP and P-AKP pretreatment could dose-dependently improve superoxide dismutase (SOD) and catalase (CAT) activities through attenuating the accumulation of reactive oxygen species (ROS) and malondialdehyde (MDA) production. Moreover, AKP and P-AKP prevented oxidative stress-induced down regulation of alkaline phosphatase (ALP) activity and matrix mineralization. Particularly, the promoting effects of P-AKP on the enzymatic antioxidant defense system, differentiation and mineralization was higher than that of AKP. These results suggested that phosphorylation might be a promising approach to improve the antioxidant and osteogenic activity of AKP, and P-AKP could be a beneficial agent for attenuating oxidative stress-related bone loss.

Keywords: phosphorylation; Antarctic krill peptides; antioxidant activity; oxidative damage; MC3T3-E1 cells.

Practical Application: Phosphorylated Antarctic krill peptide exhibits better bone health-promoting effects against oxidative damage and could be a potential effective osteogenic agent applied in functional and nutritional food industry.

1 Introduction

Bone remodeling is a dynamic and continuous process that maintains bone health through a tight coupling between the activity of bone-forming osteoblasts and bone-resorbing osteoclasts (Drissi & Sanjay, 2016). An imbalance in this remodeling process may lead to weak bone formation which ultimately results in osteoporosis, one of the most common skeletal diseases characterized by reduced bone mineral density (BMD) and deteriorated bone microstructure (Cauley, 2017). Osteoporotic fractures are a serious health problem that affects approximately 140 million women and 20 million men worldwide (aged 50 or older), usually followed by hospitalization, impaired quality of life, disability and even death (Li et al., 2017). Due to the increased life expectancy, osteoporosis has become a severe socio-economic issue and a major public health problem (Odén et al., 2015; Sözen et al., 2017). Thus, the need for prevention of osteoporosis has been receiving increasing attention globally than ever. As side effects of current therapeutic agents have been reported, some alternative protective and preventive methods are highly desirable, especially the use of safe bioactive substances with osteogenic effects (Huang et al., 2015).

Oxidative stress, resulting from excessive reactive oxygen species (ROS), was recognized as a crucial factor associated with the development and progression of osteoporosis (Suh et al., 2013). Many defined risk factors for osteoporosis, including estrogen deficiency, smoking, excessive alcohol intake, long-term exposure to drugs and chronic diseases such as hypertension and diabetes, are connected with the prolonged state of oxidative stress (Warnholtz et al., 2004). Accumulating evidence has demonstrated that increased oxidative stress is able to impair bone tissue hemostasis, leading to cellular dysfunction by inhibiting osteoblast differentiation, decreasing its viability and promoting osteoclastic development that eventually reduce overall bone formation (Jin et al., 2020). Therefore, the administration of osteogenic substances that possess antioxidant activity may be a good strategy for protecting against bone loss by preventing oxidative damage in bone metabolism. Bioactive peptides produced by enzymatic hydrolysis of food proteins have been shown to possess osteoprotective and antioxidant activities. For example, casein-derived peptide has been reported to ameliorate oxidative stress-induced osteoblasts dysfunction and increase cell viability, superoxide dismutase (SOD) and catalase (CAT) activities (Mada et al., 2018).

Antarctic krill (*Euphausia superba*), an economically important marine species in the Antarctic Ocean, is a novel biological resource with an estimated biomass of 379 million tons. It provides a sustainable resource of high-quality protein, which contains all the essential amino acids and meets the requirements of FAO/WHO/UNU (Atkinson et al., 2009). Recently, Antarctic krill peptide (AKP) has gathered much attention due to the broad spectra of bioactivities, and its effects on human health are of great interest. Scholars reported that AKP could ameliorate aging-induced osteoporosis by promoting

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bone formation *in vivo* (Wang et al., 2017a). Phosphorylation is one of the most common chemical modification methods of proteins. It has been proven that many functional properties and some physiological activities of food proteins were improved or given after phosphorylation (Li et al., 2010). Ovalbumin, egg white protein, casein peptide as well as Antarctic krill peptide have been phosphorylated by dry-heating method in the presence of pyrophosphate. It is speculated that two types of phosphate bonds are introduced to phosphorylated Antarctic krill peptide (P-AKP): tyrosine phosphate with O-P bonds, and phosphodiesters or polyphosphates with O=P bonds (Lebetwa et al., 2017; Wang et al., 2015; Yin et al., 2014). Previous studies have indicated that P-AKP could dose-dependently preserve BMD in ovariectomized rats by increasing bone mineralization and decreasing bone resorption degree. However, whether P-AKP could exhibit anti-osteoporosis effects via reducing oxidative stress-induced osteoblast injury are still unclear (Xia et al., 2015). In the present study, we evaluated the effects of P-AKP as an antioxidant agent, as well as a protective agent on H_2O_2-induced oxidative injury model in MC3T3-E1 cells. The results of this paper provide a theoretical support for the potential application of Antarctic krill protein and its phosphorylated peptide in the development of functional food for the prevention of bone formation disturbances.

2 Materials and methods

2.1 Materials

Defatted powder of Antarctic krill was provided by Dalian Ocean Fishery Group of Corporations (Dalian, Liaoning, China). MC3T3-E1 cells were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). α-Modified minimum essential medium (α-MEM) and fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Grand Island, NY, USA). Phosphate buffered saline (PBS, pH 7.2), trypsin (4.0×10^3 U/g), penicillin and streptomycin, β-glycerophosphate, ROS assay kit and DPPH were purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). Alkaline phosphatase (ALP) activity kit, bicinchoninic acid (BCA) protein assay kit, superoxide dismutase (SOD), malondialdehyde (MDA) and catalase (CAT) assay kit were obtained from Jiancheng Biotech Co., Ltd. (Nanjing, China). Other reagents used were of analytical grade.

2.2 Preparation of Antarctic krill peptide

Antarctic krill peptide was prepared as described by Hou et al. (2018) with slight modifications. Defatted krill powder was dispersed in distilled water with a solid to liquid ratio of 1:10 (w/v). According to previous study, the hydrolysis condition was set as: trypsin concentration of 3%, pH of 8.0, time of 4 h, temperature of 50 °C. After that, the hydrolysis was stopped by heating for 15 min in boiling water. The hydrolysate was centrifuged at 8000 g for 15 min, and the resulting supernatant was collected after de-fluorination, vacuum drying, freeze-drying, and stored at -20 °C for further analysis.

Ten milliliters 100 mg/mL lyophilized hydrolysate solution was applied to sieve chromatography column (2.6 × 60 cm) loading with Sephadex G-15 gel and eluted with distilled water at a flow rate of 1.5 mL/min. The absorbance was detected at 280 nm. According to the elution curve, the main fractions were collected as AKP (Li et al., 2020).

2.3 Preparation of phosphorylated Antarctic krill peptide and determination of phosphorus content

The phosphorylated peptide was prepared based on the dry-heating method reported by Lebetwa et al. (2017) with slight modifications. AKP sample was dissolved with a solid to liquid ratio of 1:100 (w/v) in 0.1 mol/L sodium pyrophosphate buffer at pH 4.0, and the solution was spray-dried. The collected mixture was incubated at 85 °C for up to 48 h. After reaction, the dry-heated sample was dissolved and dialyzed against deionized water for 3 days to remove free pyrophosphate. The sample was then lyophilized and stored for further use.

Peptide sample was digested with nitric acid and perchloric acid (4:1, v/v). The phosphorus content in the digest was regarded as the total phosphorus (P_t). For the determination of inorganic phosphorus (P_i), 5 mL of 10% trichloroacetic acid (TCA) was added to the equal volume of sample solution, and the mixture was centrifugated at 5000 g for 20 min. The phosphorus in the supernatant was regarded as inorganic phosphorus (Pi). The amount of phosphorus bound to peptide was estimated by subtracting the Pi content from the Pt content (Li et al., 2018).

2.4 In vitro antioxidant activity assays

HO• scavenging activity: Total reaction volume was 4.5 mL. Peptide sample was incubated with safranine O (0.23 μM), EDTA-Fe^{2+} (220 μM) and H_2O_2 (60 μM) in phosphate buffer (150 mM, pH 7.4) for 30 min at 37 °C. The absorbance was measured at 520 nm, using GSH as a positive control.

DPPH• scavenging activity: 1 mL of peptide sample was mixed with 2 mL of DPPH solution (150 μM in ethanol) followed by standing room temperature for 20 min in the dark. The absorbance was measured at 517 nm, using GSH as a positive control (Negrão et al., 2020).

2.5 Cell culture and treatment with H_2O_2

The murine osteoblastic MC3T3-E1 cells were cultured in a 5% CO_2 atmosphere at 37 °C in α-MEM supplemented with 10% FBS and 1% penicillin-streptomycin. MC3T3-E1 cell was cultured into 96-well plates at a density of 1.2×10^5 cells/mL for 24 h until 80-90% confluence. H_2O_2 served as an exogenous ROS source. H_2O_2 (final concentration of 0, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mM) was added into the cells and incubated for 4 h. The optimal H_2O_2 concentration was confirmed when the cell viability was close to 50%. Cell viability was measured by cck-8 (Cell counting Kit-8) assay.

2.6 Cell viability assay

The protective effect of peptide samples on MC3T3-E1 cells was determined by establishing H_2O_2-induced oxidative stress model. Briefly, cells (1.2×10^5 cells/mL) were seeded into 96-well

plate and incubated for 24 h. Afterwards, cells were incubated with various concentrations of peptide samples (100, 200, 400 and 600 μg/mL) for 48 h, respectively. Subsequently, cells were washed with phosphate-buffered saline (PBS) followed by the addition of 0.3 mM H_2O_2 for 4 h. Finally, cell viability was measured by cck-8 assay.

2.7 Determination of SOD, CAT, MDA and ROS levels

MC3T3-E1 cells were cultured in 12-well plates (1.2×10^5 cells/mL) and incubated for 24 h. Afterwards, cells were pretreated with different concentrations of peptide samples for 48 h followed by treatment with 0.3 mM H_2O_2 for 4 h. Then, cell monolayers were washed twice with PBS, lysed and then centrifuged at 4000 × *g* for 10 min. SOD, CAT and MDA contents were determined as described by the instruction of assay kits, total protein content was measured using a bicinchoninic acid (BCA) protein assay kit. The activities of SOD and CAT were normalized to protein content (Jin et al., 2020). Intracellular ROS content was evaluated by quantifying the fluorescence emitted by 2',7'-dichlorofluorescein, the oxidized form of 2',7'-dichlorofluorescein diacetate (DCFH-DA) according to the instruction of a ROS assay kit. The fluorescent intensity was measured at excitation wavelength 485 nm and emission wavelength 530 nm using a multi-mode detection plate reader (Mada et al., 2018).

2.8 Determination of Alkaline phosphatase (ALP) activity

MC3T3-E1 cells were cultured in differentiation medium containing different concentrations of peptide samples for 7 days followed by treatment with 0.3 mM H_2O_2. After indicated treatment, cells were washed twice with PBS and lysed with 0.1 M Tris buffer (containing 0.1% Triton X-100) on ice. The ALP activity was determined according to the instruction of an ALP activity assay kit, and the total protein content was measured using a BCA protein assay kit. ALP activity was normalized to protein content (Jin et al., 2020).

2.9 Mineralization assay

MC3T3-E1 cells were cultured in differentiation media supplemented with different concentrations of peptide samples for 18 days followed by treatment with 0.3 mM H_2O_2. Upon harvesting, the cells were washed twice with PBS and fixed in 4% paraformaldehyde for 10 min. After washing with distilled water, the cells were stained with 40 mM Alizarin Red S for 30 min. To quantify the bound dye, the stain was solubilized with 10% cetylpyridinium chloride in the dark for 1 h. The absorbance of the solubilized stain was measured at 570 nm using a microplate reader (Mada et al., 2018).

2.10 Statistical analysis

Data were reported as means ± standard deviation calculated from data of three different experiments. One-way analysis of variance (ANOVA) was used for statistical analysis of the data by using SPSS software (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant at $p < 0.05$ and $p < 0.01$.

3 Results and discussion

3.1 Preparation and phosphorylation of Antarctic krill peptides

Gel filtration chromatography is an effective method that is based on molecular size and has been widely applied to isolate target fractions from protein hydrolysates. The elution profile of Antarctic krill hydrolysates using the size exclusion chromatography of Sephadex G-15 was shown in Figure 1. The major subfraction, named as AKP, was collected and lyophilized for subsequent phosphorylation.

Previous studies reported that proteins with hydroxyl groups could be phosphorylated when they were dried in a presence of phosphate buffer. Therefore, dry-heating method has been applied to peptide phosphorylation to improve their functional and physiological activities (Tarelli & Wheeler, 1994). As the reaction time prolonged, the phosphorylation level increased, more phosphate groups were contained at 24 h compared to the shorter heating time, and then remained almost unchanged (Figure 2). Thus the final reaction time was fixed to be 24 h, and the phosphate level in the obtained phosphorylated Antarctic krill peptide (P-AKP) was 14.08 mg/g.

3.2 Determination of antioxidant activity

OH• is a highly active and damaging species in free-radical pathology due to its strong electron-gathering ability, which

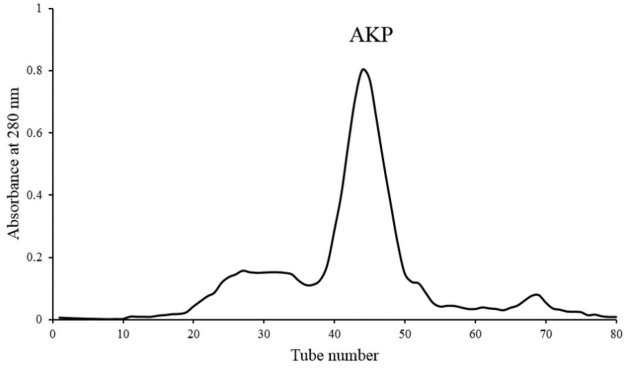

Figure 1. Elution curve of Antarctic krill hydrolysate on Sephadex G-15 gel.

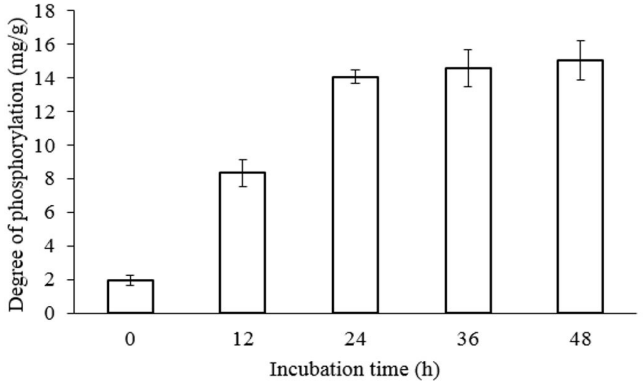

Figure 2. Effect of dry-heating time on the phosphorylation degree.

can react with almost every biological molecule found in living cells and cause tissue damage through oxidizing proteins, carbohydrates, nucleic acids and lipids. DPPH• is another strong reactive free radical which is stable in ethanolic solutions and has a maximum absorbance at 517 nm because of its odd electron ion (Li et al., 2020).

As shown in Figure 3, it is interesting to note that the scavenging activity of P-AKP was superior to that of AKP at the same concentration. AKP and P-AKP inhibited OH• oxidation by 76.26% and 92.43% at a concentration of 10 mg/mL, and their DPPH• scavenging rate was 72.84% and 87.13%, respectively, suggesting the antioxidant capacity of AKP was markedly increased by phosphorylation. The data also indicated that P-AKP had excellent OH• scavenging ability similar to that of GSH. It has been reported that peptides with more hydrophobic amino acids exhibited stronger antioxidant activity (Wang et al., 2017b). During phosphorylation, the buried hydrophobic residues become more exposed to the peptide surface by repulsion of negative charges of introduced phosphate groups (Li et al., 2004). Thus, the improved antioxidant property can be attributed to the electrostatic interactions of negatively charged phosphate groups introduced into P-AKP, and the more exposed hydrophobic groups after phosphorylation process (Yin et al., 2014).

3.3 Cell viability assay

As shown in Figure 4a, the viabilities of MC3T3-E1 cells exposed to H_2O_2 drastically decreased in a dose-dependent manner. 300 μmol/L was found to be the most suitable concentration for use in the cell injury model, achieving around 50% cell viability rate. Therefore, the appropriated dose of H_2O_2 was selected to be 300 μmol/L for subsequent experiments. Cell viability data (Figure 4b) suggested that pretreatment of AKP and P-AKP led to a positive response to the oxidative stress injured cells' survival rate. The viability rate of cells treated with 400 μg/mL AKP was obviously increased compared to the H_2O_2 group (p<0.05). Pretreatment with 100-600 μg/mL P-AKP also resulted in markedly increased cell viability (p<0.05). This result revealed that both AKP and P-AKP reduced cytotoxicity and exhibited certain protective effects against H_2O_2-induced damage, and the concentrations of 100 and 400 μg/mL were ascertained for further experiments.

3.4 Attenuation of H_2O_2-induced oxidative stress in MC3T3-E1 cells

Based on the good antioxidant capacities of phosphorylated peptides in chemical assays, the protective effects of AKP and P-AKP against H_2O_2-induced damage was investigated, including the levels of ROS, MDA, SOD and CAT in H_2O_2-damaged MC3T3-E1 cells. In Figure 5a, the ROS level in H_2O_2 exposure group was 234.98%, which was significantly higher than that of control group (p<0.01). As expected, pretreatment with different concentrations of AKP and P-AKP markedly decrease H_2O_2-induced ROS over-accumulation in a dose-dependent manner, similar to the reports of peptides from casein and *Mytilus coruscus* (Mada et al., 2018; Zhang et al., 2020). And the inhibiting effect of P-AKP was better than AKP. MDA is an oxidative metabolite of intracellular lipid peroxidation, which further damage the integrity and biological functions of cell membrane (Zhang et al., 2020). Figure 5b showed that the MDA content in H_2O_2-damaged group (22.21 nmol/mgprot) was significantly increased compare with the blank control group (p<0.01) After pretreatment with tested concentrations of AKP and P-AKP, the MDA level was markedly lower than that of H_2O_2 group. The results suggested that the intracellular ROS and MDA level in H_2O_2-damaged cells was greatly increased, but it can be prominently reduced with the addition of tested samples.

SOD and CAT are the first line in natural antioxidant defense system which can rapidly decompose the oxygen free radicals to prevent the generation of oxidative damage. Compared with the control group, the levels of SOD and CAT were significantly decreased by H_2O_2 in MC3T3-E1 (p<0.01) (Figure 5c and 5d). However, pretreatment with AKP and P-AKP could prominently elevate the SOD and CAT levels than that of the H_2O_2-damaged group. Similar results were noticed for the antioxidant peptides from *Moringa oleifera* seeds (Liang et al., 2020). At the concentration of 400 μg/mL, P-AKP strongly promote the SOD (21.61 U/mgprot) and CAT (13.49 U/mgprot) content (p<0.01), which was superior to AKP group at the same concentration (p<0.05). These results indicated that P-AKP treatment effectively inhibited ROS and MDA accumulation, and elevated the cellular endogenous enzyme activities to protect MC3T3-E1 cells from oxidative injury.

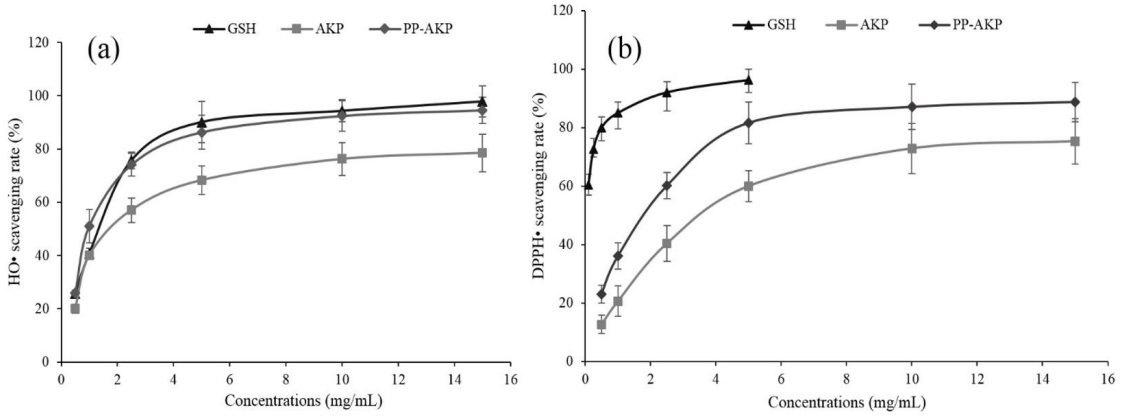

Figure 3. OH• (a) and DPPH• (b) scavenging rate of AKP and P-AKP.

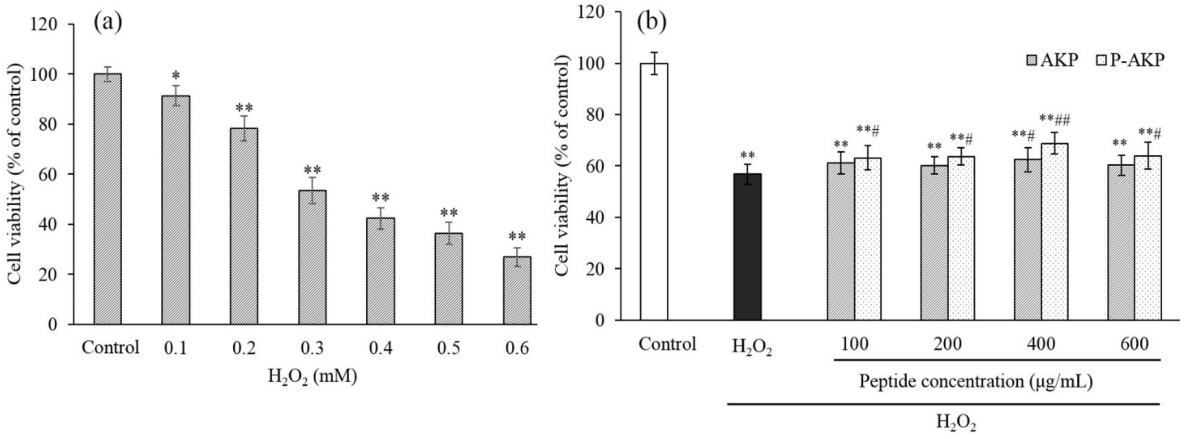

Figure 4. Effect of H_2O_2 (a); AKP and P-AKP (b) on the cell viability. *p<0.05 and **p<0.01 versus Control group; #p<0.05 and ##p<0.01 versus H_2O_2 group.

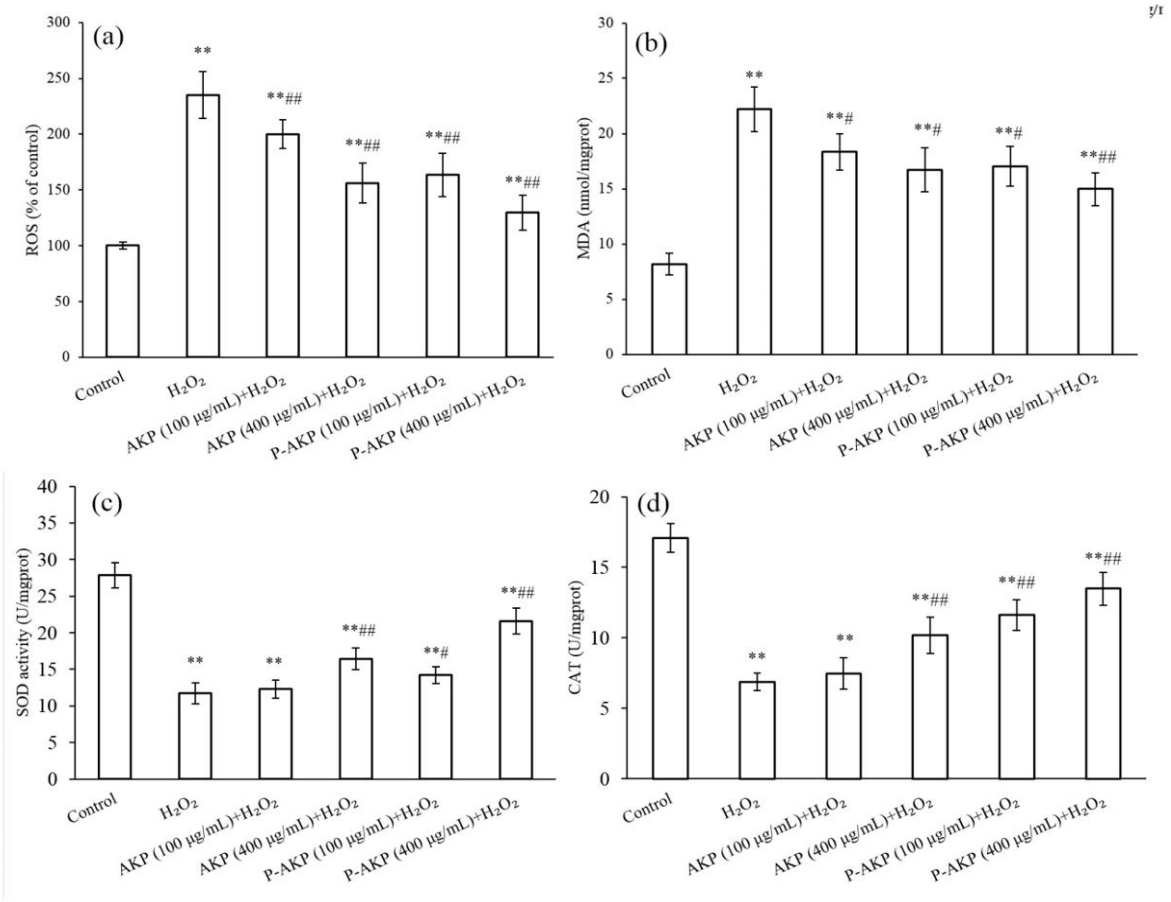

Figure 5. Effect of AKP and P-AKP on the ROS (a), MDA (b), SOD (c) and CAT (d) level in H_2O_2-induced MC3T3-E1 cells. **p<0.01 versus Control group; #p<0.05 and ##p<0.01 versus H_2O_2 group.

3.5 Attenuation of H_2O_2-suppressed differentiation and mineralization in MC3T3-E1 cells

ALP is an important marker of early stage differentiation in osteoblasts and is an essential enzyme for further mineralization, the augment of ALP content is highly correlated with the progressive osteogenesis of osteoblasts (Mada et al., 2018). As depicted in Figure 6a, results of ALP activity showed that H_2O_2 dramatically decreased the osteogenic differentiation of MC3T3-E1 cells compared to the control group (p<0.01). However, pretreatment with increasing concentrations of AKP and P-AKP exerted a positive effect on the stimulation of ALP activity, and the promotion effects improved as the concentration increased. Particularly, P-AKP groups exhibited better effectiveness than that of AKP groups at the same concentration (p<0.05). Previous studies reported that non-collagenous phosphoproteins in

extracellular matrix (ECM) of osteoblasts could stimulate bone formation (Jie et al., 2018; Boskey et al., 2008). It is speculated that more phosphate groups in P-AKP could enhance the interaction between the phosphate groups and the surface receptors on MC3T3-E1 cells, then triggered the signal transform and further stimulated the differentiation via the receptor-mediated mechanisms (Qin et al., 2004).

Mineralization is the late-stage marker in osteoblast differentiation and can visually reflect the bone formation extent. The calcium complex in mineralized nodules can be visualized after stained with Alizarin red S (ARS) (Figure 7), and the absorbance is proportional to the amount of calcium deposit. As we had predicted, pretreatment with AKP and P-AKP obviously alleviated the subsequent H_2O_2-induced reduction of calcium deposit number (Figure 6b) ($p<0.05$). Notably, the promoting mineralization ability of P-AKP was higher than AKP, which was consistent with the results of ALP activity assay. It may be that the calcium binding ability of P-AKP was better than AKP due to the enhanced interactions between calcium ions and phosphate groups through electrostatic interaction. The phosphorylated peptide-calcium complex is speculated to provide high-density nucleation sites, reduce the interfacial energy and then promote the mineralization process. The AKP-Ca complex could be incorporated into the nucleation sites and become a part of the mineralized nodules (Zhang et al., 2015; Jie et al., 2018).

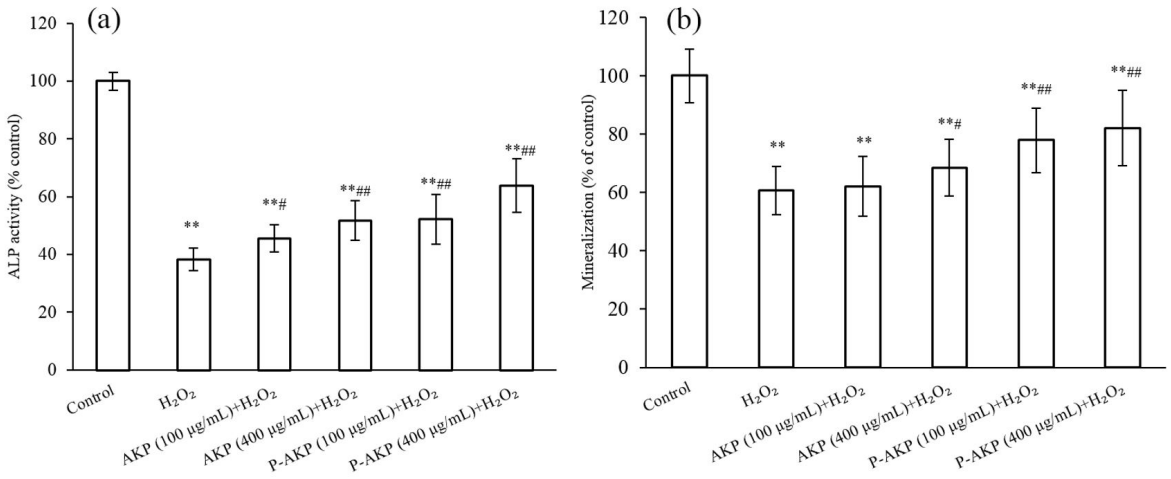

Figure 6. Effect of AKP and P-AKP on the ALP activity (a) and mineralization degree (b) in H_2O_2-induced MC3T3-E1 cells. $^{**}p<0.01$ versus Control group; $\#p<0.05$ and $\#\#p<0.01$ versus H_2O_2 group.

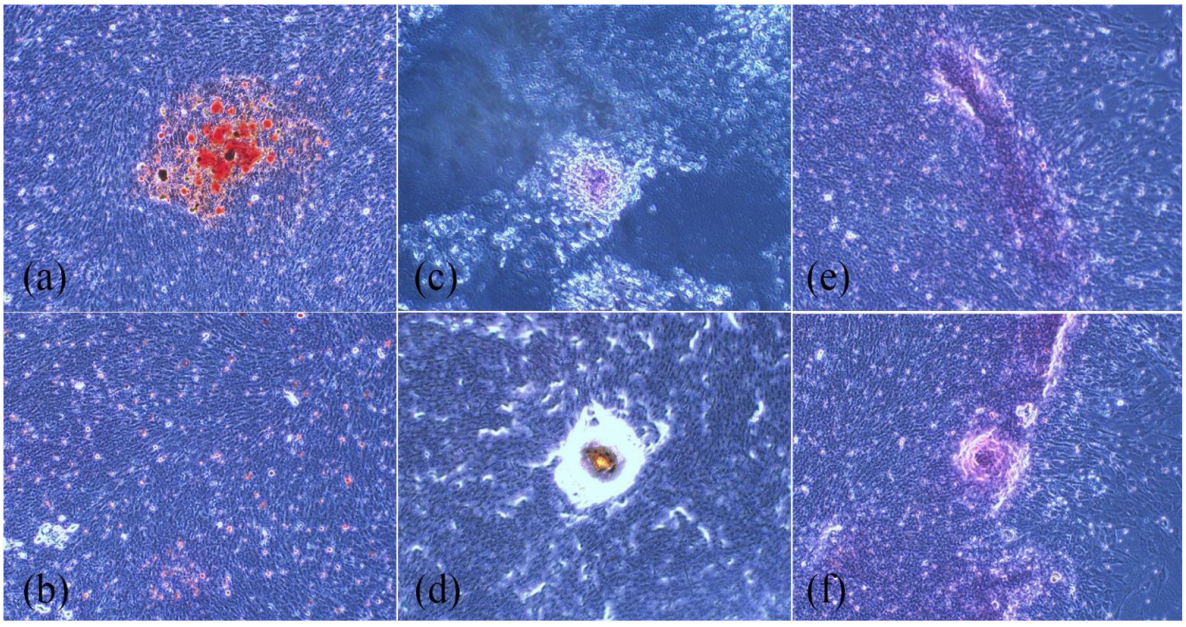

Figure 7. Effect of AKP and P-AKP on mineralization of MC3T3-E1 cells after Alizarin red S staining. (a) Control group; (b) H_2O_2 group; (c) AKP (100 μg/mL) + H_2O_2 group; (d) AKP (400 μg/mL) + H_2O_2 group; (e) P-AKP (100 μg/mL) + H_2O_2 group; (f) P-AKP (400 μg/mL) + H_2O_2 group.

4 Conclusion

The present study showed that the antioxidant activity of P-AKP was enhanced by the introduction of additional phosphates by dry-heating method. Both AKP and P-AKP pretreatment resulted in improved osteoblast survival rate, differentiation and mineralization degree by conferring resistance to H_2O_2-induced cell injury. The key enzymes within the cellular antioxidant defense enzyme system, such as SOD and CAT, was up-regulated before oxidative damage occurred. Notably, pretreatment with P-AKP exhibited a more significant beneficial effect on the promotion of osteogenesis in comparison with native AKP. These results indicated that phosphorylation is a promising method to improve the antioxidant capacity of food-derived peptides, and P-AKP may be a potential nutritional component in functional food development for the prevention and treatment of bone formation disturbances induced by oxidative injury. Further investigation is desirable to explore the underlying mechanism of how P-AKP attenuates oxidative stress-induced bone loss, and *in vivo* activity must be executed in the future.

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