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Casein-derived antioxidative peptide prevents oxidative stress-induced dysfunction in osteoblast cells

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ABSTRACT

Oxidative stress has been linked to osteoblast cells dysfunction and plays a crucial role in the pathogenesis of postmenopausal osteoporosis. The present study was designed to investigate the effects casein-derived antioxidative peptide VLPVPQK (PEP) against hydrogen peroxide (H_2O_2)-induced dysfunction and oxidative damage in osteoblast cells. The present data demonstrated that treatment of osteoblast cells with PEP increases cell viability, superoxide dismutase (SOD) and catalase (CAT) activities. PEP also reduces reactive oxygen species (ROS) production, cell death and capsase-3/9 activities. Moreover, PEP prevent oxidative stress-induced down regulation of osteogenic genes (ALP, OCN, COL-I) expression and matrix mineralization. In addition, PEP decreases the expression of bone resorbing and inflammatory cytokines. Thus, our data demonstrated that PEP increases osteoblast cells differentiation through augmentation of osteogenic genes expression and antioxidant enzymes activities. Altogether, PEP exhibits bone health-promoting effect and could be beneficial agent for the management of postmenopausal osteoporosis.

1. Introduction

Postmenopausal osteoporosis is characterized by decrease in bone mineral density (BMD), increase fracture risks, and subsequent bone loss [1]. Estrogen is a key regulator of bone metabolism responsible for maintaining delicate balance between bone forming-osteoblast cells and bone degrading-osteoclast cells [2]. Several millions of postmenopausal women are at risk of developing osteoporosis due to estrogen deficiency. Estrogen deficiency impairs antioxidant defense system, increases production of reactive oxygen species (ROS), diminishes bone formation by affecting osteoblasts maturation and survival [3,4]. Also, excessive ROS production stimulates nuclear damage and programmed cell death through activation of caspases [5]. Osteoblast differentiation can be regulated by the interactions between ligand and receptor that acquired signal conciliation by growth factors and cytokines, bone-specific transcription factors and hormones [6,7]. Differentiation of osteoblast cell is characterized by three successive stages including proliferation, differentiation and matrix mineral deposition. The main proteins which plays crucial role in osteoblast differentiation are alkaline phosphatase (ALP), osteocalcin (OCN) and collagen type-1 (COL-1) [8]. Furthermore, the expression of bone resorbing cytokines; RANKL, IL-6 and TNF-α protein by osteoblasts were reported to enhanced under oxidative stress condition [9,10].

Milk has been regarded as nature's most complete food. Milk and milk derived bioactive peptides offers a wide range of health beneficial effects in humans [11,12]. These beneficial effects include immunomodulatory, antihypertensive, antioxidative, antimicrobial and osteogenic activities [13–16]. Recently, a novel bioactive peptide VLPVPQK (PEP) derived from buffalo milk casein was isolated in our laboratory. We have previously reported that PEP improves osteoblast cells proliferation, exhibits anti-osteopenic in ovariectomized rats and was bioavailable up to 1% [17–19]. However, the present study was designed to investigate the effect of casein-derived antioxidative peptide VLPVPQK (PEP) against hydrogen peroxide-induced oxidative damage and dysfunction in osteoblast cells.

2. Materials and methods

2.1. Chemicals

Peptide (VLPVPQK) was custom synthesized by (Bioconcepts Pvt.

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Ltd., India), *N*-acetyl-L-cysteine, alpha-minimal essential medium (α-MEM), penicillin, streptomycin and amphotericin, (NAC), ascorbic acid, β-glycerophosphate, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT), Alizarin Red S Stain (ARS), para-nitrophenyl phosphate (pNPP), reactive oxygen species (ROS) assay kit [2',7'-dichlorofluorescein -diacetate (DCFH-DA)], Propidium Iodide (PI) assay kit, MTT dye, dimethyl sulfoxide (DMSO), Ac-DEVD-para-nitroanilide, Ac-LEHD-para-nitroanilide, Pyrogaloll, hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich Chemical Co. St. Louise (MO., USA.). RevertAid™ First strand cDNA synthesis kit, SYBR-Green-I master mix (Thermo Scientific). Rat sandwich ELISA assay kits for osteocalcin (OCN) was purchased from Bioassay Technology Laboratory (Shanghai, China), receptor activator of NF-κB ligand (RANKL) was purchased from Cusabio Biotech. Co., Ltd (Hubei Province, China), interleukin-6 (IL-6) was purchased from RayBiotech, (Norcross, GA, USA), and tumor necrosis factor-α (TNF-α) was purchased from Leinco Technologies, inc., (Fenton, MO, USA). All other chemicals and materials used in this study were of analytical grade and purchased from local suppliers.

2.2. Isolation of osteoblast cells and establishment of cell injury model

Two days old rat pups were procured from the small animal house after approval from the Institutional Animal Ethics Committee (IAEC). Rat calvarial osteoblast cells were obtained by sequential digestion according to the method described previously [20,21]. On confluence, osteoblast cells were detached using 0.25% trypsin-EDTA and the released osteoblast cells (1×10^5cells/well) were seeded in a 96 well plate and cultured in α-MEM media for 24 h. Then, cells were cultured in differentiation media (α-MEM, 10% FBS, 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid and 10 nM dexamethasone) containing H_2O_2 at different concentrations (0.1–0.5 mM) for 24 h. Osteoblast cell viability was determined by MTT Method. Briefly, 20 μl MTT (5 mg/ml) solution was added to each well and the plate was incubated at 37 °C for 4 h. Then, the media containing MTT dye was aspirated gently and discarded. The dark blue formazan crystals were dissolved in 200 μl acidic-isopropanol with intermittent shaken for 5 min. Absorbance was measured at 540 nm by microplate reader (BioTek model, UK). The concentration of H_2O_2 that causes the decrease in cell viability by 50% was determined and used in all subsequent experiments in the present study.

2.3. Cell viability assay

Osteoblast cells were cultured for 24 h to allow the cells to attach to the wells. Subsequently, cells were cultured with differentiation media containing different (50–200 ng/ml) concentrations of PEP for 24 h, followed by treatment with 0.3 mM H_2O_2 for 24 h. Cell viability was determined by MTT method as described above.

2.4. Measurement of serum superoxide dismutase (SOD) and catalase (CAT) activity

Osteoblast cells were cultured with differentiation media supplemented with different concentration of PEP for 14 and 21 days followed by treatment with 0.3 mM H_2O_2 for 24 h. Then, cells monolayers were washed twice with ice-cold PBS, the cells were lysed in cell lysis buffer and centrifuged at 4000 × *g* for 10 min. The lysate obtained was used to measure SOD and CAT activities as described previously [22,23], using Specord 200 double beam UV/visible spectrophotometer (Analytikjena, Germany). The activities of SOD and CAT were normalized by total protein content from each sample [24].

2.5. Measurement of reactive oxygen species (ROS)

Osteoblast cells were cultured in a 6-well plate containing round coverslip with differentiation media supplemented with PEP for 14 days followed by treatment with 0.3 mM H_2O_2 for 24 h. Intracellular ROS production was determined using DCFH-DA (2′,7′-dichlorofluorescein-diacetate), ROS assay kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Fluorescent images were taken using a fluorescent microscope (Olympus, USA). Quantification of intracellular ROS production was carried out at 485 nm excitation and 535 nm emission wavelengths using a multi-mode detection plate reader (TECAN, Switzerland).

2.6. Cell death assay by PI staining

Osteoblast cells were cultured in a 6-well plate containing round coverslip with differentiation media containing PEP for 14 days followed by treatment with 0.3 mM H_2O_2 for 24 h. Osteoblast cell death was investigated by using PI staining kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions as previously described [25]. Fluorescent images were taken using fluorescent microscope (Olympus, USA), the PI-positive cells (dead cells) were quantified at 535 nm excitation and 617 nm emission wavelengths, using a multi-mode detection plate reader (TECAN, Switzerland).

2.7. Measurement of caspase-3 and 9 activities

Osteoblast cells were cultured in differentiation media containing different concentration of PEP for 14 and 21 days followed by treatment with 0.3 mM H_2O_2 for 24 h. On harvesting, caspase-3 and 9 activities were measured using colorimetric assay kits (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions.

2.8. Quantitative real-time PCR of osteoblast differentiation marker genes

Osteoblast cells were cultured in differentiation media containing different concentration of PEP for 7, 14 and 21 days followed by 0.3 mM H_2O_2 treatment for 24 h. At the end of the treatment, total RNA was extracted from the cells by single-step RNA isolation as previously described [26], using TRI™ Reagent. Total RNA was converted into cDNA (RevertAid™ First strand cDNA synthesis kit). Relative expression of osteoblast differentiation marker genes (ALP, COL-I and OCN) were determined by quantitative real-time PCR (SYBR-Green chemistry, Thermo Scientific) using ABI PRISM 7500 sequence detection system (Applied Biosystems). Specific primers were designed using Primer Express Software (Version 1.0, PE Applied Biosystems). Primers used are listed in Table 1. Relative abundance of cDNA of each sample was quantified relative to GAPDH. The $2^{-\Delta\Delta Ct}$ method was applied to calculate relative target gene expression.

Table 1
Sequences of primers used for qRT-PCR.

Gene	Accession no.	Forward primer Sequence	Reverse primer Sequence	bp
ALP	J03572	CGTCTCCATGGTGGATTATGC	TGGCAAAGACCGCCACAT	101
COL-I	Z78279	TTCACCTACAGCACGCTTGTG	GATGACTGTCTTGCCCCAAGTT	65
OCN	X04141	GAGCTAGCGGACCACATTGG	CCTAAACGGTGGTGCCATAGA	63
GAPDH	NM017008	GACAACTTTGGCATCGTGGA	ATGCAGGGATGATGTTCTGG	133