Experimental and Toxicologic Pathology 69 (2017) 485–495

Contents lists available at ScienceDirect

Experimental and Toxicologic Pathology

journal homepage: www.elsevier.com/locate/etp

Full Length Article

Buffalo casein derived peptide can alleviates H_2O_2 induced cellular damage and necrosis in fibroblast cells

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ARTICLE INFO

Keywords:
Fibroblasts
Oxidative stress
Antioxidative
Reactive oxygen species
Hydrogen peroxide

ABSTRACT

Oxidative stress is one of a critical pathogenic factor in the progression of aging and chronic diseases such as cancer, myocardial inflammation and diabetes. In the present scenario, peptides with short half life and more biological specificities are gaining much attention as prodrugs. Thus, the present investigation carried out to screen potential antioxidative peptide, VLPVPQK to cope with the cellular oxidative damage. Our results showed that treatment of rat fibroblast cells with 0.2 mM H_2O_2 for 6 h significantly declined different oxidative stress biomarkers such as SOD, CAT, GSH, and promoted LDH activity. In addition, ROS and TNF-α levels were also increased upon H_2O_2 exposure for 6 h and thereby, it induced cell death. Amazingly, pretreatment of the peptide (VLPVPQK) significantly elevated cell survivability, by reversing all H_2O_2 induced alterations in fibroblast cells. Therefore, our results indicated that, the peptide (VLPVPQK) acted as a potential cytoprotective agent, who restored redox balance and cell homeostasis in cultured fibroblast cells, even after H_2O_2 exposure, suggesting that the peptide can be valuable as an effective remedy in treatment of oxidative stress related diseases and skin inflammation related disorders.

1. Introduction

Fibroblasts are the main cell type present in the dermis of skin responsible for the synthesis of extracellular matrix, thus play a crucial role in the protection against the development of patho- physiological conditions of the skin (Gillitzer and Goebeler, 2001). Human body consists of a well developed antioxidative defense system against metabolic oxidative stress (Simic, 1988). Oxidative stress can be defined as elevated ROS production above the critical point in impaired antioxidative defense of cell, which leads to the development of oxidative stress and inflammation related diseases, such as cancer, atherosclerosis, hypertension and arthritis (Frenkel, 1992; Halliwell and Gutteridge, 1984; Sliwa-joźwik et al., 2002; Crujeiras et al., 2008; Laveti et al., 2013). Hydrogen peroxide (H_2O_2) has been demonstrated as an intra and extracellular stimulant of oxidative stress, due to its stable and diffusible nature through the membranes of many cell types (Yuan et al., 2003). Therefore, H_2O_2-induced oxidative stress model served as a potential tool for screening antioxidative compounds (Feng et al., 2013). SOD, catalase and GSH are the major regulators of antioxidative defense, which neutralizes the excessive ROS, produced in the metabolic process. Several studies have reported that SOD, catalase and GSH were markedly decreased in fibroblast and other different cells upon H_2O_2 treatment (Troyano et al., 2003; Dash et al., 2007; Feng et al., 2013). LDH is a soluble protein which is involved in the cellular anaerobic biochemical process and is released out of the cell, thus, it can be used as a cell injury marker. Moreover, Dash and colleagues, demonstrated the use of LDH as cell damage marker in fibroblast cell (Dash et al., 2007). ROS and the consequential cellular redox imbalance work as strong stimulants in apoptotic signal transduction (Kannan and Jain, 2000). Caspases are the major candidates, responsible in cellular programmed cell death (Troyano et al., 2003). Apart from nutritional resource, milk proteins also possess several health benefits including antioxidative, antihypertensive, antimicrobial, anti-inflammatory, and osteogenic effects which can be attributed to the encrypted bioactive peptides (Kumar et al., 2010; Shanmugam et al., 2015; Reddi et al., 2016a,b,c). Recently, four novel peptides from buffalo milk casein were

Abbreviations: VLPVPQK, Valine Leucine-Proline-Valine-Proline-Glutamine-Lysine; SOD, superoxide dismutase; LDH, lactate dehydrogenase; ROS, reactive oxygen species; DMEM, Dulbecco's modified minimal essential medium; FBS, Fetal bovine serum; DCFH-DA, 2',7'-dichlorodihydrofluorescein–diacetate; PI, propidium iodide; MTT, 3–(4, 5–dimethylthiazol–2–yl)–2, 5–diphenyl tetrazolium bromide; DMSO, dimethyl sulfoxide; DAPI, 4', 6-diamidino-2-phenylindole; ABTS, 2, 2 azinobis (ethylbenzthiazoline-6-sulfonic acid); AAPH, 2, 2'-azobis (2-amidino-propane) dihydrochloride; FITC, fluorescein isothiocyanate; NAC, N-acetyl-L-cysteine

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http://dx.doi.org/10.1016/j.etp.2017.04.009
Received 2 December 2016; Received in revised form 15 March 2017; Accepted 16 April 2017
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isolated, purified and sequenced in our laboratory. Hepta peptide (VLPVPQK) derived from pepsin-trypsin hydrolyzate of β-casein displayed highest antioxidant activity as evaluated by ABTS method (Shanmugam et al., 2015). Moreover, this peptide was bio-accessible up to 1% using Caco-2 monolayer cells (Vij et al., 2016). However, the role of VLPVPQK in protection against cellular oxidative damage in fibroblasts has not yet been investigated. Therefore, to cope with oxidative stress-related diseases, searching for natural antioxidants, especially from food sources becomes imperative due to the adverse effects associated with existing synthetic antioxidants. Hence, in the present study an *in-vitro* oxidative cell injury model induced by H_2O_2 was established to investigate the cytoprotective effect of the peptide, VLPVPQK in fibroblast cell.

2. Material and methods

2.1. Materials

Peptide VLPVPQK derived from buffalo β-Casein was custom synthesized with purity > 98%, molecular weight, 779.48 (Techno concept pvt. Limited India)) and used in present study. Dulbecco's modified minimal essential medium (DMEM), Fetal bovine serum (FBS), N-acetyl-L-cysteine (NAC), penicillin, streptomycin, amphotericin, 2′,7′-dichlorofluorescein-diacetate (DCFH-DA), propidium iodide (PI) dye, 3–[4,5–dimethylthiazol–2–yl]–2, 5–diphenyl tetrazolium bromide (MTT) dye, dimethyl sulfoxide (DMSO), 4′, 6-diamidino-2-phenylindole (DAPI), 2, 2 azino bis (ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2, 2′-azobis (2-amidino-propane) dihydrochloride (AAPH), trolox, Fluorescein diacetate, hydrogen peroxide (H_2O_2) and secondary antibody conjugated with fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich Chemical Co., St. Louise, M.O., USA. Catalase, Superoxide dismutase (SOD), Glutathione and lactate dehydrogenase (LDH) were purchased from cayman chemical assay kit (Ann Arbor, MI.USA). Anti- vimentin antibody and CD 86 were obtained from Santacruz Biotechnology, Paso Robles, CA, USA. All other reagents used in the present study were of analytical grade.

3. Methods

3.1. Antioxidative property of the peptide

The antioxidative property of the peptide VLPVPQK was evaluated by using free radical scavenging assay ABTS and ORAC.

3.2. Radical scavenging activity

The radical scavenging activity of the peptide was examined by ABTS as described previously (Re et al., 1999) with some modifications. Briefly, from the stock solution of 7 mM $ABTS^{\bullet+}$ and 140 mM potassium persulfate, working concentration was prepared by mixing the two stock solutions in equal quantities and then allow them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml $ABTS^{\bullet+}$ solution with PBS to obtain an absorbance of 0.70 ± 0.02 units at 734 nm using the spectrophotometer (Infinite Model, F200 PRO, Austria). Twenty micro liter of various concentration of the peptide 30, 50, 100, 250, 500 and 1000 ng/ml prepared in double distilled water was added to 180 µl of diluted $ABTS^{\bullet+}$ and then absorbance was measured at 734 nm at 30 °C for 10 min. The percentage inhibition was calculated using the following formula:

$$\text{Percentage inhibition} = (\text{Initial } A_{734\,nm} - \text{Final } A_{734\,nm})/\text{Initial } A_{734\,nm}) \times 100$$

The percentage of inhibition was plotted as function of concentration of Trolox (µM) and taken as standard. Results are expressed in µM Trolox equivalents.

3.3. Oxygen radical absorbance capacity (ORAC)

Oxygen radical absorbance capacity was measured using 2, 2′-azobis (2-amidino-propane) dihydrochloride (AAPH) (Ou et al., 2001) for generating peroxyl radicals. Fluorescein diacetate was used as substrate. Twenty microliter of different concentration of the peptide (30–1000 ng/ml of medium) was added to 120 µl fluorescein diacetete (117 nM) and incubated at 37 °C for 15 min in 75 mM phosphate buffer pH 7.4. Then, 60 µl of AAPH (12 mM final concentration) was added to the mixture. The microplate was shaken before the first reading and the fluorescence was recorded every 1 min and the whole assay lasted for 80 min. Fluorescence intensity was measured using a fluorescence microplate reader (Model Infinite F200 Pro, Tecan, Austria) at 485 nm excitation and 520 nm emission.

3.4. Isolation of fibroblast cells from rat skin and primary culture

This study was conducted after getting approval from the Institutional Animal Ethical Committee (IAEC) of ICAR-National Dairy Research Institute, Karnal. Primary fibroblast cells were isolated from 3- days' old rat pup skin using collagenase enzyme (Wang et al., 2004). Briefly, three days' old rat pup skin was cleaned with 70% alcohol and rinsed thrice with PBS containing antibiotic solution in the petri plate with simultaneous removal of hairs and fat from the skin. The minced tissue was subjected to enzymatic digestion (1 ml of 0.1% collagenase), and this process was carried out in a shaking water bath at 140 rpm at 37 °C for 10 min. After a 10-min digestion, enzyme activity was inhibited by adding an equal volume of Dulbecco's modified minimal essential medium (DMEM) with 10% fetal bovine serum and the released cells were recovered from the supernatant by filtering through a cell strainer 100 µm (BD Falcon™). The filtrate was designated as Digest I, left over tissue was washed with PBS to remove the traces of serum and fresh enzyme was added to continue the second round of digestion. The filtrate obtained after the second round of digestion was designated as Digest I. Digestion process was repeated to get Digest III, IV and V respectively. Digest I was discarded while Digests II to V were pooled and centrifuged at 1800 rpm at 4 °C for 5 min. At the end of the centrifugation, the supernatant was discarded, and the remaining pellet was suspended in 1 ml of complete growth medium and cultured further in CO_2 incubator at the cell density of 1×10^6 cells per ml of medium.

3.5. Immunocytochemical characterization of fibroblast cells

Previously, it has been indicated that homogeneous population of fibroblast cells can be obtained after two or third passages (Liu et al., 2014). Vimentin is a cytoskeleton protein, mostly expressed in fibroblast cells and thus, has been commonly used as marker for characterization of fibroblast cells (Priya et al., 2012). In this study, homogenous rat skin fibroblast cells were isolated and characterized at third passage by immunocytochemistry using anti-vimentin as a positive marker and CD-86 as a negative marker as described previously (Priya et al., 2012). In brief, cells were grown on the coverslip in 35 mm petri-plate till about 80% confluency was achieved. Then, cells were fixed with ice chilled methanol for 10–15 min at room temperature and permeabilized by 0.25% Triton X-100 in PBS for 10 min after washing with PBS. Then, blocking was done with 5% BSA for 30 min. At end of blocking, cells were incubated with primary antibodies (1:150; rabbit anti-vimentin and 1:250 rabbit anti-CD 86 diluted with 1% BSA solution (Santacruz Biotechnology, Paso Robles, CA, USA) for 24 h at 4 °C. The contents were washed three times with washing buffer. Then, cells were incubated with secondary antibody (1:250; anti-rabbit) diluted with 1% BSA solution (Sigma St. Louis, MO, USA) conjugated with fluorescein isothiocyanate (FITC) for 1 h in dark at room temperature. After washing, cells were treated with DAPI (4′, 6-diamidino-2- phenylindole) for 10 min. Then, cells were washed again three times with

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