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A Novel Buffalo Casein Derived Bioactive Peptide Protects Against Oxidative Stress Induced Dysfunctions

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Abstract—*Today's demanding life style pushing the people towards getting oxidative stress related metabolic diseases and also an incredible factor in the development of aging, cancer, diabetes, inflammatory diseases and myocardial infections. Bioactive peptides from natural sources with antioxidative potential are of utmost importance and highly attractive as prodrug. So, present study focuses on establishing an in-vitro cellular oxidative stress model system and screening the antioxidative potential of milk derived bioactive peptide (VLPVPQK) in resisting cellular death. Our results demonstrated that, 0.2mM H_2O_2 for 6 h significantly suppressed the cellular anti-oxidative enzymatic system and promoted LDH, ROS and caspases' activity and thereby, it promoted cell death. Amazingly, peptide pretreatment significantly enhanced the survivability by reducing cellular necrosis, maintaining redox homeostasis and, also reversed all the related dysfunctions. So, our results suggest that peptide can be effective as a potential remedy in treatment of inflammatory and oxidative stress related diseases.*

Keywords*: Fibroblasts, oxidative stress, hydrogen peroxide, peptide, reactive oxygen species.*

Abbreviations
VLPVPQK, Valine-Leucine-Proline-Valine-Proline-Glutamine-Lysine; SOD, superoxide dismutase; LDH, lactate dehydrogenase; DCFH-DA, 2',7'-dichlorodihydrofluorescein –diacetate; DMSO, dimethyl sulfoxide; DAPI, 4', 6-diamidino-2-phenylindole; ABTS, 2, 2 azinobis (ethylbenzthiazoline-6-sulfonic acid)

1. INTRODUCTION

Our cellular system has well developed anti-oxidative enzymatic and non-enzymatic defense mechanism to combat against metabolic oxidative stressors [1]. But elevation in reactive oxygen species (ROS) production due to burdened metabolism leads to cell death and development of oxidative stress associated diseases such as atherosclerosis, cancer, arthritis and hypertension [2, 3, 4]. Hydrogen peroxide (H_2O_2) is the most commonly used precursor of oxidative stress in cells and thus, can serve as a potential way to screen antioxidative compounds including bioactive peptides [5]. Antioxidative cellular enzymes include SOD and catalase, was markedly declined upon exposure to elevated ROS, LDH and caspases production in fibroblast cells [5, 6, 7]. Inspite of antioxidative, antihypertensive, antimicrobial, anti-inflammatory, and osteogenic effects of milk derived bioactive peptide; studying overall cytoprotective effect of these peptides is very attractive in preventing oxidative stress related disorders. Recently, four peptides isolated, purified and sequenced in our laboratory, among which heptapeptide (VLPVPQK) derived from pepsin-trypsin hydrolysates of β-casein, displayed highest antioxidative as evaluated by ABTS method and also, possesses osteoanabolic activity and was also bio-accessible up to 1% in cellular system [8, 9, 10]. However, its cytoprotective antioxidative effect has not yet been studied in oxidatively stressed *invitro* fibroblast cells.

2. MATERIALS AND METHODS

2.1 Materials

The All the reagents used in the present study were of cell culture or analytical grade.

2.2 Methods

2.2.1 Evaluation of antioxidative activity by radical scavenging assays

Antioxidative potential of the peptide was estimated by ABTS and ORAC method depending on free radical scavenging activity of the peptide. Radical scavenging activity of peptide was measured by ABTS as described previously [11] with little modifications. Briefly, twenty micro liter of various concentration of peptide 30, 50, 100, 250, 500 and 1000ng/ml prepared in double distilled water was added to 180µl of diluted $ABTS^{\bullet+}$ and then absorbance was measured at 734nm. Oxygen radical absorbance capacity was evaluated using 2, 2'-azobis (2-amidino-propane) dihydrochloride (AAPH) as described previously [12].

2.2.2 Isolation and primary culture of fibroblast cells

Primary fibroblast cells were isolated from 3-days old rat pup skin using collagenase enzyme [13] and cultured further in CO_2 incubator at the density of 1 x 10^6cells per ml medium.

2.2.3 Establishement of *in vitro* oxidative stress model

Fibroblast cells oxidative stress model was established by the method of Mada *et al*. (2016) [10]. Cells were treated with DMEM medium containing 5% FBS with different (0.2, 0.4, 0.6, 0.8 and 1.0mM) concentrations of H_2O_2 except control cells which were plated with DMEM medium containing 5% FBS and incubated for 3, 6 and 24 hours in CO_2 incubator. After incubation, cell survivability was measured by MTT method as previously described [14].

2.2.4 Measurement of oxidative cytotoxicity

2.2.4.1 Estimation of intracellular ROS production

Intracellular ROS was evaluated using DCFH-DA (Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions.

2.2.4.2 Evaluation of cell mortality

Cell mortality was evaluated by propidium iodide (PI) staining [15]. Further, to assess the mortality signaling factor, caspase-3/-9 activities were measured using caspase-3 and -9 Colorimetric assay kits (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions.

2.2.5 Evaluation of cytoprotective effect of the peptide

2.2.5.1 Assessment of cell viability assay

Fibroblast cell viability was measured using MTT method as described earlier in note 2.2.3. In brief, cells were cultured with DMEM containing different concentrations of peptide (10, 50, 100, 150, 200, 250, 500, 1000ng) or 5mM N-acetyl cysteine (NAC) for 24 hours, followed by treatment with 0.2mM and 1mM H_2O_2 (IC_{50}) for 6 hours. Then, cells were incubated with 20 µl MTT (5mg/ml) solution for maximum of 4 h at 37°C to allow the MTT to be metabolized. Then cell viability was determined by measuring absorbance at 540 nm using microplate reader (BioTek ELISA reader).

2.2.5.2 Estimation of oxidative cell injury

The oxidative cell injury was assessed using LDH activity as determined by LDH cytotoxicity assay kit (Cayman, Ann Arbor, MI. USA) according to manufacturer's protocol.

2.2.5.3 Measurement of cellular oxidative status

Peptide cytoprotective effect was determined by evaluating antioxidative defense biomarkers such as superoxide dismutase (SOD), catalase (CAT) and MDA level. SOD, CAT activity and MDA level was determined after peptide and H_2O_2 treatment using cayman chemical assay kit (Ann Arbor, MI. USA) following manufacturer's instructions.

3. DATA ANALYSIS

GraphPad Prism 5.01 version (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis. Results are compared using one way ANOVA and dunnett's test. Each experiment (n=3) was observed in triplicates. $P<0.05$ was considered as statistically significant.

4. RESULT

4.1 Antioxidative activity of peptide

Our result showed that, both $ABTS^{\cdot+}$ free radical scavenging and ORAC activity of peptide was increased in a dose dependent manner, suggesting the antioxidative potential of peptide as shown in Fig. 1.

4.2 H_2O_2-induced cellular necrosis

4.2.1 Reduction of cell viability

Reduction in cell viability was observed on treatment of fibroblast cells with different concentration of H_2O_2 for 3 h. But treatment of 0.2mM H_2O_2 for 6 h significantly ($P<0.05$) decreased cell survival rate to about 50% (IC_{50}) (Fig. 2) and is used throughout the present study. A variable response was observed in case of 24 h with respect to different concentration of H_2O_2.

4.2.2 ROS production and cell mortality

DCFH-DA staining revealed that ROS level was significantly increased at all the concentration from 0.2 to 1mM H_2O_2 (Fig. 3A). Also, a significant ($P<0.05$) dose dependent increase in PI stained cells was observed from (10155±1259) relative units to (23118±1429) relative units on H_2O_2 exposure compared to control as shown in Fig. 3B and C, indicated the enhanced cell mortality in oxidative stress conditions.

4.2.3 Caspase-3/-9 activity

A significant ($P<0.05$) elevation in apoptotic activity of both executioner and initiator caspases i.e. caspase-3/-9 was observed on H_2O_2 treatment, suggesting that H_2O_2 induced cell death via these caspases (Fig. 4).

4.3 Effect of the peptide pretreatment on cell survivability

4.3.1 Enhanced cell viability by the peptide

In addition, cells pretreated with different (30-500ng/ml) concentrations of peptide for 24h before H_2O_2 exposure significantly reversed the cytotoxicity induced by both lowest chosen experimental dose i.e. 0.2mM H_2O_2 as well as highest dose i.e. 1mM H_2O_2 compared to untreated cells. 100ng/ml of the peptide was most significantly effective in improving cell survivability ($P<0.05$) in both the conditions as shown in Fig. 5.

4.3.2 Improvement of antioxidative enzymatic system

Our data showed that peptide pretreatment significantly ($p<0.05$) prevented elevation in LDH activity and MDA level (Fig. 6) and, also an alteration in SOD and CAT activities in H_2O_2 treated cells, further ascertaining the cytoprotective effect of peptide as shown in Fig. 7.

5. DISCUSSION

Fibroblasts are the main cell type in the skin responsible for the synthesis of extracellular matrix [15]. Previous studies have demonstrated the antioxidative potential of several isolated biomolecules using ABTS and ORAC methods [8, 16]. In the present study, peptide (VLPVPQK) exhibits a significant antioxidative property by both of these methods. Hydrogen peroxide (H_2O_2) has been demonstrated as a stimulant of oxidative stress, due to its strong diffusible nature through cell membranes. [5]. Our results demonstrated that 0.2mM H_2O_2 treatment for 6 h reduced cell survival to about 50% (IC_{50}). So, this concentration is used throughout the study for generating oxidative stress. Oxidative damage leads to decreased cell viability and death via increased caspases activity stimulated by elevated ROS production in fibroblast cells [6]. Hence, to further gain insight into reduced survival rate, ROS production, cell mortality and caspase-3/-9 activities were quantified. H_2O_2 exposure significantly induced the apoptosis through enhanced intracellular ROS and apoptotic caspase-3/-9 activities. Production of LDH and MDA level are the potential hallmark of oxidative stress induced injury in fibroblast cells [7]. However, antioxidative peptide pretreatment for 24 h before H_2O_2 exposure significantly reversed the oxidative dysfunctions induced by H_2O_2 as interpreted through reduced intracellular LDH and MDA level comparable to standard antioxidant NAC (5mM) in H_2O_2 stressed cells. 100ng/ml peptide was even effective in case of higher (1mM) doses of H_2O_2. Cellular antioxidative enzymatic system consists of SOD and CAT; acts as the primary line of defense against oxidative stress or excessive ROS produced in cell metabolic process [17]. In the present study, peptide pretreatment increased the CAT activity and prevented H_2O_2-induced elevation of SOD activity. Thus, the peptide restored the redox homeostasis of cellular system. In confirmation with existing evidence, our results suggest that peptide pretreatment prevented necrotic death promoted by H_2O_2 in fibroblasts.

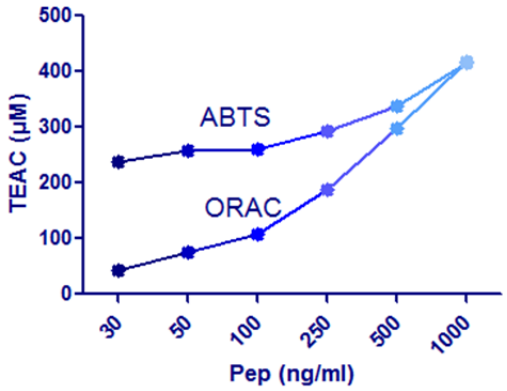


Fig. 1: Peptide exerts antioxidative property as revealed by ABTS and ORAC activity. A concentration dependent effect was exhibited by the peptide. The results are expressed as mean±S.E.M. (n=3)

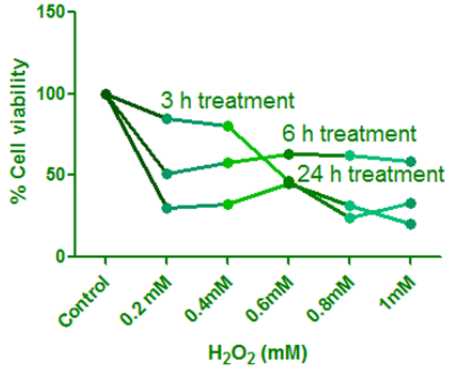
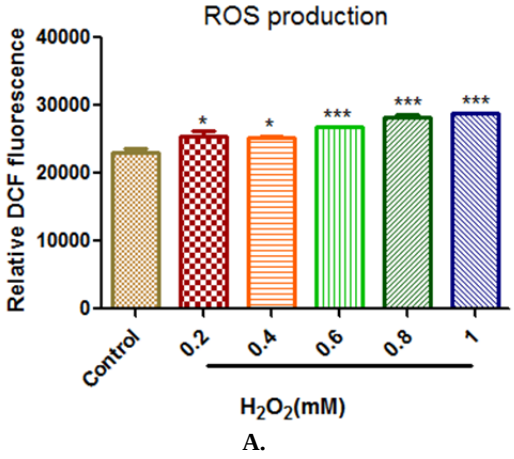
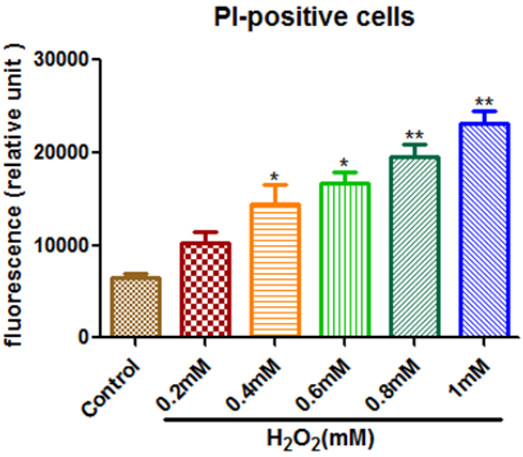


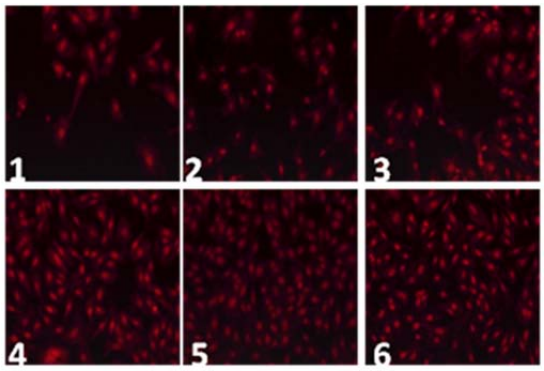
Fig. 2: Effect of different concentration of H_2O_2 on fibroblasts survival rate at different treatment time by MTT colorimetric assay. % cell viability was calculated by considering control as 100%. The results are expressed as mean±S.E.M. (n=3)



A.



B.



C.

Fig. 3: Quantitative estimation of A. ROS production B. PI-positive cells. C. A representative photomicrograph of PI stained cells. 1: Control cells; 2: 0.2mM H_2O_2 treated cells for 6 h; 3: 0.4mM H_2O_2 treated cells for 6 h; 4: 0.6mM H_2O_2 treated cells for 6 h; 5: 0.8mM H_2O_2 treated cells for 6 h; 6: 1mM H_2O_2 treated cells for 6 h. Cell mortality rate was increased in dose dependent manner. The results are expressed as mean±S.E.M. (n=3). *P<0.05, **P<0.01 and ***P<0.001 compared with control group.

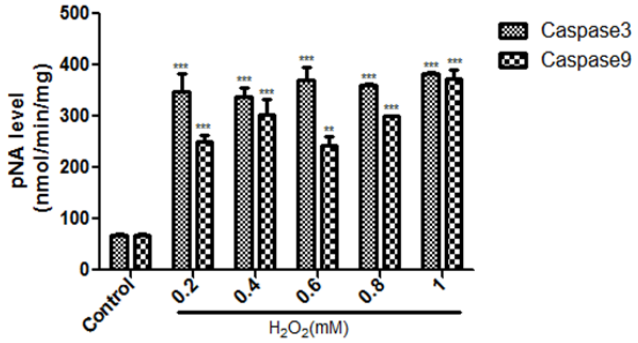


Fig. 4: Relative quantitative analysis of caspase-3/-9 activity. A significant (p<0.05) increase in apoptotic caspase-3/-9 activity was observed on exposure to different concentration of H_2O_2 compared to control group. % release was calculated by considering control as 100%. The results are expressed as mean±S.E.M. (n=3). **P<0.01 and ***P<0.001 compared with control group.

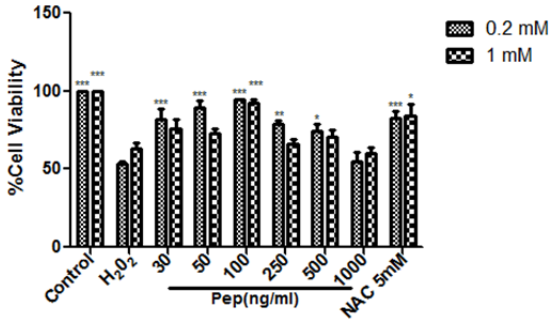


Fig. 5: Comparative effect of the different concentration of peptide at different doses of H_2O_2 i.e. 0.2 AND 1mM cell viability. 100ng/ml of peptide was most significant (P<0.05) for attenuating the H_2O_2 cytotoxicity. % cell viability was calculated by considering control as 100%. The results are expressed as mean±S.E.M. (n=3). *P<0.05, **P<0.01 and ***P<0.001 compared with control group.

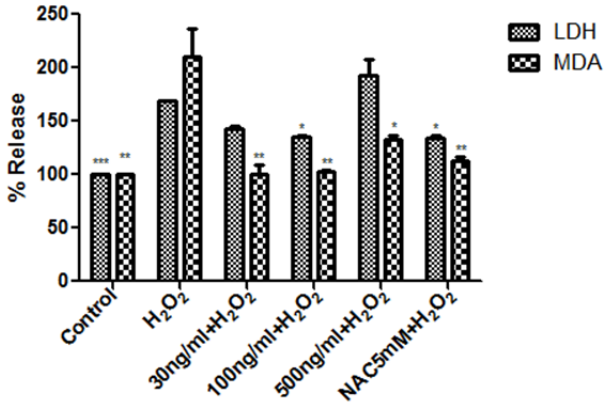


Fig. 6: Peptide, VLPVPQK significantly (P<0.05) attenuated the lactate dehydrogenase and MDA level. % release was calculated by considering control as 100% in cell medium. The results are expressed as mean±S.E.M. (n=3). * P<0.05, **P<0.01 and ***P<0.001 compared with control group.

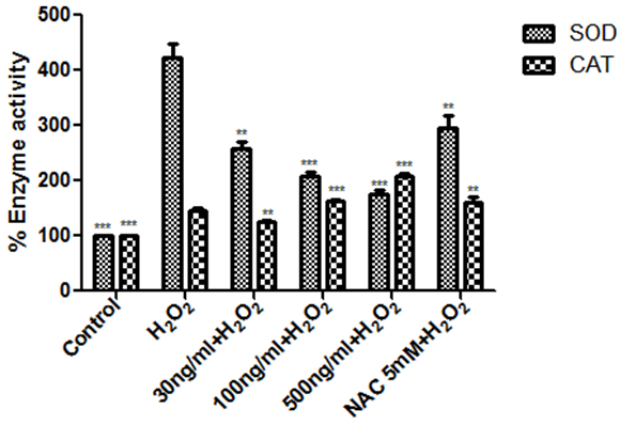


Fig. 7: Effect of the peptide pre-incubation on the antioxidative enzymes; SOD and catalase. % enzyme activity was calculated by considering control as 100%. The results are expressed as mean±S.E.M. (n=3). **P<0.01 and ***P<0.001 compared with control group.

6. CONCLUSION

The present study demonstrated that a novel peptide (VLPVPQK) prevents oxidative stress induced cellular death via its cytoprotective antioxidative effect.

7. ACKNOWLEDGEMENTS

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