Journal of Functional Foods 15 (2015) 365–375

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Antioxidant peptides from "Mozzarella di Bufala Campana DOP" after simulated gastrointestinal digestion: *In vitro* intestinal protection, bioavailability, and anti-haemolytic capacity

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ARTICLE INFO

Article history:
Received 19 January 2015
Received in revised form 24 March 2015
Accepted 26 March 2015
Available online 14 April 2015

Keywords:
Mozzarella di Bufala Campana DOP
In vitro gastrointestinal digestion
Antioxidant peptides
In vitro intestinal protection
Bioavailability
Anti-haemolytic capacity

ABSTRACT

The bioactive properties of milk and milk-products are largely attributed to the peptides released during gastrointestinal digestion. Nevertheless, no similar studies on "Mozzarella di Bufala Campana DOP" (MBC), the European name given to a unique protected origin designation buffalo milk product, are available so far. A novel antioxidant peptide (MBCP) after MBC gastrointestinal digestion was identified and its *in vitro* intestinal protection, bioavailability, and anti-haemolytic capacity were assayed. A 0.2 mg/mL MBCP incubation dose made H_2O_2-stressed CaCo2 cell line proliferation increase by about 100%. Less than 10% hydrolysis in the apical solution and about 10% concentration in the basolateral solution indicated for MBCP good stability and bioavailability, respectively. A 0.2 mg/mL MBCP incubation dose reduced H_2O_2-induced human erythrocyte haemolysis by 91.25%. Our data indicated MBC as a potential functional food and MBCP as a novel food ingredient, food additive and pharmaceutical, relevant in health promotion and disease prevention.

1. Introduction

In addition to its major nutritional role, the bioactive potential of milk is now well accepted. It has been recognised that much of the bioactivity can be attributed not only to intact caseins and whey proteins but also to many milk protein-derived peptides (Korhonen, 2009). Such peptides are inactive within the sequence of the parent protein and can be released by digestive enzymes during gastrointestinal transit or by fermentation or ripening during food processing (Kitts & Weiler, 2003). They can have a beneficial effect on a variety of biological systems including the cardiovascular, gastrointestinal, immune and nervous systems (Murray & FitzGerald, 2007).

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http://dx.doi.org/10.1016/j.jff.2015.03.048
1756-4646/

Reactive oxygen species (ROS), the by-products of oxygen metabolism, are among the main causes of a wide range of human degenerative pathologies, such as cardiovascular diseases, diabetes, inflammation, cancer, neurodegeneration, and accelerated aging, by a process named oxidative stress (Di Bernardini et al., 2011; Mendis, Kim, Rajapakse, & Kim, 2007). Peptides contribute to the body antioxidant protection. Peptides occurring in the blood can on average scavenge 20% (range 10–50%) of the peroxyl radicals of plasma (Wayner, Burton, Ingold, Barclay, & Locke, 1987). Glutathione (GSH), a tripeptide synthesised predominantly within the liver, mainly scavenges free radicals and ROS (Powers & Jackson, 2008); carnosine, a dipeptide found in skeletal muscle, can act as a radical scavenger and metal ion chelator (Chan, Decker, & Feustman, 1994). Several milk-derived peptides have been found to possess antioxidant activity (Pihlanto, 2006). Particularly, caseinphosphopeptides (CPPs) are derived from enzymatic hydrolysis of casein and are rich in phosphoserine residues. The proposed mechanism of CPP antioxidant activity is linked to the presence of phosphate groups originating from the phosphoserine residues in close proximity to the peptide chain (Kitts, 2005). CPPs have been shown to possess radical scavenging (Chiu & Kitts, 2003) and metal chelating activity (Kim, Jang, & Kim, 2007), whereas high amounts of CPP have been reported to be pro-oxidative (Díaz & Decker, 2004).

Peptides can be absorbed in the intestinal tract by a number of mechanisms including the paracellular and transcellular routes, the lymphatic system and via basolateral transporters. The molecular size and structural properties such as peptide hydrophobicity will determine the mechanism of transport (Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernandez- Escalante, 2011). Nevertheless, a bioactive peptide may not need to be absorbed in order to elicit the antioxidant function. Binding to an intestinal receptor may be sufficient to trigger the bioactive (*i.e.* antioxidant) response (Xiong, 2010). The intestine is at the interface between the organism and its luminal environment, thus, representing a critical defence barrier against luminal toxic agents. Therefore, in addition to being exposed to luminal nutrients, the intestinal mucosa is constantly challenged by diet-derived oxidants, mutagens, and carcinogens as well as by endogenously generated ROS (Aw, 1999). Thus, antioxidant peptides may also elicit a beneficial biological effect by reducing oxidative damage within the gastrointestinal tract (Xiong, 2010).

Buffalo milk is a very ancient product, typical of the tropical and sub-tropical countries, such as Southeast Asia. It is also intensely produced in Italy, especially in the southern regions, where it is entirely addressed to cheese making due to its high dry residue value similar to that of sheep milk. Buffalo mozzarella cheese is the most famous and consumed buffalo milk product in Southern Italy. Particularly, the so called Mozzarella di Bufala Campana is listed as a protected origin designation (POD) product from the European Council [Commission Regulation (EC) No. 1107/1996)]. Its disciplinary of production identifies 96 municipalities in Southern Italy as the only places of production of the so called Mozzarella di Bufala Campana DOP. It indicates a peculiar manufacturing process that contemplates exclusively fresh whole buffalo milk characterised by a specific macronutrient composition, and precise chemical–physical and organoleptic properties of the final product.

Buffalo milk proteins have been reported to have a high homology to their cow counterparts (D'Ambrosio et al., 2008). Nevertheless, few studies on proteins from buffalo milk and its products as sources of bioactive peptides are available so far. ACE-inhibitory and antimicrobial peptides after enzymatic digestion of buffalo milk casein have been described (De Simone et al., 2011), while buffalo mozzarella cheese whey has been indicated as a major source of antiproliferative and cytomodulatory peptides (De Simone et al., 2009). Rizzello et al. (2005) found that the peptides extracted from Italian buffalo mozzarella cheese exhibited a specific antibacterial activity in comparison to peptides from other cheeses. However, no studies on bioactive buffalo mozzarella cheese peptides derived from gastrointestinal digestion are available to date. Thus, the aim of the present study was to evaluate the peptide production after simulated gastrointestinal digestion of "Mozzarella di Bufala Campana DOP" (MBC) and their potential antioxidant activity by *in vitro* assays.

2. Materials and methods

2.1. *Reagents and standards*

All chemicals and reagents used were either analytical-reagent or HPLC grade. The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA) before use. Chemicals and reagents used to simulate the GI digestion were: potassium chloride (KCl), potassium thiocyanate (KSCN), monosodium phosphate (NaH_2PO_4), sodium sulphate (Na_2SO_4), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), urea, α-amylase, hydrochloric acid (HCl), pepsin, pancreatin, bile salts (Sigma Chemical Co., St. Louis, MO, USA). Chromatographic resins Sephadex G-50, G-25 and G-10, DPPH (1,1-diphenyl-2-picrilhydrazyl), 2,4,6-tris-2,4,6-tripiridyl-S-triazine (TPTZ), iron (III) chloride (dry), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), purchased from Sigma Aldrich (Sigma Chemical Co.). All organic solvents were purchased from Carlo Erba (Milano, Italy). 2′,7′-Dichlorofluorescin diacetate (DCFH-DA, Sigma Chemical Co.) was dissolved in dimethylsulphoxide (DMSO) to obtain 100 mM stock solution (aliquoted and stored at –20 °C). Work solutions of DCFH-DA were produced by diluting aliquots in 1% phosphate buffer saline (PBS, 10 mM, pH 7.4) at different concentrations.

2.2. *Sample collection and preparation*

"Mozzarella di Bufala Campana DOP" (MBC) was purchased in Caserta (Campania, Italy) in a local dairy. An aliquot (250 g), approximately 1 h after its preparation, was stored at –80 °C. Sample was freeze-dried and, then, subjected to lipid extraction according to AOAC (1995) method 948.16, by using a 6-place units Extraction Unit E-816 Soxhlet (Buchi, Flawil, Switzerland). After centrifugation at 3000 *g* for 5 min, pellets were transferred into a pre-weighed scintillation vial, dried under nitrogen, and kept at –80 °C until analyses.

2.3. In vitro *gastrointestinal digestion*

The assay was performed according to the procedure described by Raiola, Meca, Mañes, and Ritieni (2012), with slight

modification. GI digestion was distinguished into salivary, gastric and duodenal digestive steps. For the salivary digestion, defatted sample (80.5 g) was mixed with 6 mL of artificial saliva composed of: KCl (89.6 g/L), KSCN (20 g/L), NaH_2PO_4 (88.8 g/L), Na_2SO_4 (57.0 g/L), NaCl (175.3 g/L), $NaHCO_3$ (84.7 g/L), urea (25.0 g/L) and 290 mg of α-amylase. The pH of the solution was adjusted to 6.8 with 0.1 M HCl. The mixture was introduced in a plastic bag containing 40 mL of water and homogenised in a Stomacher 80 Microbiomaster (Seward, Worthing, UK) for 3 min. Immediately, 0.5 g of pepsin (14,800 U) dissolved in HCl 0.1 N was added, the pH was adjusted to 2.0 with 6 M HCl, and then incubated at 37 °C in a Polymax 1040 orbital shaker (250 rpm) (Heidolph, Schwabach, Germany) for 2 h. After the gastric digestion, the pancreatic digestion was simulated as follows: the pH was increased to 6.5 with 0.5 M $NaHCO_3$ and then 5 mL of a mixture pancreatin (8.0 mg/mL) and bile salts (50.0 mg/mL) (1:1; v/v), dissolved in 20 mL of water, were added and incubated at 37 °C in an orbital shaker (250 rpm) for 2 h. Intestinal digested sample was freeze-dried and stored at –80 °C until further analysis.

2.4. *Gel filtration chromatography of MBC gastrointestinal digest*

2.4.1. *Fraction separation*

The freeze-dried intestinal digested sample was dissolved with water (50 mg/mL) and purified on a Sephadex G-50 gel filtration column (2 × 75 cm), by eluting with distilled water at 0.5 mL/min. Aliquots (3 mL) were collected and pooled into fractions by monitoring absorbance at 280 nm. Fractions were lyophilised, dissolved with water (4 mg/mL), and analysed for their antioxidant properties (see Section 2.4.2). The most active fraction was lyophilised, dissolved with water (50 mg/mL), and loaded on a Sephadex G-25 gel filtration column (2 × 75 cm), by eluting, monitoring, and pooling into fractions as described earlier. Similarly, the fraction with the highest antioxidant activity was further purified on a G-10 gel filtration column (2 × 75 cm), by eluting, monitoring, and pooling into fractions as described earlier. The most antioxidant fraction was analysed by RP-HPLC (see Section 2.4.3).

2.4.2. *Fraction antioxidant activity*

For each antioxidant assay, a trolox aliquot was used to develop a 50–500 μmol/L standard curve. All data were then expressed as Trolox Equivalents (μmol TE/100 mL).

2.4.2.1. *DPPH•-scavenging assay*. The test was performed according to Brand-Williams, Cuvelier, and Berset (1995). Fraction aliquots (20 μL) were added to 3 mL of DPPH solution (6×10^{-5} mol/L) and the absorbance was determined at 515 nm every 5 min until the steady state.

2.4.2.2. *Ferric reducing antioxidant power (FRAP) assay*. The assay was performed as previously described (Benzie & Strain, 1996). A solution of 10 mmol/L TPTZ in 40 mmol/L HCl and 12 mmol/L ferric chloride was diluted in 300 mmol/L sodium acetate buffer (pH 3.6) at a ratio of 1:1:10. Fraction aliquots (20 μL) were added to 3 mL of the FRAP solution and the absorbance was determined at 593 nm every 5 min until the steady state.

2.4.3. *Fraction purity evaluation*

Sephadex G-10 fraction showing the strongest antioxidant capacity was filtered through a Phenex-PVDF 17 mm Syringe Filter 0.45 μm (Phenomenex, Torrance, CA, USA) and analysed by RP-HPLC. Analyses were run on a Finnigan HPLC system (Thermo Electron Corporation, San Jose, CA, USA) provided with photodiode array detector (DAD). The column selected was an Aeris PEPTIDE 3.6 μm XB-C18 New Column 250 × 4.6 mm (Phenomenex). Elution conditions consisted in 2% acetic acid (solvent A) and 2% acetic acid in methanol (solvent B) gradient at a flow rate of 1.0 mL/min. The gradient conditions were: 0–20 min, 0–100% B; 20–23 min, 100% B; 23–27 min, 0% B, followed by 5 min of maintenance. Chromatograms were recorded at 280 nm and indicated that the fraction analysed was a high purity peptide (96%), that was named as MBCP and subjected to characterisation.

2.5. *Characterisation of MBCP*

MBCP was collected, and its purity and molecular weight were determined by ultra performance liquid chromatography electrospray ionisation tandem mass spectrometry (UPLC ESI–MS/MS). A Waters ACQUITY UPLC system (Waters Corporation, Milford, MA, USA), coupled with a Synapt Mass Quadrupole Time-of-Flight Mass Spectrometer (UPLC-ESI Q-TOF MS) (Waters Corporation), was used to identify the purified peptide. MBCP was dissolved in mobile phase, and 5 μL of peptide solution was loaded onto an ACQUITY BEH C18 column (100 mm × 2.1 mm, 1.7 μm) (Waters Corporation). Elution conditions consisted in 0.5% acetic acid (eluent A) and methanol (eluent B) gradient at a flow rate of 0.3 mL/min. The gradient conditions were: 0–3 min, 5% B; 3–15 min, 5%–30% B; 15–17 min, 30% B. Following molecular mass determination, the peptide was automatically selected for fragmentation, and sequence information was obtained by tandem mass spectroscopy analysis. MS/MS analyses were performed in the positive electrospray ionisation mode by using CID. Collision energy was selected from 10 to 35 eV. Argon was introduced as the collision gas at a pressure of 10 psi. Sequencing of peptide was acquired over the *m*/*z* range 50–2000 using the Biolynx software.

2.6. *Gel filtration chromatography of non-digested MBC protein extract*

In order to ascertain the presence of MBCP in the non-digested MBC, a MBC aliquot (250 g) was subjected to preparation as described in Section 2.2. Then, the prepared sample was purified as described in Section 2.4 and fraction characterisation was performed as described in Section 2.5.

2.7. *Peptide synthesis*

MBCP was synthesised using the solid-phase method and purified via HPLC after deprotection. Synthetic peptide was used to confirm MBCP identity. Both synthetic and isolated MBCP were used for *in vitro* assays and results were compared.

2.8. CaCo2 cell line tests

2.8.1. Cell culture and proliferation assay

The assay was performed according to previous authors (De Simone et al., 2009). CaCo2 cells (American Type Culture Collection, Rockville, MD) were grown in HEPES buffered Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose and supplemented with 12.5% fetal calf serum (FCS) (Flow, McLean, VA, USA), 1% (v/v) MEM non-essential amino acids, 5 mM L-glutamine, 1% sodium pyruvate, 40 U/mL penicillin, 100 μg/mL gentamycin, and 40 μg/mL streptomycin (DMEMc). The cells (17–21 passages) were grown in a humidified atmosphere of CO_2/air (5:95) at 37 °C and were plated in 12 multi-well plates at different densities. After incubation for 4 h, the cells were washed with PBS to remove unattached dead cells. The cells were pre-treated for 30 min with 50 μM H_2O_2 in PBS solution (H-CaCo2). Then, the pre-confluent H-CaCo2 (21 passages) cells were seeded in 12 multi-well plates and incubated at 37 °C for 24 h with 0.0125–1 mg/mL of MBCP in water solution. The cell number was measured with a haemocytometric counter and cell proliferation was evaluated by CyQuant® cell proliferation assay Kit (Invitrogen™) (Thermo Fisher Scientific, San Jose, CA, USA) with dye fluorescence measurement at 480 nm excitation maximum and 520 nm emission maximum. The control sample consisted in H-CaCo2 cells not added of peptide (untreated cells). Cell proliferation was expressed in percentage of proliferation compared with the control. All experiments were performed on triplicate cultures.

2.8.2. Preparation of cell extract

The assay was performed as previously described (Tenore et al., 2014). H-CaCo2 cells were collected by centrifugation and then resuspended in ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 2 mM ethylenediaminetetraacetic acid (EDTA). The cells were sonicated, followed by centrifugation at 13,000 × *g* for 10 min at 4 °C. The resulting supernatants were collected and kept on ice for immediate measurements, to be described later.

2.8.3. Measurement of intracellular ROS accumulation

The assay was performed as previously described (Gomez-Monterrey et al., 2013). The thiobarbituric acid reactive substance (TBARS) assay was performed on membranes extracted from Caco-2 and H-Caco2 cells after 48 h incubation with 0.0125–1 mg/mL of MBCP in water solution, and from the untreated control cells, using an ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 1% Triton) supplemented with a mixture of protease inhibitors. The homogenate was centrifuged at 1200 × *g* for 10 min to separate the cytosol (supernatant) from the membranes (pellet). The pellet was dissolved in 50 mM Tris, 150 mM NaCl, and 10 mM EDTA. Aliquots (10 μL) of the membrane preparation were added to 2 mL thiobarbituric acid (TBA) trichloroacetic acid (TCA) (15% TCA, 0.3% TBA in 0.12 M HCl) solution at 100 °C for 30 min. The reaction was stopped by cooling the sample in cold water. The samples were centrifuged at 5000 × *g* for 10 min and the chromogenic (TBARs) was quantified by spectrophotometry at a wavelength of 532 nm. The quantity of TBARs was expressed as μM/μg proteins.

2.8.4. Measurement of cellular superoxide dismutase activity

The assay was performed as previously described (Tenore et al., 2014). Total cellular superoxide dismutase (SOD) activity was measured as follows (Kirshenbaum & Singal, 1992). Briefly, a reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 1.33 mM diethylenetriaminepentaacetic acid, 1.0 U/mL catalase, 70 μM nitroblue tetrazolium, 0.2 mM xanthine, 50 μM bathocuproinedisulphonic acid, and 0.13 mg/mL bovine serum albumin (BSA). A 0.8 mL aliquot of the reaction mixture was added to each cuvette, followed by addition of 100 μL of lysate. The cuvettes were pre-warmed at 37 °C for 3 min. The formation of formazan blue was monitored at 560 nm, 37 °C for 5 min. The sample total SOD activity was calculated using a concurrently run SOD (Sigma) standard curve, and expressed as units per mg of cellular protein. Cellular protein content was quantified with Bio-Rad protein assay dye (Hercules, CA) based on the method which makes use of BSA as the standard.

2.9. In vitro intestinal transepithelial transport studies

The assay was performed as previously described (Tenore et al., 2014). The human colon carcinoma cell line CaCo2 (HTB-37) was obtained from the American Type Culture Collection (LGC Promochem, Molsheim, France). Cells were cultured (17–21 passages) in HEPES buffered Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose and supplemented with 12.5% fetal calf serum (FCS), 1% nonessential amino acids, 5 mM L-glutamine, 40 U/mL penicillin, 100 μg/mL gentamycin, and 40 μg/mL streptomycin (DMEMc). Cells were maintained at 37 °C in a humidified atmosphere of CO_2/air (5:95, v/v) and passaged every 7 days by trypsinisation. They were seeded in Transwell (Transwell® inserts of 3 μm pore size and 24 mm diameter) at 6×10^4 cells/cm^2. The medium (15 mL DMEM containing 12.5% FCS) was changed every 2 days until cells reached confluence (7–8 days). The integrity of the monolayers (cultured for 14–15 days) was evaluated by measurement of the transepithelial electrical resistance (TEER) using a Millicell-ERS device (Millipore, Zug, Switzerland). Only CaCo2 monolayers showing TEER higher than 300 Ω × cm^2 were used for the experiments. The integrity of the monolayers was checked before, during and after the experiment. Then, CaCo2 cells monolayers were gently rinsed twice with PBS, medium was removed from the apical and basal sides of the cultures, transport medium (TM, Hank's balanced salt solution supplemented with 25 mM glucose and 10 mM HEPES) was added to the apical (2 mL) and to the basolateral (2 mL) compartments, and pH was adjusted to 6 or 7.4. After 30 min of incubation, medium at the apical side was replaced with fresh TM containing MBCP 0, 0.5, 1, 2 or 4 mM. After 4 h of incubation at 37 °C, apical and basal solutions were collected, and aliquots (5 mL) were filtered on Phenex-PVDF 17 mm Syringe Filter 0.45 μm (Phenomenex, Torrance, CA). Samples were stored at −20 °C until LC–MS/MS analyses to measure the concentration of MBCP in both compartments (for LC–MS/MS operating conditions, see Section 2.5).

2.10. Human erythrocyte test

2.10.1. Haemolysis inhibition assay

The inhibitory capacity of MBCP on erythrocyte haemolysis was determined according to the procedures reported by Helmerhorst,

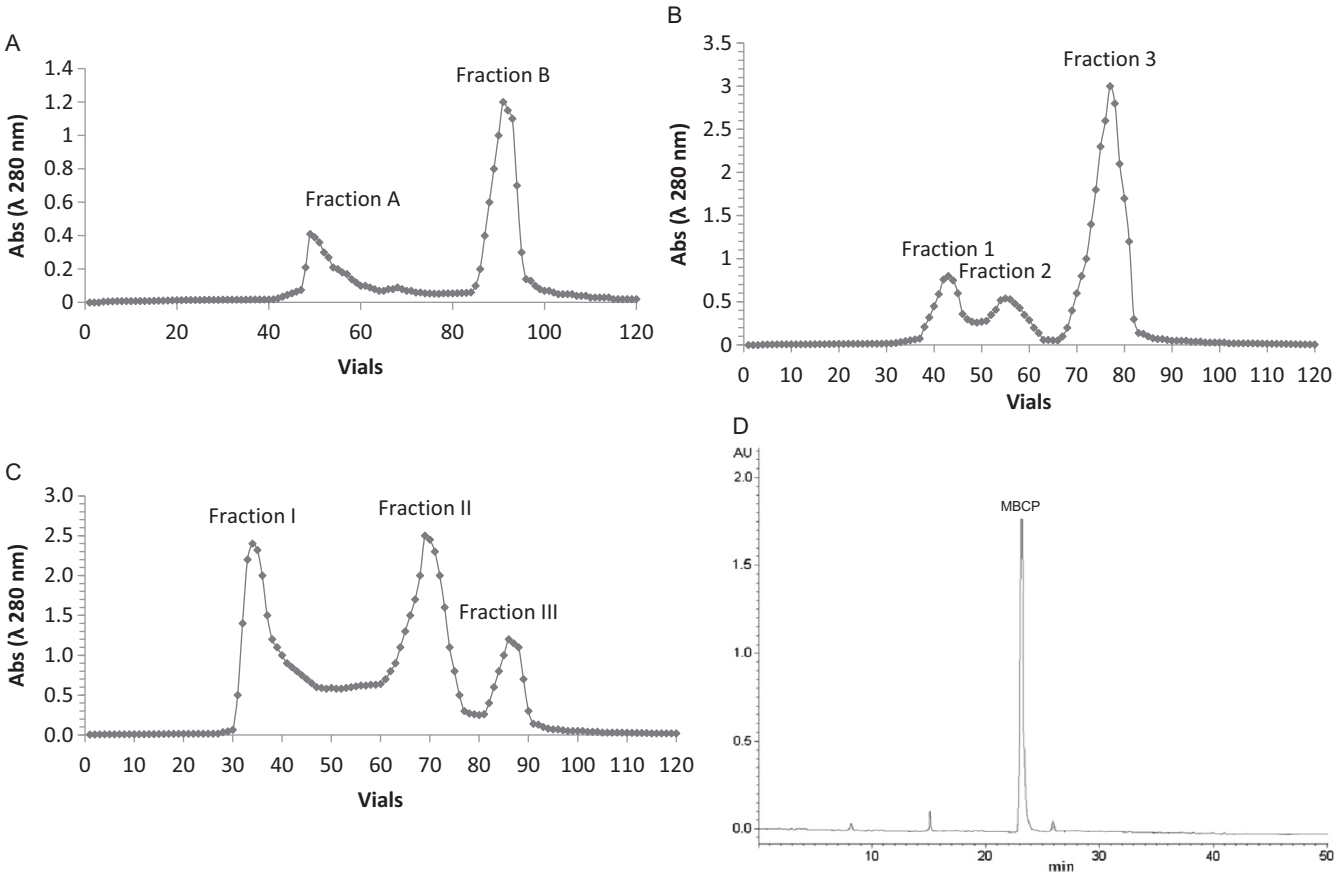

Fig. 1 – Chromatographic isolation of MBCP. (A) Fractionation of MBC gastrointestinal digest by Sephadex G-50 gel filtration chromatography; (B) fractionation of fraction B by Sephadex G-25 gel filtration chromatography; (C) fractionation of fraction 3 by Sephadex G-10 gel filtration chromatography; (D) RP-HPLC analysis of fraction III. MBC: "Mozzarella di Bufala Campana DOP"; MBCP: "Mozzarella di Bufala Campana DOP" peptide.

Reijnders, van't Hof, Veerman, and Nieuw Amerongen (1999) with slight modification. Blood was obtained from healthy volunteers. The experiment was carried out in accordance with the guidelines issued by the Ethical Committee of the Istituto Superiore di Sanità (2003). Erythrocytes were separated from plasma by centrifuging at 1200 *g* for 10 min at 4 °C, and washed three times with PBS. Then, erythrocyte suspension (0.2 mL, 20%, *v*/*v*, dissolved with PBS) was mixed with MBCP solution (0.2 mL, 0.1–2 mg/mL), and was gently shaken and incubated at 37 °C for 30 min. At the end of incubation, 0.2 mL of 100 mM H_2O_2 in PBS solution was added. The mixture was further incubated at 37 °C for 2 h. After incubation, the mixture was diluted with 3.2 mL PBS (pH 7.4). After centrifuging at 1200 *g* for 10 min at 4 °C, the absorbance of the supernatant was read at 540 nm. Distilled water was used as control. The inhibition of erythrocyte haemolysis was calculated as $(1\text{-}A_{\text{sample 540}}/A_{\text{control 540}}) \times 100\%$.

2.10.2. *Effect on intracellular ROS generation*

In order to clarify the anti-haemolytic mechanisms, the potential effects of MBCP against H_2O_2-induced oxidative stress in human erythrocytes were evaluated.

Erythrocyte suspension was obtained and incubated with MBCP solutions as described in Section 2.10.1. After addition of 0.2 mL of 100 mM H_2O_2 in PBS solution and incubation at 37 °C

Table 1 – Near equilibrium steady state antioxidant capacity of size exclusion chromatographic fractions from MBC intestinal digest[i].

Fractions	Assay method	
	DPPH	FRAP
Sephadex G-50*		
A	137.53 ± 2.10[a]	98.25 ± 1.86[a]
B	161.39 ± 2.61[b]	125.77 ± 1.72[b]
Sephadex G-25**		
1	157.53 ± 1.93[c]	120.18 ± 2.76[c]
2	143.09 ± 3.11[d]	112.43 ± 2.16[d]
3	163.98 ± 2.72[e]	126.37 ± 2.58[e]
Sephadex G-10***		
I	139.09 ± 3.21[f]	102.43 ± 2.35[f]
II	129.64 ± 2.83[g]	86.59 ± 1.96[g]
III	164.84 ± 3.25[h]	114.76 ± 1.93[h]

* The concentration of all fractions was 4 mg/mL.
** The concentration of all fractions was 0.1 mg/mL.
*** The concentration of all fractions was 0.05 mg/mL.
[a,b,c,d,e,f,g,h] Mean values in columns with different superscript letters are significantly different by the Tukey–Kramer multiple comparison test ($P < 0.05$).
[i] Expressed as TEs (μmol/L) of fractions measured by FRAP and DPPH assays at the steady state (DPPH, 45 min; FRAP, 55 min). Data are mean values ± SD ($n = 5$; $P < 0.05$).

for 2 h, the mixture was centrifuged at 1200 *g* for 10 min at 4 °C. The residue erythrocytes were washed 3 times with PBS (pH 7.4) and re-suspended with 5 volumes of PBS (pH 7.4). An aliquot (100 μL) of erythrocytes suspension was firstly centrifuged at 1200 *g* for 10 min at 4 °C and the supernatant was discarded. Then, DCFH-DA (200 μL, 10 μmol/L) was added to suspense the erythrocytes. After incubation at 37 °C for 25 min in the dark, the mixture was washed with PBS to completely remove the DCFH-DA outside the erythrocytes. At the end of washing, the erythrocytes containing the fluorescent probe were re-suspended with 600 μL PBS. Intracellular ROS generation was measured by recording the fluorescence intensity of erythrocytes by a Varioskan Flash Spectral Scan Multimode Plate Reader (Thermo Fisher Scientific, Waltham, MA), with the excitation and emission wavelengths at 485 and 525 nm, respectively. PBS-treated erythrocytes were considered as the blank control with 100% of the fluorescence intensity. The results were reported as the percentage of DCF fluorescence intensity of control (% control) calculated as following: DCF Fluorescence (% control) = Fsample × 100%/Fblank, where Fsample and Fblank represent the fluorescence intensity of sample and blank control, respectively.

2.11. *Statistics*

Unless otherwise stated, all of the experimental results were expressed as mean ± standard deviation (SD) of at least five replications. Statistical analysis of data was performed by Student's *t* test or two-way ANOVA followed by the Tukey–Kramer multiple comparison test to evaluate significant differences between a pair of means. The level of significance (α-value) was 95% in all cases ($P < 0.05$).

3. Results and discussion

3.1. *Isolation and identification of MBC peptides after gastrointestinal digestion*

The gel filtration chromatography was used for the fractionation of MBC intestinal digest. Two fractions (fractions A and B) were obtained from Sephadex G-50 column (Fig. 1A) and their antioxidant activity was evaluated. Since natural antioxidants are characterised by complex reactivity and different

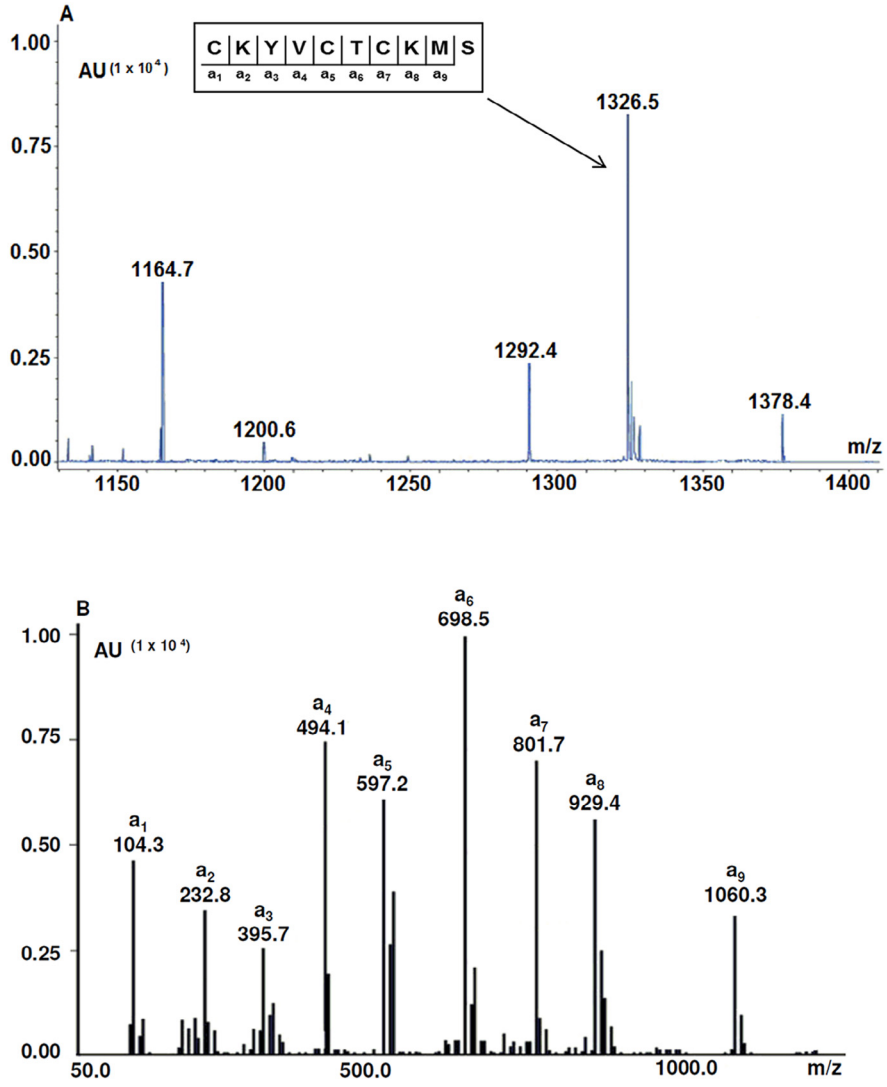

Fig. 2 – Q-TOF MS analysis of MBCP. (A) Molecular mass of MBCP; (B) fragmentation pattern of ion at *m/z* 1326.5. MBCP: "Mozzarella di Bufala Campana DOP" peptide.

mechanisms of action, the antioxidant capacity of food and food extracts cannot be assessed by using a single method (Schlesier, Harwat, Bohm, & Bitsch, 2002). Then, two different spectrophotometric assays, DPPH and FRAP tests, were executed, and antioxidant activities were expressed as trolox equivalents (TEs). The sample ability to scavenge free radicals is indicated by the decrease in DPPH absorption, while the FRAP test evaluates the reducing power of sample constituents.

As shown in Table 1, all the fractions showed antioxidant capacity, and fraction B exhibited the highest values. Fraction B was further purified on a Sephadex G-25 column (Fig. 1B) to obtain three fractions (fractions 1–3). The most active one (fraction 3) was further fractionated on Sephadex G-10 column (Fig. 1C) to yield three fractions (fractions I–III) (Table 1). Fraction III showed the strongest antioxidant capacity (Table 1) and was chosen to be further purified by HPLC. As shown in Fig. 1D, HPLC analysis showed four peaks of which peak at Rt 23.4 min represented about 96% of the fraction. Thus, HPLC analysis indicated that fraction III was a high purity peptide that was named as MBCP and subjected to characterisation.

Q-TOF MS analysis indicated for MBCP a molecular mass of 1326.5 Da (Fig. 2A). The observed molecular mass was in agreement with the calculated molecular mass of the peptide. MS/MS analysis (Fig. 2B) showed that the major sequence of MBCP was Cys-Lys-Tyr-Val-Cys-Thr-Cys-Lys-Met-Ser (CKYVCTCKMS), which was referred to a novel peptide, in accordance to databases available online (http://www.prospector.ucsf.edu; http://www.expasy.ch).

Pure MBCP was used as external standard for HPLC–DAD quantitative analysis. A stock solution was prepared in water and dilutions were made to cover a concentration range of 0.012–0.36 mg/mL. MBCP was quantified as 180.89 mg/100 g MBC fw.

In order to ascertain the presence of MBCP in the non-digested MBC, a MBC aliquot (250 g) was subjected to preparation, purification, and fraction characterisation, as reported earlier. The absence of such peptide in the native MBC corroborated the hypothesis that MBCP was the enzymatic product of MBC simulated GI digestion.

3.2. *MBCP effects on H_2O_2-stressed CaCo2 cell lines*

CaCo2 cell lines derived from human intestinal epithelial adenocarcinoma are regarded as a suitable model system for the *in vitro* evaluation of intestinal functions and nutrient absorption. Particularly, they are reported to reproduce several of the normal physiological responses to various modulatory agents, in a model which mimics the damages deriving from exposition to endogenous and exogenous oxidative agents (Levy, Mehran, & Seidman, 1995).

Thus, the potential protective effects of MBCP against H_2O_2-induced oxidative stress in CaCo2 cell lines were evaluated. Cells were exposed to 50 μM H_2O_2 for 30 min (H–CaCo2) and then treated for 24 h with 0.0125 to 1 mg/mL of MBCP. A linear correlation between incubation dose and cell proliferation was revealed, and the maximum result was achieved with a 0.2 mg/mL MBCP dose which made the stressed cell line proliferation increase by about 100% (Fig. 3A). We examined the effect on free radical and manganese superoxide dismutase levels in the stressed cell line exposed to increasing doses

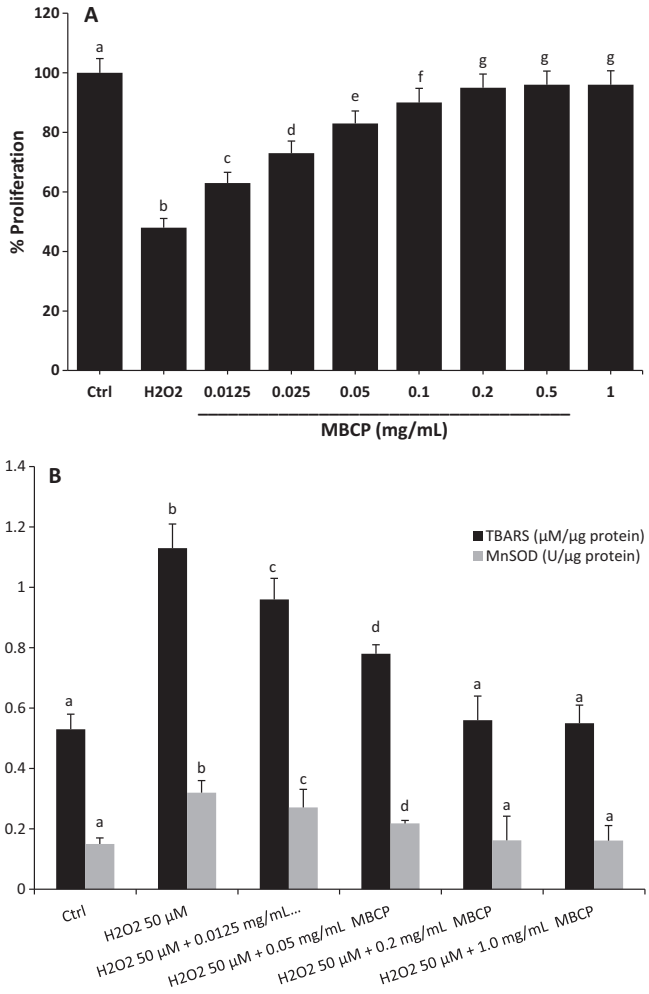

Fig. 3 – Effects of MBCP on H_2O_2-stressed CaCo2 cell lines. (A) Enhancement of cell proliferation; (B) protection against H_2O_2-induced ROS generation. MBCP: "Mozzarella di Bufala Campana DOP" peptide. Ctrl: untreated cell lines. TBARS: thiobarbituric acid reactive substances; MnSOD: manganese superoxide dismutase. Values are expressed as means ± SD ($n = 5$; $P < 0.05$). ^{abcd}Mean values with different superscript letters are significantly different by the Tukey–Kramer multiple comparison test.

(0.0125–1 mg/mL) of MBCP (Fig. 3B). Our data demonstrated that MBCP at a maximum dose of 0.2 mg/mL was able to directly scavenge free radicals without interfering with cell antioxidant defensive system involving enzymes and proteins for self-protection.

The structure–function relationship and the mechanism of peptide-induced antioxidant activity have not been fully elucidated. The mechanism of action has been referred to the physicochemical properties of the peptide quali-quantitative amino acid profile. As regards our peptide composition, amino acids, such as Tyr, Met, Lys, and Cys, are generally accepted to be antioxidants (Wang & Gonzalez de mejia, 2005). Particularly, residues with an aromatic ring structure (such as Tyr) can donate a proton to electron deficient radicals, while non-polar residues (Val and Met) can enhance the solubility of peptides in a lipid matrix improving the accessibility to

hydrophobic radical species or polyunsaturated fatty acids (Power, Jakeman, & FitzGerald, 2013). Further structural properties, which are important predictors of a peptide antioxidant activity, refer to the polar domain of the C terminal amino acid, and the low hydrophobicity, together with steric hindrance, of the second amino acid adjacent to the C terminus. These aspects make amino acids, such as Ser and Met, respectively, frequently present in these positions (Power et al., 2013). Nevertheless, the sulphydryl (R-SH) group in Cys has a unique antioxidant activity and interacts with the radical species by hydrogen donation from the SH group (Elias, Kellerby, & Decker, 2008).

All of the reported results were in full agreement with those obtained by performing the same assays on the synthetic MBCP.

3.3. In vitro *MBCP intestinal stability and bioavailability*

The intestinal stability and bioavailability of MBCP were evaluated by using single layers of CaCo2 cells as a model of absorption in the small intestine.

LC–MS analysis highlighted no significant hydrolysis (less than 10%) of MBCP in the apical solution by the brush border exopeptidases after 120 min incubation and regardless of the peptide concentration (Fig. 4A). LC–MS analysis of basolateral solution revealed that MBCP was absorbed intact through CaCo2 monolayer, with a concentration-dependent transport following a saturable pattern described by a linear curve (Fig. 4B). For LC–MS operating conditions, see Section 2.5. Interestingly, the actual amount of MBCP transepithelially transported was about 10%, thus, higher than what generally reported for different

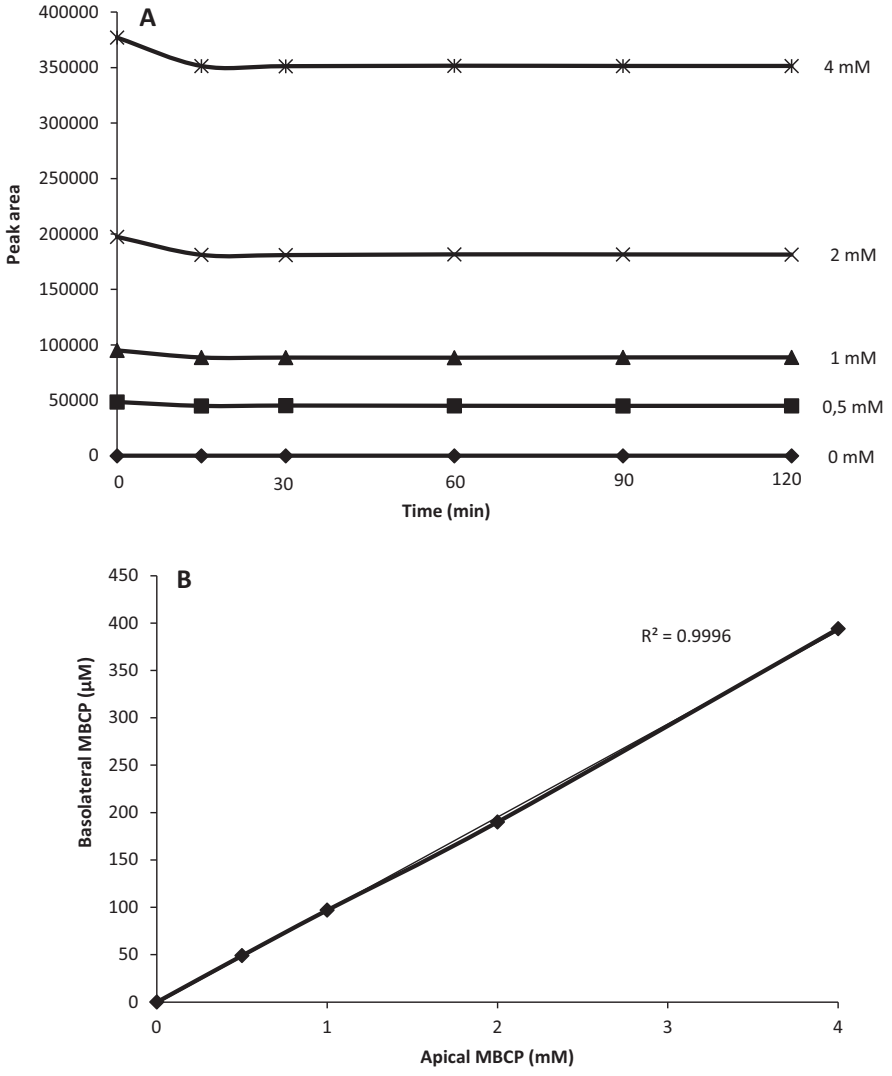

Fig. 4 – *In vitro* MBCP intestinal stability and bioavailability. (A) Stability of MBCP to the brush border exopeptidases measured as change in LC–MS chromatogram peak area of MBCP introduced at different concentrations in the apical compartment of CaCo2 cell monolayer. (B) Quantification of MBCP in CaCo2 cell monolayer apical and basolateral solutions using a five-point calibration curve of pure MBCP as standard analysed by LC–MS. MBCP: "Mozzarella di Bufala Campana DOP" peptide.

size (3–17 amino acid units) and polarity peptides transported from CaCo2 monolayer apical to basolateral side (Pauletti, Okumu, & Borchardt, 1997; Regazzo et al., 2010; Satake et al., 2002). Among the many different transport pathways, the carrier-mediated transport systems, such as the H^+-coupled PepT1 transporter, may be excluded as main pathway involved in the transport of MBCP since they are active and saturable symporters specific for intestinal absorption of charged di- and tripeptides (Brandsch, Knutter, & Bosse-Doenecke, 2008). The average polar properties of MBCP would also exclude a possible passive transcellular diffusion since a vesicular-mediated internalisation, the main mechanism involved, would imply absorption by apical cell membrane through hydrophobic interactions (Knipp, Velde, Siahaan, & Borchardt, 1997). Actually, the low level of degradation of MBCP during its transepithelial transfer strongly corroborates that passive transcellular diffusion would not be the main pathway involved in its transport. Nevertheless, the capacity of amino acids to form hydrogen bonds with lipid phosphates of cell membranes, like that of most of MBCP amino acid moieties (Cys, Lys, Tyr, Thr, Ser), has been described as a main physicochemical feature favoring the translocation process via transcytosis route (Pauletti et al., 1996), so that the passive diffusion would not be completely excluded. Overall, a possible involvement of the paracellular route in the transport of MBCP could be hypothesised. In fact, the passive paracellular transport via tight junctions has been usually reported to be normally applicable to the absorption of water soluble low-molecular-weight and short-chain peptides and, in general, it is specific for positively charged molecules because tight junctions are on average negatively charged (Salamat-Miller & Johnston, 2005).

All of the reported results were in full agreement with those obtained by performing the same assays on the synthetic MBCP.

3.4. *MBCP effects on H_2O_2-induced human erythrocyte haemolysis*

Since a significant amount (about 10%) of MBCP was shown to be transported from CaCo2 monolayer apical to basolateral side, MBCP would be expected to be potentially able to reach blood circulation and exert systemic effects. Primary targets could be red blood cells; interestingly, recent studies have reported on both erythrocyte protecting and haemolytic capacities of food deriving peptides (Ghribi et al., 2015; Hong, Chen, Hu, & Wang, 2014; Xue et al., 2009).

MBCP was observed to possess a strong ability to inhibit H_2O_2-induced human erythrocyte haemolysis (Fig. 5A). The inhibition ratio was 41.38% at 0.1 mg/mL of MBCP, and reached 91.25% when the concentration increased to 2.0 mg/mL. The estimated half-inhibitory concentration (IC50) was 0.12 ± 0.01 mg/mL.

In order to clarify the anti-haemolytic mechanisms, the potential effects of MBCP against H_2O_2-induced oxidative stress in human erythrocytes were evaluated. The nonpolar DCFH-DA was used as a probe to investigate the generation of ROS. DCFH-DA can rapidly diffuse into cells and is hydrolysed by cellular esterases into DCFH. The intracellular DCFH can be easily oxidised by free radicals into fluorescent DCH. The fluorescence intensity increases with the increase of intracellular ROS (Wolfe & Liu, 2007). Our data clearly indicated for MBCP a significant capacity to counteract H_2O_2-induced oxidative stress in red blood cells (Fig. 5B). Particularly, ROS generation was reduced by 43.28% at 0.1 mg/mL of MBCP, and reached 92.18% when the concentration increased to 2.0 mg/mL.

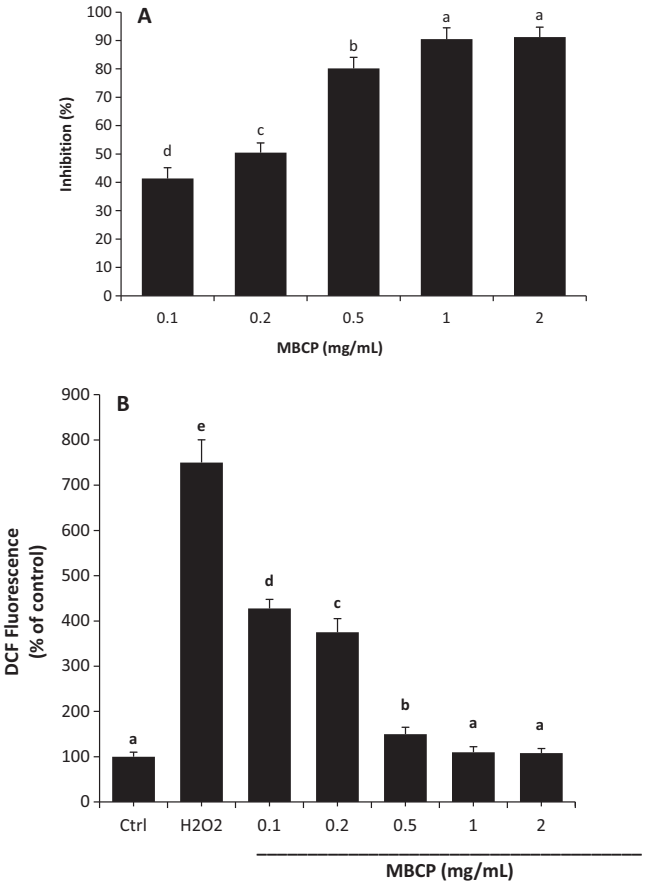

Fig. 5 – Protective effect of MBCP against H_2O_2-induced human erythrocyte haemolysis. (A) Inhibition of erythrocyte haemolysis; (B) effect on ROS generation in erythrocytes. MBCP: "Mozzarella di Bufala Campana DOP" peptide. Values are expressed as means ± SD ($n = 5$; $P < 0.05$). [abcd]Mean values with different superscript letters are significantly different by the Tukey–Kramer multiple comparison test. Ctrl: untreated cell lines.

All of the reported results were in full agreement with those obtained by performing the same assays on the synthetic MBCP.

4. Conclusions

The present study indicated MBC as a good source of bioactive peptides after GI digestion. Specifically, a novel antioxidant peptide (MBCP) was detected in the intestinal digest and its *in vitro* intestinal protection, bioavailability, and anti-haemolytic capacity were assayed. A potential intestinal protection against induced oxidative stress was revealed. MBCP demonstrated a good stability to brush border exopeptidases and a surprisingly higher bioavailability than what generally reported by *in vitro* experiments for different sizes and polarity peptides. Concerning the significant amount of MBCP transepithelially transported, MBCP would be expected to be potentially able to

reach blood circulation and exert systemic effects. According to recent studies reporting on erythrocyte protecting capacities of food deriving peptides, MBCP was observed to possess a strong ability to inhibit H_2O_2-induced human erythrocyte haemolysis. Our experimental results would suggest MBC as a potential functional food and MBCP as a novel food ingredient, food additive and pharmaceutical, relevant in health promotion and disease risk reduction.

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